- Pg. 19 bioluminescent imaging: Is it possible to administer anesthesia with isoflurane during scans rather than injectable ketamine/xylazine?

- Pg. 22 Cyclosporin A: Would it be worth monitoring effective plasma cyclosporin levels in the rat to ensure you are reaching effective therapeutic levels?

- Pg. 31 Survival Surgery: If you expect procedure to last 40 minutes, what are your plans for re-dosing general anesthesia? Please include language to reflect possibility of multiple doses.

- Pg. 31 Survival surgery: Is it possible to provide a local anesthetic for extraction? E.g. maxillary nerve block?

- Pg. 31 Survival surgery: How will you plan to prevent aspiration of the copious saline used?

- Pg. 32 Survival surgery: In rodent dental extraction models, is it possible to provide softened food as post-operative support?

- Pg. 56 Micro CT imaging: Is it possible to administer anesthesia with isoflurane during CT scans rather than injectable ketamine/xylazine?

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Thursday, June 11, 2020 5:31 PM
To: Daniel Eldridge; Jourdan E. Brune; Kristin Zabrecky
Cc: Leandra Mosca
Subject: assignment for first protocol review on 6/18/20
Attachments: Print_ PROTO202000003 - 4174-03_ Magnesium Stem Cell.pdf; ZhangH_cited_paper.pdf

Hi all,

Here is the first assignment. It's a pdf I saved of the "print version" of a new protocol that is currently being reviewed. *Please do not look this protocol up in Hoverboard*, as my first round of questions are in there and the protocol has probably changed since then (edited), so that would defeat the purpose of our exercise. Just use this pdf. I also attached a pdf of a paper that the lab attached for your reference. You're welcome to look at whatever you want in Pubmed/online, just sending this paper to you to save you a step. You can also look at other protocols in Hoverboard if you want to, just not this particular protocol.

I don't love the print version format, but right now it is the only way to "freeze" a protocol in a certain state, and I wanted you to see it as I was seeing it on my first review, but I didn't want to delay it moving through the review process.

Please write up your review questions in a Word doc and email them to me as an attachment no later than 8pm on Wednes 6/17 (earlier is fine!), so I have some time to look over what you came up with before we meet via Zoom on Thurs AM. Remember to address your questions/comments to the group (not to me). And please be prepared to discuss, it will be small and informal and I don't want to do all of the talking. If you can use video I think it would be nice.

Let me know if you have questions and have fun ☺
Molly

HoverBoard

Date: Tuesday, June 2, 2020 3:37:32 PM Print Close

View: SF: Basic Information

Basic Information

1. *** Select research team:**
   Zhang, H
2. *** Title of protocol:**
   Effect of magnesium, I50 and iPSC on rat extraction socket preservation
3. *** Short title:**
   4174-03: Magnesium Stem Cell
4. *** Summary of research:**
   This research uses rat molar extraction model to determine the effects of magnesium ion, induced pluripotent stem cells (iPSCs) and Tie2 super agonist I50 on socket preservation. The general approach is as followed: magnesium ion, osteogenic pre-induced rat derived iPSCs (abbreviated as riPOBs, which will be generated from rat peripheral blood mononuclear cells) and I50 will be mixed with deproteinized bovine bone mineralized matrix (BMM) and applied in the maxillary first molar extraction sockets of 12-week-old SD rats individually and in combination. Bone formation in the sockets and the dimension of alveolar ridge in height and width will be evaluated by high-resolution micro-CT at 2 weeks (live animals) and 6 weeks (sacrificed animals). At 6 weeks, the alveolar bone samples will be harvested and submitted for histology. New blood vessel formation will be evaluated by H&E staining and immunohistochemical staining.
5. *** Principal investigator:**
   Hai Zhang
6. *** What is the intention of the animal protocol?**
   Experimental Research

View: SF: Experimental Research Protocol Addition

Experimental Research Protocol Addition

1. * **Will the protocol include breeding?**
   ○ Yes ● **No**

View: Custom SF: Protocol Team Members

Protocol Team Members

1. Identify each additional person involved in the design, conduct, or reporting of the research:

Name	Role	Involved in Animal Handling	Authorized To Order Animals	E-mail	Phone
[redacted]	Graduate Student	yes	yes	[redacted]	

FERPA

RCW 42.56.070(1)

2. If veterinary care will be provided by individuals outside of DCM or WaNPRC, provide the name, credentials and contact information below:

N/A

View: Custom SF: Funding Sources

Funding Sources

1. Identify each organization supplying funding for the protocol:

	Funding Organization	eGC1 Number(s)
View	Restorative Dentistry	N/A

Scientific Aims

1. * Scientific aims of the research:

One aim is to establish a rat derived induced pluripotent stem cell (riPSC) cell line from rat peripheral blood mononuclear cell (rPBMC) and induce its initial differentiation towards riPSCs derived pre-osteoblasts (riPOBs). Another aim is to determine the effects of magnesium ion, riPOBs and I50 in a socket preservation model *in vivo*.

We hypothesize that the riPSC cell line will be successfully established, and magnesium ion can promote the proliferation and osteogenic differentiation of riPOBs in the rat socket preservation model. In addition, I50 can accelerate new blood vessel ingrowth in socket, thus prompting bone formation to achieve better socket preservation outcome.

2. * Using language understandable to non-scientists, describe the goals and significance of the protocol to humans, animals and science:

This research addresses a critical clinical problem (bone loss after tooth extraction resulting in deficient foundation tissue for implant or prosthetic treatment) that has a significant impact in the field of restorative dentistry. Many patients still need an operation to acquire enough bone for implant placement. The entire treatment is lengthy, costly and accompanied with morbidity. This has significantly affected patient's acceptance of implant therapy and quality of life during the treatment.

In recent years, magnesium, induced pluripotent stem cells (iPSCs, which is a new kind of stem cell that can differentiate to different body cells) and agent promoting vessel growth showed promising potential in bone regeneration. This research will generate iPSCs, and evaluate the effect of magnesium, iPSCs and an agent promoting vessel growth (which is called I50 in this study) on bone growth in a rat extraction socket. The results of this research will provide insights for new approaches either by applying them individually or combined. The outcome of the bone formation in the socket will be much improved and the need of additional surgical procedure will be significantly reduced. In the meantime, patient's satisfaction will be significantly improved due to the reduced surgical procedures and treatment time.

3. * Provide a statement to address the potential harm to the animals on this study (e.g., pain, distress, morbidity, mortality) relative to the benefits to be gained by performing the proposed work:

The animals in this study will lose one maxillary first molar, and experience post-operation local pain which can be controlled by analgesics, just as the human patients who undergo tooth extraction do. The chewing efficiency will decrease at the beginning, but will be gradually compensated by other teeth. The results of this research will provide insights for new approaches of socket preservation by evaluating the effect of magnesium, riPSCs and I50 on bone formation in rat extraction socket as well as the ridge dimension.

View: Custom SF: Experiments

Experiments

Note: If you will be administering cells, cell lines, sera or other biologicals to rodents, contact the Rodent Health Monitoring Program (RHMP, rhmp@uw.edu). Testing may be required prior to administration to rodents.

1. * Define the experiments to be used in this protocol:

Name	Species	USDA	Count	Count by Pain Category	Procedures	Husbandry Exception Types
01. Blood Collection for riPSC Cell Line Generation	Rats	no	2	B: 0 C: 0 D: 2 E: 0	▪ Other: Body Condition Score (Standard) ▪ Substance Administration: Anesthesia, Terminal, Ketamine and Xylazine (Standard) ▪ Tissue/Blood Collection: Zhang: Intracardiac Blood Collection Under Anesthesia (Team)	Rats - No husbandry or enrichment exceptions.

Name	Species	USDA	Count	Count by Pain Category	Procedures	Husbandry Exception Types
02. Socket Preservation - Pilot Study	Rats	no	6	B: 0 C: 0 D: 6 E: 0	▪ Euthanasia: CO2 followed by Secondary Method (>10 days of age) (Standard) ▪ Imaging: Zhang: Bioluminescence Imaging (Team) ▪ Other: Body Condition Score (Standard) ▪ Substance Administration: Analgesia, Meloxicam (SC, 72 hours) (Standard) ▪ Substance Administration: Zhang: Administration of Ampicillin (Team) ▪ Substance Administration: Zhang: Administration of Cyclosporine (Team) ▪ Substance Administration: Anesthesia, Ketamine and Xylazine (Standard) ▪ Substance Administration: Zhang: Filling Reagents in Tooth Socket (Team) ▪ Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team)	Rats - No husbandry or enrichment exceptions.

Name	Species	USDA	Count	Count by Pain Category	Procedures	Husbandry Exception Types
03. Socket Preservation - Full Study	Rats	no	72	B: 0 C: 0 D: 72 E: 0	▪ Euthanasia: CO2 followed by Secondary Method (>10 days of age) (Standard) ▪ Imaging: Zhang: Micro CT Imaging (Team) ▪ Other: Body Condition Score (Standard) ▪ Substance Administration: Anesthesia, Ketamine and Xylazine (Standard) ▪ Substance Administration: Zhang: Filling Reagents in Tooth Socket (Team) ▪ Substance Administration: Zhang: Administration of Ampicillin (Team) ▪ Substance Administration: Zhang: Administration of Cyclosporine (Team) ▪ Substance Administration: Analgesia, Meloxicam (SC, 72 hours) (Standard) ▪ Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team)	Rats - No husbandry or enrichment exceptions.

2. Will any single animal undergo more than one survival surgery? (include any animal that underwent surgery prior to use on this protocol) ○ Yes ● **No**

View: SF: Procedure Personnel Assignment

Procedure Personnel Assignment

1. * Select the team members who will be performing each procedure:

Procedure	Species	Is USDA Species	Team Members
Euthanasia: CO2 followed by Secondary Method (>10 days of age), ver. 2 (Standard)	Rats	no	
Imaging: Zhang: Bioluminescence Imaging, ver. 1 (Team)	Rats	no	
Imaging: Zhang: Micro CT Imaging, ver. 1 (Team)	Rats	no	
Other: Body Condition Score , ver. 1 (Standard)	Rats	no	
Substance Administration: Analgesia, Meloxicam (SC, 72 hours), ver. 1 (Standard)	Rats	no	
Substance Administration: Anesthesia, Ketamine and Xylazine, ver. 1 (Standard)	Rats	no	
Substance Administration: Anesthesia, Terminal, Ketamine and Xylazine, ver. 2 (Standard)	Rats	no	
Substance Administration: Zhang: Administration of Ampicillin, ver. 1 (Team)	Rats	no	
Substance Administration: Zhang: Administration of Cyclosporine, ver. 1 (Team)	Rats	no	
Substance Administration: Zhang: Filling Reagents in Tooth Socket, ver. 1 (Team)	Rats	no	
Survival Surgery: Zhang: Rat Tooth Extraction and Implantation, ver. 1 (Team)	Rats	no	
Tissue/Blood Collection: Zhang: Intracardiac Blood Collection Under Anesthesia, ver. 1 (Team)	Rats	no	

FERPA
RCW 42.56.070(1)

2. Team member training:

First Name Last Name Training

Course	Category	Source	Stage	Stage Number	Completion Date	Expiration Date
No experience data to display						

Course	Category	Source	Stage	Stage Number	Completion Date	Expiration Date
Annual DCM Facility Access Training (Rodent)	General	Online	Basic Course	Stage 1	2/13/2020	2/28/2021
Animal Use Medical Screening	General	Online	Basic Course	Stage 1	2/19/2020	2/28/2023
Rat Hands-On Laboratory	Animal Handling	In Person	Basic Course	Stage 1	3/5/2020	
Surgery Laboratory Part 2	Surgery	In Person	Basic Course	Stage 1	3/6/2020	
Surgery Laboratory Part 1A	Surgery	In Person	Basic Course	Stage 1	3/2/2020	
Animal Use Laws & Regulations	General	Online	Basic Course	Stage 1	10/8/2019	10/8/2024

Hai Zhang

Course	Category	Source	Stage	Stage Number	Completion Date	Expiration Date
Animal Use Laws & Regulations	General	Online	Basic Course	Stage 1	3/8/2017	3/8/2022
Foege Facility Orientation	Orientation	In Person	Basic Course	Stage 1	7/18/2014	
Annual DCM Facility Access Training (Rodent)	General	Online	Basic Course	Stage 1	3/1/2020	3/31/2021
Rat Online Course: Working with Rats at UW	Animal Handling	Online	Basic Course	Stage 1	3/5/2020	
Cervical Dislocation, Mouse Anesthetized	Procedure	In Person	Basic Course	Stage 1	1/19/2011	
Cervical Dislocation, Mouse Unanesthetized	Procedure	In Person	Basic Course	Stage 1	1/19/2011	
Mouse Hands-On Laboratory	Animal Handling	In Person	Basic Course	Stage 1	1/19/2011	
Animal Use Medical Screening	General	Online	Basic Course	Stage 1	1/17/2019	1/31/2022

No experience data to display

View: Custom SF: Animal Details

Animal Details

1. *** How are animals acquired?**
   Purchased
2. **Describe the acquisition for:**
   a. **Not purchasing through DCM or WaNPRC:**
      N/A
3. **Identification of individual animals (other than cage cards):**
   a. **Method(s) (e.g., ear punch/tag, tattoo, tagging/banding, radio collar, etc.) (Note: If method is implantation (e.g. PIT tag), create or select an Implant procedure to describe the details. If method is surgical (e.g., satellite tag), create or select Survival Surgery procedure to describe the details):**
      Ear tag
   b. **Will external identification be replaced if it falls off/out? If yes, describe the plan for replacement:**
      No
   c. **Will external identification be removed as part of the protocol (e.g., radio collars on field animals)? If yes, describe the plan for removal:**
      No
4. **Identify strain/stock for rodents and genetically modified animals:**

	Species	Is USDA Species	Strain	Genetically Modified Strain	Phenotype Description
View	Rats	no	Crl:CD(SD); Hsd:SD; NTac:SD (Sprague-Dawley)	no	No anticipated deleterious phenotypes.

View: Custom SF: Animal Number Adjustments

Animal Number Adjustments

"Animals Identified in Experiments" is the total number of animals per pain category listed in all experiments on this protocol. If more or fewer animals will be used on the protocol (see Help Text for examples), click Update to enter this new number in the corresponding "Adjusted Animal Count" column. **Only input numeric values in this field; 0 is acceptable.**
If no adjustment is required, the values in the "Animals Identified in Experiments" and "Adjusted Animal Count" columns must match. Click Update in each Pain Category row to input the matching value.
For questions about adjusting animal numbers, contact OAW.

1. * Click Update to adjust the number of animals to be used or produced for this protocol:

	Species	USDA Covered Species	Pain Category	Animals Identified in Experiments	Adjusted Animal Count
View	Rats	no	Pain Category B	0	0
View	Rats	no	Pain Category C	0	0
View	Rats	no	Pain Category D	80	80
View	Rats	no	Pain Category E	0	0

2. If you adjusted the number of animals for this protocol, explain why:

N/A

3. If you will be using animals to train personnel or to practice procedures included in this protocol, describe below:

N/A

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Alternatives and Duplication Searches

Alternatives and Duplication Searches

Display Procedures that cause pain or distress:

- Survival Surgery: Zhang: Rat Tooth Extraction and Implantation, ver. 1 (Team)

1. Record all searches for any previous research that this protocol might duplicate:

	Search Date	Searched Databases	Other
View	5/11/2020	EMBASE (searches multiple databases) Web of Science (searches multiple databases)	N/A
View	5/11/2020	EMBASE (searches multiple databases) Web of Science (searches multiple databases)	N/A

2. Briefly describe the results of your searches and why you can or cannot incorporate the findings. Or, if a literature search was not performed, describe the methods used to determine that alternatives are not available or feasible:

I50 used in this protocol is a newly synthesized compound, which hasn't been reported by any former articles. The effect of magnesium on riPSC hasn't been investigated, and riPSCs haven't been applied in animal ridge preservation model in combination with magnesium. So there's no duplicate of this protocol. The results of searches for alternatives don't yield any practical methods for this protocol. The results include culturing cells in various kinds of scaffolds. However, the in vitro models cannot totally mimic in vivo microenvironment of tooth socket, and cannot simulate bone maturation and angiogenesis at the same time. Thus, the animal experiments in this protocol cannot be replaced by in vitro studies.

3. Confirm that you have made every effort to ensure that this protocol is not unnecessary duplication of previous research: ☑

View: Custom SF: Housing and Use

Housing and Use

Housing and use outside of the vivarium is not allowed without strong scientific justification.

1. Identify each location where animals will be housed:

	Facility	Species	Justification for Housing Outside Vivarium
View	ARCF ABSL1	Rats	N/A

2. Identify each location where animals will be used:

	Facility Use	Species	Justification for Use Outside Vivarium
View	ARCF ABSL1 All procedures will be performed here. A cleared place will be used for operation and a power source will be needed for portal dental drill motor.	Rats	N/A

View: Custom SF: Disposition

Disposition

1. **Disposition plans for the animals when this research is complete:**
   (check all that apply)
   Euthanasia
2. **If other, provide an animal disposition description:**
   N/A
3. **If protocol involves fixing tissues, list agents (e.g., paraformaldehyde, formalin):**
   4% paraformaldehyde for fixing the bone tissue that is collected after euthanasia.

View: Custom SF: Refinement, Replacement and Reduction

Refinement, Replacement and Reduction

1. Describe below how the three R's (refinement, replacement and reduction) have been employed on this project. Include alternatives that were considered for the procedures above that cause pain or distress:

* Refinement (use of methods to decrease animals' sensitivity to pain)

Surgical refinements to reduce animal discomfort and stress will always be considered. Analgesia will begin before the surgery, and anesthesia will be given properly during tooth extraction to reduce pain to the largest extent.

* Replacement (include in vitro tests, use of less sentient animals)

Established iPSC cell line will be used prior to the animal experiments, and in vitro cell culture constitutes the bulk of the research done for our project. We will use in vitro experiment to determine the optimal concentrations of the MgCl2 solution for iPSCs osteogenic differentiation, instead of testing the concentrations in animal model. When strong and positive results are observed in vitro, these results must then will be confirmed in animal models- the living system. In vitro bone cultures have not been successful in our lab and others, so animal model is still irreplaceable to date.

* Reduction (use of fewer animals to attain statistical significance)

Larger sample size will provide higher statistical power for the study, however, use of power analysis (based on pilot work) helped us identify the minimal sample size needed to draw a valid conclusion. Whenever a reduction is possible without compromising the findings, it is undertaken.

2. Describe the rationale for using animals and the appropriateness of the species proposed:

The in vitro cell experiments can't totally mimic the environment of live animal. In this study, bone formation is influenced not only by the reagents filled in the bony defect, but also by the cytokines circulating with the blood and microenvironment in 3D live tissue. So the animal model is non-substitutable. Rat and mouse are most commonly used animals in dentistry experiment. In our research, rats are more suitable because the larger size of molar makes it easier to perform the operation.

View: SF: Supporting Documents

Supporting Documents

1. Attach supporting files:

Document Name	Date Modified
flow chart (1).pptx	5/11/2020 1:04 PM

Procedures Appendix:

HoverBoard

View: Custom SF: Procedure Identification

Procedure Identification: Body Condition Score

1. * Name of the procedure or surgery:
Body Condition Score

2. * Select procedure type:
Other

3. * Species:
Rats

4. * Will administering this procedure cause any more than momentary pain or distress? Yes **No**

If yes,

i. Identify expected symptoms from administering this procedure:
N/A

ii. Identify criteria under which animals will be removed from research:
N/A

View: Custom SF: Other

Other

1. Description of Procedure:

Rats are handled gently during palpation of bony prominences over the shoulders, spinal column, and pelvis. This is usually performed with rats standing comfortably on the wiretop of the cage with minimal tail-base restraint.

A numerical Body Condition Score (BCS) (see attached diagram from Hickman and Swan, 2010) is assigned for each individual animal. Frequency of BCS assessment is described in the experiment.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
Hickman and Swan, 2010.pdf	10/6/2016 5:25 PM

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Bioluminescence Imaging

1. *** Name of the procedure or surgery:**
   Zhang: Bioluminescence Imaging
2. *** Select procedure type:**
   Imaging
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Imaging

Imaging

1. **Imaging types:**
   Other
2. **If Other, specify:**
   Bioluminescence imaging
3. **Select the anesthesia and analgesia procedures to be used:**

Anesthesia, Ketamine and Xylazine	Substance Administration	1 Standard

4. **Frequency, including minimum time between imaging sessions and the maximum number of sessions (enter specific, detailed procedure timing in the Experiment):**
   See experiment for timing and frequency.
5. **Duration of imaging session:**
   Approximately 15-30 minutes
6. **Purpose:**
   To detect transplanted cell survival with bioluminescence imaging
7. **Will supportive care of animals be necessary during the imaging session?**
   Yes No
8. **If yes, describe:**
   Upon removal from the scanner, rats are placed in a recovery cage until they resume normal ambulation. The recovery cage is warmed to 37°C with a small animal heating pad.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Administration of Cyclosporine

1. *** Name of the procedure or surgery:**
   Zhang: Administration of Cyclosporine
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)	Standard	Subcutaneous	10mg/kg	N/A	250-500 uL	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Cyclosporine A will be diluted to the appropriate concentration and then administered via SC injection. The daily administration of cyclosporine A (10mg/kg) begins at three days before grafting and continue daily until sacrifice.

3. Describe the intended effects of administering the substance(s):

To suppress transplant rejection.

4. Describe any potential adverse reactions to administering the substance(s):

High doses cause renal and hepatic toxicity.

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

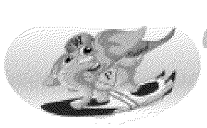
1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)
2. **Route:**
   Subcutaneous

   If you indicated Other, specify the route:
3. **Dose:**
   10mg/kg
4. **Frequency and duration of dosages:**
   Once per day; see experiment for duration
5. **Volume (for rodents or intracranial injections):**
   250-500 uL
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Cyclosporine A will be pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A



View: Custom SF: Procedure Identification

Procedure Identification: Anesthesia, Ketamine and Xylazine

1. *** Name of the procedure or surgery:**
   Anesthesia, Ketamine and Xylazine
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Ketamine (Ketaset, Ketaflo, Vetalar)	Standard	Intraperitoneal	45 – 68.2 mg/kg	N/A	Up to 10 microliters per gram of body weight	N/A
View	Xylazine	Standard	Intraperitoneal	1.1 – 4.4 mg/kg	N/A	Up to 10 microliters per gram of body weight	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Ketamine/Xylazine is mixed together and diluted in sterile pharmaceutical grade saline. The cocktail is administered IP to induce 25-30 minutes of general anesthesia. Appropriate depth of anesthesia is monitored by respiratory rate, corneal reflex, and response to front toe pinch. Heat support and eye lubrication will be provided.

3. Describe the intended effects of administering the substance(s):

General anesthesia

4. Describe any potential adverse reactions to administering the substance(s):

Respiratory and cardiac depression, including bradycardia and hypotension

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Needles must not be recapped unless a recapping device is used.

Gloves must be worn when handling these agents.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Ketamine (Ketaset, Ketaflo, Vetalar)
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   45 – 68.2 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Up to 10 microliters per gram of body weight
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Ketamine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   Xylazine
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   1.1 – 4.4 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Up to 10 microliters per gram of body weight
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Xylazine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Rat Tooth Extraction and Implantation

1. *** Name of the procedure or surgery:**
   Zhang: Rat Tooth Extraction and Implantation
2. *** Select procedure type:**
   Survival Surgery
3. *** Species:**
   Rats

4. * Will administering this procedure cause any more than momentary pain or distress? **Yes** No

If yes,

i. **Identify expected symptoms from administering this procedure:**
This procedure is expected to cause discomfort that should be relieved by anesthesia and/or analgesia. Please see procedure description and/or experimental description for monitoring plan, including specific behavioral and clinical signs to be monitored.

ii. **Identify criteria under which animals will be removed from research:**
Please see experimental description for end point criteria.

View: Custom SF: Survival Surgery

Survival Surgery

1. *** Surgery Type:**
   Major
2. *** Describe how the animal, surgeon, and instruments will be prepared for surgery:**
   Surgical instruments including dental explorer and dental bur will be autoclaved prior to the initial surgery. Several sets of sterile instruments that are sufficient for one day use will be prepared.

   Sterile surgical gloves and face mask will be used. Surgical gloves will be changed between animals. No food/water restriction will be needed. Animal will be weighed prior to surgery.

   Extraction site will be scrubbed by sterile gauze soaked with 1% chlorhexidine and dried using sterile gauze prior to tooth extraction. Rats will receive eye lubricant in each eye to prevent corneal drying. Rats will be anesthetized through injection of ketamine and xylazine cocktail mixed with sterile 0.9% saline and mounted on a jaw retraction board.
3. *** Describe the surgical procedure, including any deficits expected as a result of the surgery:**

   The rats will be kept warm on a warm-water pad during the surgical procedure. After anesthetized by intraperitoneal injection with ketamine and xylazine, 1 mg/kg meloxicam will be injected subcutaneously, and sterile eye lubricant will be placed in each eye.

   The left maxillary first molars will be extracted, and a standardized bone defect (approximately 3 mm in length, 2.6 mm in width and 2 mm in depth) will be created in the extraction area with approximately 0.5 mm to the mesial of maxillary second molar using a sterilized round bur and copious amount of sterilized saline for cooling. Depending on the experiment/group, the defects will be (1) left unfilled, or (2) filled by bone mineralized matrix (Bio-Oss®) solely, or (3) filled by bone mineralized matrix (Bio-Oss®) mixed with different reagents ($MgCl_2$ solution, Tie2 super agonist, osteogenic pre-induced riPSCs (riPOBs) solely or in combination). See related experiments for filling reagents in each group. Collagen membrane (Bio-Gide®, approximately 3×3 mm^2) will be sutured to gingival margin by 5-0 absorbable suture to seal the wound.

   Duration of procedure: 40 minutes.

   Expected Deficits: difficulty with eating during 24-48 hours post-surgery. Soft food will be provided during this period.
4. *** Select associated substance administration procedures, including anesthesia and analgesia procedures to be used:**

Analgesia, Meloxicam (SC, 72 hours)	Substance Administration	1 Standard
Anesthesia, Ketamine and Xylazine	Substance Administration	1 Standard
Zhang: Administration of Ampicillin	Substance Administration	1 Team

5. **Describe how animals will be monitored during the procedure:**
   Front toe pinch will be used to monitor depth of Ketamine/Xylazine anesthesia. Heart

rate, respiration, temperature, movement, relaxed jaw, corneal reflex will be monitored until the rats recover from anesthesia.

6. Describe the routine for postoperative care: (including removal of sutures, if applicable)

The rats will be placed in the recovery cage and monitored every 5 minutes for breathing rate and body temperature. After righting reflex has been regained, rats will be placed into a clean cage with gel on the cage floor and immediately placed back into the rack in the housing room.

We anticipate that subcutaneous injections of meloxicam will last for 20-24 hours, so we will administer meloxicam every 20-24 hours for 72 hours post-surgery. If rats are exhibiting signs of pain or distress, we will consult with Vet Services.

All animals will also be under penicillin/ampicillin treatment (8WU for intraperitoneal injection daily) for 5 days, starting on the day of the operation to prevent infection. If prominent reduction of iPOB longevity is proved in the pilot study, immunosuppressant (10mg/kg cyclosporine A daily) will be administered in iPOB-transplanted groups in formal experiment beginning three days before grafting and continuously until sacrifice. See related procedures for details.

Animals will be monitored daily for 7 days post-surgery for body weight, signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. The surgical site will be monitored daily for 3 days post-surgery for bleeding, dislodgement of suture and any signs of infection such as redness, swelling and pus. Because the wound will be closed by absorbable sutures, the removal of sutures will not be needed.

7. Describe how postoperative pain and distress will be assessed:

(including need for further care)

The analgesic (subcutaneous injections of meloxicam) will be administered for 72 hours as described in the related procedure. Animals will be monitored daily for 7 days post-surgery for body weight, signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. If rats are exhibiting signs of pain or distress, we will consult with Vet Services.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
diagram for rat socket preservation model.docx	2/18/2020 3:40 PM

View: Custom SF: Procedure Identification

Procedure Identification: Anesthesia, Terminal, Ketamine and Xylazine

1. *** Name of the procedure or surgery:**
   Anesthesia, Terminal, Ketamine and Xylazine
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Ketamine (Ketaset, Ketaflo, Vetalar)	Standard	Intraperitoneal	≥68.2 mg/kg	N/A	Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.	N/A
View	Xylazine	Standard	Intraperitoneal	≥4.4 mg/kg	N/A	Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Ketamine/Xylazine is mixed together and diluted in sterile pharmaceutical grade saline or water. The cocktail is administered IP to induce anesthesia appropriate for a short (<20 minutes) terminal procedure such as perfusion.

Deep anesthesia is confirmed by lack of response to toe pinch, change in respiratory character and decreased respiratory rate.

3. Describe the intended effects of administering the substance(s):

Anesthesia for short (<20 minutes) terminal procedure

4. Describe any potential adverse reactions to administering the substance(s):

N/A

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Needles must not be recapped unless a recapping device is used.

Gloves must be worn when handling this agent.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA)**

paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Ketamine (Ketaset, Ketaflo, Vetalar)
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   ≥68.2 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Ketamine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   Xylazine
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   ≥4.4 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Xylazine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: CO2 followed by Secondary Method (>10 days of age)

1. *** Name of the procedure or surgery:**
   CO2 followed by Secondary Method (>10 days of age)
2. *** Select procedure type:**
   Euthanasia
3. *** Species:**
   Rats

4. * Will administering this procedure cause any more than momentary pain or distress? Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

View: Custom SF: Euthanasia

Euthanasia

1. *** Method of euthanasia:**
   CO2 Overdose
2. **Describe procedure:**
   CO2 will be administered from a compressed commercial cylinder utilizing a flow meter to deliver 30-70% of the chamber volume per minute. Total gas exposure will be at least 10 minutes, with gas flow being maintained for at least 1 minute after apparent clinical death. A timer will be used to ensure adequate length of exposure.

   Secondary method will be one of the following: placed in a bag filled with CO2, decapitation, exsanguination, thoracotomy/tissue collection.
3. *** Will anesthesia be used?** Yes **No**
4. **Describe how death will be confirmed:**
   Death will be confirmed by lack of respirations and heartbeat.
5. **Is this method approved by the AVMA Guidelines on Euthanasia (2013)?**
   Yes No

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Intracardiac Blood Collection Under Anesthesia

1. *** Name of the procedure or surgery:**
   Zhang: Intracardiac Blood Collection Under Anesthesia
2. *** Select procedure type:**
   Tissue/Blood Collection
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Live Tissue/Blood Collection

Live Tissue/Blood Collection

1. *** Identify tissues to be collected:**
   Blood from heart
2. **Describe timing and frequency of collection and amount to be collected:**
   Volume: 2-4 ml. Once during terminal procedure under anesthesia.
3. **Select the anesthesia and analgesia procedures to be used:**

Anesthesia, Terminal, Ketamine and Xylazine	Substance Administration	2 Standard

4. **If withholding anesthesia/analgesia when normally required, provide scientific justification:**
   N/A
5. **Describe any potential complications from collection:**
   None anticipated.
6. *** Describe the collection procedure:**
   The rat is anesthetized. Before thorax opening, front toe pinch will be used to monitor depth of ketamine/xylazine anesthesia. The thorax is then opened to expose the heart. A 5-ml syringe with a 15G needle are primed with EDTA to prevent clotting. The needle is introduced into a heart ventricle and a terminal blood sample is collected.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Administration of Ampicillin

1. *** Name of the procedure or surgery:**
   Zhang: Administration of Ampicillin
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Penicillin (Ampicillin)	Standard	Intraperitoneal	80,000 IU	N/A	0.2 ml	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Penicillin G sodium will be injected intraperitoneally 1 hour before surgery and daily for 4 days after surgery.

Restrain the rats appropriately in the head-down position. Injections are performed with a small gauge needles (22 to 27 gauge), which is inserted to the depth in which the entire bevel is within the abdominal cavity. The barrel of the syringe is drawn back to make certain that the needle is not in a blood vessel. The injection is delivered slowly but steadily, checking for leakage around needle.

3. Describe the intended effects of administering the substance(s):

Broad-spectrum antibiotic to reduce the risk of infection as a result of biomaterials and reagents implantation.

4. Describe any potential adverse reactions to administering the substance(s):

No side effects have been seen with this antibiotic.

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Penicillin (Ampicillin)
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
3. **Dose:**
   80,000 IU
4. **Frequency and duration of dosages:**
   Daily for 5 days
5. **Volume (for rodents or intracranial injections):**
   0.2 ml
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Pharmaceutical grade will be obtained
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Filling Reagents in Tooth Socket

1. *** Name of the procedure or surgery:**
   Zhang: Filling Reagents in Tooth Socket
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Bone mineralized matrix (Bio-Oss®)	Team	Other	N/A	N/A	Approximately 16 cubic milliliter	N/A
View	I53-50 (abbreviated as I50)	Team	Other	N/A	1000ng/ml of F-domains	20µl	N/A
View	magnesium chloride	Team	Other	N/A	0.8, 1.8, 5, 10 or 20 mM (decided by in vitro experiments)	20µl	N/A
View	riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)	Team	Other	20,000 cells per site in media	N/A	N/A	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Bone mineralized matrix (Bio-Oss®) alone or together with one or more of the below reagents will be implanted into the tooth socket of rats (prepared into a box-like defect) during a survival surgery (see related survival surgery for details):

- riPOBs (riPOBs used in pilot study will be labelled with luciferase prior to implantation)
- magnesium chloride solution
- I50

See the treatment of each group in experiment protocol for combination of the reagents in each group.

Note: The optimal concentration of magnesium chloride will be verified by cell proliferation and osteogenic differentiation experiments on riPOBs (riPSCs derived pre-osteoblasts) in vitro, and will be applied in grafting procedure. The concentration will be chosen from 0.8, 1.8, 5, 10 and 20 mM.

3. Describe the intended effects of administering the substance(s):

riPOBs labelled with luciferase: To test the longevity of transplanted riPOBs in rat tooth socket with or without immunosuppressive therapy.

riPOBs used in full study: To promote bone growth in rat tooth socket.

I50 is a Tie2 super agonist that can activate Ang-1/Tie2 pathway, thus to accelerate angiogenesis and promote bone growth in rat tooth socket.

Magnesium chloride: To reduce bone resorption after tooth extraction, and promote bone growth in tooth socket.

4. **Describe any potential adverse reactions to administering the substance(s):**
   No
5. **If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.**
6. *** Does this procedure include the use of a paralytic agent?**
   Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Bone mineralized matrix (Bio-Oss®)
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket
3. **Dose:**
   N/A
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   Approximately 16 cubic milliliter
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Bone mineralized matrix (Bio-Oss®) is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   I53-50 (abbreviated as I50)
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket together with bone matrix
3. **Dose:**
   N/A
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   20µl
6. **Concentration:**
   1000ng/ml of F-domains
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   I50 is an investigational new compound, not available in pharmaceutical grade. It will be dissovled in Dulbecco's Modified Eagle Medium (or similar media) and sterile filtered prior to use.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   magnesium chloride
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket together with bone matrix
3. **Dose:**
   N/A
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   20µl
6. **Concentration:**
   0.8, 1.8, 5, 10 or 20 mM (decided by in vitro experiments)
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Magnesium chloride is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket together with bone matrix
3. **Dose:**
   20,000 cells per site in media
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   N/A
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Not available pharmaceutical grade; cell mixture will be prepared in sterile culture hood prior to administration.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Micro CT Imaging

1. *** Name of the procedure or surgery:**
   Zhang: Micro CT Imaging
2. *** Select procedure type:**
   Imaging
3. *** Species:**
   Rats

4. * Will administering this procedure cause any more than momentary pain or distress? Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

View: Custom SF: Imaging

Imaging

1. **Imaging types:**
   Computed Tomography (CT)
2. **If Other, specify:**
3. **Select the anesthesia and analgesia procedures to be used:**

Anesthesia, Ketamine and Xylazine	Substance Administration	1 Standard

4. **Frequency, including minimum time between imaging sessions and the maximum number of sessions (enter specific, detailed procedure timing in the Experiment):**
   See experiment for timing and frequency.
5. **Duration of imaging session:**
   20 minutes
6. **Purpose:**
   Within experiment assessment of bone formation in mandible defect.
7. **Will supportive care of animals be necessary during the imaging session?**
   Yes No
8. **If yes, describe:**
   Upon removal from microCT scanner, rats are placed in a recovery cage until they resume normal ambulation. The recovery cage is warmed to 37°C with a small animal heating pad.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Procedure Identification

Procedure Identification: Analgesia, Meloxicam (SC, 72 hours)

1. *** Name of the procedure or surgery:**
   Analgesia, Meloxicam (SC, 72 hours)
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Meloxicam (Metacam)	Standard	Subcutaneous	1 mg/kg	N/A	Total volume will not exceed 5 mL/kg of body weight.	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

One dose of meloxicam will be injected subcutaneously (SC) at least 30 minutes prior to recovery from the procedure, and then administered every 20-24 hours for 72 hours.

If signs of pain are noted despite meloxicam administration or following this period, Veterinary Services will be consulted.

If dilution is necessary, it will be done with sterile water or saline for injection (pharmaceutical grade).

Note: Many category 2 and 3 procedures require multimodal analgesia and more than one type of analgesic is ideally administered. Please consult with Veterinary Services if questions. Please refer to the IACUC policy on "Analgesia in Research Animals," for more information on what types of procedures fall into this category.

3. Describe the intended effects of administering the substance(s):

Provide analgesia for 72 hours

4. Describe any potential adverse reactions to administering the substance(s):

Gastrointestinal ulceration or bleeding, renal toxicity

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Needles must not be recapped unless a recapping device is used.

Gloves must be worn when handling this agent.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA)**

paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

1. Supporting documents:

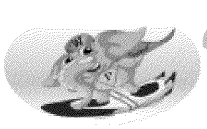
Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Meloxicam (Metacam)
2. **Route:**
   Subcutaneous

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   1 mg/kg
4. **Frequency and duration of dosages:**
   Once at the time of the procedure, then every 20-24 hours for 72 hours
5. **Volume (for rodents or intracranial injections):**
   Total volume will not exceed 5 mL/kg of body weight.
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Meloxicam is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

Substances Appendix:



View: Custom SF: Substance Information

Substance Information: riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)

1. *** Name:**
   riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)
2. *** Substance types:** (select all that apply)
   Cell, Cell Line, or Tissue - Other

3. *** Is this a hazardous agent:** Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: I53-50 (abbreviated as I50)

1. *** Name:**
   I53-50 (abbreviated as I50)
2. *** Substance types:** (select all that apply)
   Chemical Agent
3. *** Is this a hazardous agent:** Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: magnesium chloride

1. *** Name:**
   magnesium chloride
2. *** Substance types:** (select all that apply)
   Chemical Agent
3. *** Is this a hazardous agent:** Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Bone mineralized matrix (Bio-Oss®)

1. * Name:

Bone mineralized matrix (Bio-Oss®)

2. * Substance types: (select all that apply)

Other

3. * Is this a hazardous agent: Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Ketamine (Ketaset, Ketaflo, Vetalar)

1. * Name:

Ketamine (Ketaset, Ketaflo, Vetalar)

2. * Substance types: (select all that apply)

Anesthetic
Reproductive Hazard/Teratogen

3. * Is this a hazardous agent: **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Xylazine

1. * Name:

Xylazine

2. * Substance types: (select all that apply)

Anesthetic

3. * Is this a hazardous agent: **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Meloxicam (Metacam)

1. * Name:

Meloxicam (Metacam)

2. * Substance types: (select all that apply)

Analgesic
Reproductive Hazard/Teratogen
Other

3. * Is this a hazardous agent: **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions,**

contact EH&S Research and Occupational Safety at 206-221-7770 or ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Penicillin (Ampicillin)

1. * Name:

Penicillin (Ampicillin)

2. * Substance types: (select all that apply)

Antibiotic
Reproductive Hazard/Teratogen

3. * Is this a hazardous agent: **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)

1. * Name:

Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)

2. * Substance types: (select all that apply)

Analgesic
Carcinogen
Immunosuppressant
Reproductive Hazard/Teratogen

3. * Is this a hazardous agent: **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create and Edit

1. *** Select the funding organization:**
   Restorative Dentistry

   If Other was selected in question 1, provide Funding Organization:

2. *** All animal use projects must be reviewed for scientific merit prior to initiating animal use. Choose the required reviews for this project:**
   Has already been conducted and approved by a funding agency

3. **Provide name of the committee or the department reviewer** (Required if "Has been conducted by my department or school and has been found to be scientifically meritorious" was selected):
   Dr. Marty Anderson, Margaret Spencer Fund Committee Chair

4. **eGC1 Number(s):**(assigned internally)
   N/A

View: Custom: Create and Edit

Experiments Appendix:

01. Blood Collection for riPSC Cell Line Generation

1. *** Experiment name:**
   01. Blood Collection for riPSC Cell Line Generation
2. *** Species:**
   Rats
3. **If other was selected, provide a species:**
4. **What is the scientific goal of this experiment:**
   To establish a rat induced pluripotent stem cell (riPSC) cell line with rat peripheral blood mononuclear cells (rPBMC).
5. *** Describe the animal experience in the experiment, from enrollment in the study to the final endpoint, including all procedures in chronological order and the minimum time between procedures. We encourage using bullet points, timeline, table, or a flow chart as appropriate:**

   1. Rats will be anesthetized by ketamine/xylazine cocktail overdose before blood collection.

   2. **Thorax will be opened, and 2-4 milliliter blood will be collected from heart ventricle. Rat will be euthanized by exsanguination.**

   Animal Sex:
   Female

   Animal Ages:
   3 months

   Animal Size:
   250-300g
6. **Select experimental procedures:**

Name	Type	Version	Scope
Body Condition Score	Other	1	Standard
Anesthesia, Terminal, Ketamine and Xylazine	Substance Administration	2	Standard
Zhang: Intracardiac Blood Collection Under Anesthesia	Tissue/Blood Collection	1	Team

7. **Monitoring protocol, including frequency and specific behavioral and clinical signs to be monitored. Include humane endpoints (criteria for euthanasia):**
   The rats will be monitored once every week for weight, body condition score and

other general condition before euthanasia.

Rats will be euthanized before blood collection according to the following criteria:
1. More than 20% weight loss;
2. Body condition score of 2 or less. See Body Condition Score Procedure for detailed evaluation method.
3. Inability or reluctance to move when stimulated, or moribund condition.
4. Impairment of ability to eat, drink, or ambulate normally.
5. Labored breathing.
6. Hypothermia.
7. Ulcerated tumors.

8. If there is expected mortality (spontaneous death) in this experiment:

a. Procedure/condition associated with mortality:
N/A

b. Estimated mortality rate, i.e. percentage of animals expected to die spontaneously (not via euthanasia) or need to be euthanized as a result of the procedure. (Be sure to account for this in your animal number calculations):
N/A

c. Explain why euthanasia is not possible or appropriate:
N/A

9. Will some animals live out their natural lifespan as part of this experiment? If so, indicate their use and describe the monitoring plan for aged animals (e.g., rodents >18 months of age), including frequency, behavioral and clinical signs to be monitored and criteria for euthanasia.
No

10. * Total number of animals used in this experiment:(including all the animals to be produced)
2

a. Justify total number of animals used in this experiment:

Two milliliter blood is needed for isolation of the monocytes at one time according to manufacturer's instruction (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/General_Information/1/ge-isolation-of-mononuclear-cells.pdf). It is difficult to collect this volume of blood from other sites, and blood sample is easy to get contaminated by other survival blood collection techniques. So exsanguination by this open method is more suitable for our cell line generation purpose. One rat is needed for single blood collection procedure. One more rat will be prepared for additional blood collection in case the iPSCs generation fails.

11. Number of animals by pain and distress category:(include each animal only once in the highest pain category)
B: 0
C: 0
D: 2
E: 0

a. Justify the need for any animals in pain category E:

N/A

12. * Identify husbandry exceptions:

	Exception Type	Description and Justification
View	Rats - No husbandry or enrichment exceptions.	N/A

13. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create and Edit

1. * Exception type:

Rats - No husbandry or enrichment exceptions.

2. Description and justification:

N/A

View: Custom: Create and Edit

02. Socket Preservation - Pilot Study

1. *** Experiment name:**
   02. Socket Preservation - Pilot Study
2. *** Species:**
   Rats
3. **If other was selected, provide a species:**
4. **What is the scientific goal of this experiment:**
   To evaluate the longevity of transplanted riPSCs derived pre-osteoblasts (riPOBs) in rat tooth socket, and the efficacy of immunosuppressive therapy on survival of riPOBs.
5. *** Describe the animal experience in the experiment, from enrollment in the study to the final endpoint, including all procedures in chronological order and the minimum time between procedures. We encourage using bullet points, timeline, table, or a flow chart as appropriate:**
   1. **Three-month-old SD rats will be enrolled in this study. Female rats will be selected because the size is smaller and easier to handle compared to male ones.**
   2. **Animals will be divided into 2 groups (n=3 in each group). All rats will receive left maxillary first molar extraction and bony defect creation, with defect filled by riPOBs and** Bio-oss**. The immunosuppressive therapy group will receive immunosuppressant administration, while control group will not. See procedure** Zhang: Rat Tooth Extraction and Implantation **for detailed process of surgery, and** Zhang: Administration of Cyclosporine **for details of** immunosuppressant administration**.**
   3. **A**nalgesic **and antibiotic will be administered as described in the related procedures. Immunosuppressant will be administrated in** immunosuppressive therapy group**.** See procedures Analgesia, Meloxicam (SC, 72 hours), Zhang: Administration of Ampicillin, and Zhang: Administration of Cyclosporine for details of drug administration.
   4. **Transplanted cell survival will be monitored at 2 weeks and 6 weeks post-operative via bioluminescent imaging (BLI)**. See procedure Zhang: bioluminescence imaging for details of BLI.
   5. Euthanasia will be conducted a**t 6 weeks post-operation.**

Animal Sex:
Female

Animal Ages:
3 months

Animal Size:
250-300g

6. Select experimental procedures:

Name	Type	Version	Scope
CO2 followed by Secondary Method (>10 days of age)	Euthanasia	2	Standard
Zhang: Bioluminescence Imaging	Imaging	1	Team
Body Condition Score	Other	1	Standard
Analgesia, Meloxicam (SC, 72 hours)	Substance Administration	1	Standard
Anesthesia, Ketamine and Xylazine	Substance Administration	1	Standard
Zhang: Administration of Ampicillin	Substance Administration	1	Team
Zhang: Administration of Cyclosporine	Substance Administration	1	Team
Zhang: Filling Reagents in Tooth Socket	Substance Administration	1	Team
Zhang: Rat Tooth Extraction and Implantation	Survival Surgery	1	Team

7. Monitoring protocol, including frequency and specific behavioral and clinical signs to be monitored. Include humane endpoints (criteria for euthanasia):
Animals will be monitored daily for 7 days post-surgery for body weight, signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. The surgical site will be monitored daily for 3 days post-surgery for bleeding, dislodgement of suture and any signs of infection such as redness, swelling and pus. Then, the rats will be monitored 3 times a week for appetite, body weight, signs of pain/distress and signs of infection or tumor generation in surgical site throughout to the endpoint .

Criteria for euthanasia:
1. More than 20% weight loss;
2. Body condition score of 2 or less. See Body Condition Score Procedure for detailed evaluation method.
3. Inability or reluctance to move when stimulated, or moribund condition.
4. Impairment of ability to eat, drink, or ambulate normally.

5. Labored breathing.
6. Ulcerated tumors.
7. Severe infection in surgical site.

8. If there is expected mortality (spontaneous death) in this experiment:

a. Procedure/condition associated with mortality:
N/A

b. Estimated mortality rate, i.e. percentage of animals expected to die spontaneously (not via euthanasia) or need to be euthanized as a result of the procedure. (Be sure to account for this in your animal number calculations):
N/A

c. Explain why euthanasia is not possible or appropriate:
N/A

9. Will some animals live out their natural lifespan as part of this experiment? If so, indicate their use and describe the monitoring plan for aged animals (e.g., rodents >18 months of age), including frequency, behavioral and clinical signs to be monitored and criteria for euthanasia.
N/A

10. * Total number of animals used in this experiment:(including all the animals to be produced)
6

a. Justify total number of animals used in this experiment:

This is only a pilot study, and we only want to observe the trend of cell survival, and want to include the least number of animals possible while still drawing a valid conclusion. We will use 3 rats per group, and have 2 groups (control, immunosuppressive therapy).

We based our immunosuppressive therapy and choice in group number on similar work performed by Li et al (Li K, Javed E, Scura D, Hala TJ, Seetharam S, Falnikar A, et al. Human iPS cell-derived astrocyte transplants preserve respiratory function after spinal cord injury. Experimental Neurology 2015;271:479-92), which included 3 animals in each group for each time point, and allowed them to see positive results. We will start with this sample size. If it doesn't work, we will amend the protocol to expand the sample size (based on the acquired pilot data) and/or amend the immunosuppressive therapy as needed.

11. Number of animals by pain and distress category:(include each animal only once in the highest pain category)

B: 0
C: 0
D: 6
E: 0

a. Justify the need for any animals in pain category E:
N/A

12. * Identify husbandry exceptions:

Exception Type	Description and Justification

	Exception Type	Description and Justification
View	Rats - No husbandry or enrichment exceptions.	N/A

13. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create and Edit

1. * Exception type:

Rats - No husbandry or enrichment exceptions.

2. Description and justification:

N/A

View: Custom: Create and Edit

03. Socket Preservation - Full Study

1. *** Experiment name:**
   03. Socket Preservation - Full Study
2. *** Species:**
   Rats
3. **If other was selected, provide a species:**
4. **What is the scientific goal of this experiment:**
   To determine the effects of magnesium ion, riPOBs and I50 in a socket preservation model in vivo.
5. *** Describe the animal experience in the experiment, from enrollment in the study to the final endpoint, including all procedures in chronological order and the minimum time between procedures. We encourage using bullet points, timeline, table, or a flow chart as appropriate:**

   a. **Three-month-old SD rats will be enrolled in this study. Female rats will be selected because the size is smaller and easier to handle compared to male ones.**

   b. **Animals will be divided into 9 groups (n=8 in each group). All rats will receive left maxillary first molar extraction and bony defect creation, with defect filled by different combination of Bio-oss, magnesium chloride solution, I50 and riPOBs or left untreated. Treatment design for each group is listed in the following table. See procedure** Zhang: Rat Tooth Extraction and Implantation **for detailed process of surgery.**

Table: Groups in Animal Study

Group Number	Bone Mineralized Matrix (BMM)	riPOBs	$MgCl_2$	I50
1	Applied			
2	Applied	Applied		
3	Applied		Applied	
4	Applied			Applied
5	Applied	Applied	Applied	
6	Applied	Applied		Applied

7	Applied		Applied	Applied
8	Applied	Applied	Applied	Applied
9				

3. Analgesic and antibiotic will be given as described in the related procedures. Immunosuppressant will be administrated if immune rejection is proven to be prominent and immunosuppressant is verified to be necessary in pilot study (see Expt 02). See procedures Analgesia, Meloxicam (SC, 72 hours), Zhang: Administration of Ampicillin, and Zhang: Administration of Cyclosporine for details of drug administration.

4. We will evaluate animals at 2 weeks post-operation by micro CT under anesthesia.

5. Euthanasia will be conducted at 6 weeks post-operation.

Animal Sex:
Female

Animal Ages:
3 months

Animal Size:
250-300g

6. Select experimental procedures:

Name	Type	Version	Scope
CO2 followed by Secondary Method (>10 days of age)	Euthanasia	2	Standard
Zhang: Micro CT Imaging	Imaging	1	Team
Body Condition Score	Other	1	Standard
Analgesia, Meloxicam (SC, 72 hours)	Substance Administration	1	Standard
Anesthesia, Ketamine and Xylazine	Substance Administration	1	Standard
Zhang: Administration of Ampicillin	Substance Administration	1	Team
Zhang: Administration of Cyclosporine	Substance Administration	1	Team
Zhang: Filling Reagents in Tooth Socket	Substance Administration	1	Team
Zhang: Rat Tooth Extraction and Implantation	Survival Surgery	1	Team

7. **Monitoring protocol, including frequency and specific behavioral and clinical signs to be monitored. Include humane endpoints (criteria for euthanasia):**
Animals will be monitored daily for 7 days post-surgery for body weight, signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. The surgical site will be monitored daily for 3 days post-surgery for bleeding, dislodgement of suture and any signs of infection such as redness, swelling and pus. Then, the rats will be monitored 3 times a week for appetite, body weight, signs of pain/distress and signs of infection or tumor generation in surgical site throughout to the endpoint.

Criteria for euthanasia:
1. More than 20% weight loss;
2. Body condition score of 2 or less. See Body Condition Score Procedure for detailed evaluation method.
3. Inability or reluctance to move when stimulated, or moribund condition.
4. Impairment of ability to eat, drink, or ambulate normally.
5. Labored breathing.
6. Ulcerated tumors.
7. Severe infection in surgical site.

8. **If there is expected mortality (spontaneous death) in this experiment:**
   a. Procedure/condition associated with mortality:
   N/A
   b. Estimated mortality rate, i.e. percentage of animals expected to die spontaneously (not via euthanasia) or need to be euthanized as a result of the procedure. (Be sure to account for this in your animal number calculations):
   N/A
   c. Explain why euthanasia is not possible or appropriate:
   N/A

9. **Will some animals live out their natural lifespan as part of this experiment? If so, indicate their use and describe the monitoring plan for aged animals (e.g., rodents >18 months of age), including frequency, behavioral and clinical signs to be monitored and criteria for euthanasia.**
N/A

10. *** Total number of animals used in this experiment:**(including all the animals to be produced)
72
    a. **Justify total number of animals used in this experiment:**

    We used an effect size of 0.75 which was the effect size found in the pilot study. Using ANOVA with a significance level of 0.05, there will be 90% power to detect an effect size of 0.75. This applies to the effects of each of the 3 factors (riPOBs, $MgCl_2$ and I50) being tested. Therefore we concluded that 8 animals in each group is an appropriate sample size for our study. We have 9 groups in total, so 72 is the total sample size for this study.

11. Number of animals by pain and distress category:(include each animal only once in the highest pain category)

B: 0
C: 0
D: 72
E: 0

a. Justify the need for any animals in pain category E:

N/A

12. * Identify husbandry exceptions:

	Exception Type	Description and Justification
View	Rats - No husbandry or enrichment exceptions.	N/A

13. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create and Edit

1. * Exception type:

Rats - No husbandry or enrichment exceptions.

2. Description and justification:

N/A

View: Custom: Add Vivarium Location

1. * Identify the location where animals will be used:

ARCF ABSL1

a. For locations that are lab managed, provide justification for housing outside of the vivarium:

N/A

2. * What species will be housed in this location?

Common Name	Scientific Name
Rats	Rattus

View: UW IACUC Select Room Level

1. Campus:

Vivarium

2. Vivarium:

ARCF (Animal Research & Care Facility)

3. * BSL Level:

ARCF ABSL1

View: Custom: Add Animal Use Location

1. * Identify the location where animals will be used:

ARCF ABSL1

a. For locations that are outside of the vivarium, provide justification for the use of this space:

N/A

2. * What species will be used in this location?

Common Name	Scientific Name
Rats	Rattus

3. Describe how this location will be used:

All procedures will be performed here.

A cleared place will be used for operation and a power source will be needed for portal dental drill motor.

4. * If animals are left unattended in this location, provide an explanation and include maximum duration:

The researcher maybe leave to get equipment essential for surgery. The animals won't be under unattended longer than half an hour.

5. Describe how animals will be transported to and from this location, including container and route. (Note: use of private vehicles requires IACUC approval):

The animal will be purchased through AOps/DCM, and the animals will be transported by Vendor's vehicle in cage. When transported in buildings, the animals will be in draped cages.

View: UW IACUC Select Room Level

1. Campus:

Vivarium

2. Vivarium:

ARCF (Animal Research & Care Facility)

3. * BSL Level:

ARCF ABSL1

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Research Paper

Human iPS cell-derived astrocyte transplants preserve respiratory function after spinal cord injury

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ABSTRACT

Transplantation-based replacement of lost and/or dysfunctional astrocytes is a promising therapy for spinal cord injury (SCI) that has not been extensively explored, despite the integral roles played by astrocytes in the central nervous system (CNS). Induced pluripotent stem (iPS) cells are a clinically-relevant source of pluripotent cells that both avoid ethical issues of embryonic stem cells and allow for homogeneous derivation of mature cell types in large quantities, potentially in an autologous fashion. Despite their promise, the iPS cell field is in its infancy with respect to evaluating *in vivo* graft integration and therapeutic efficacy in SCI models. Astrocytes express the major glutamate transporter, GLT1, which is responsible for the vast majority of glutamate uptake in spinal cord. Following SCI, compromised GLT1 expression/function can increase susceptibility to excitotoxicity. We therefore evaluated intraspinal transplantation of human iPS cell-derived astrocytes (hIPSAs) following cervical contusion SCI as a novel strategy for reconstituting GLT1 expression and for protecting diaphragmatic respiratory neural circuitry. Transplant-derived cells showed robust long-term survival post-injection and efficiently differentiated into astrocytes in injured spinal cord of both immunesuppressed mice and rats. However, the majority of transplant-derived astrocytes did not express high levels of GLT1, particularly at early times post-injection. To enhance their ability to modulate extracellular glutamate levels, we engineered hIPSAs with lentivirus to constitutively express GLT1. Overexpression significantly increased GLT1 protein and functional GLT1-mediated glutamate uptake levels in hIPSAs both *in vitro* and *in vivo* post-transplantation. Compared to human fibroblast control and unmodified hIPSA transplantation, GLT1-overexpressing hIPSAs reduced (1) lesion size within the injured cervical spinal cord, (2) morphological denervation by respiratory phrenic motor neurons at the diaphragm neuromuscular junction, and (3) functional diaphragm denervation as measured by recording of spontaneous EMGs and evoked compound muscle action potentials. Our findings demonstrate that hiPSA transplantation is a therapeutically-powerful approach for SCI.

1. Introduction

Transplantation of neural stem cells (NSCs) and neural progenitor cells (NPCs) is a promising therapeutic strategy for both neurodegenerative diseases of the central nervous system (CNS) and traumatic CNS injury, including spinal cord injury (SCI), because of the ability to replace lost and/or dysfunctional nervous system cell types, promote neuroprotection, deliver gene factors of interest and provide other benefits (Gage, 2000).

Initial trauma following SCI results in immediate cell death and axotomy of passing fibers. Contusion- and compression-type injuries, the predominant forms of traumatic SCI observed in the clinical population, are followed by an extended period of secondary cell death and consequent exacerbation of functional deficits (McDonald and Becker,

Abbreviations: SCI, spinal cord injury; iPS cells, induced pluripotent stem cells; hIPSAs, human induced pluripotent stem cell-derived astrocytes; GLT1, glutamate transporter 1; PhMN, phrenic motor neuron; C3 (4 5, *etc.*), cervical spinal cord level 3 (4, 5, *etc.*); GRP, glial-restricted precursor; CMAP, compound muscle action potential; NMJ, neuromuscular junction; GFP–hIPSA, lentivirus-GFP transduced hIPSA; GLT1–hIPSA, lentivirus-GLT1 transduced hIPSA; GFP–hFibro, lentivirus-GFP transduced human fibroblast; LV-GFP, lentivirus-GFP; LV-GLT1, lentivirus-GLT1.

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0014-4886/

2003). One of the major causes of secondary degeneration following SCI is excitotoxic cell death due to dysregulation of extracellular glutamate homeostasis (Park et al., 2004; Stys, 2004). Exogenous parenchymal administration of glutamate to uninjured spinal cord results in tissue and function loss similar to SCI (Xu et al., 2005). While large increases in glutamate can occur shortly after SCI, elevation can also persist depending on injury severity (Liu et al., 1991; Panter et al., 1990; Xu et al., 2004). In addition to focal increases, levels can also rise in regions removed from the lesion site, possibly *via* a spreading mechanism involving activated glia (Hulsebosch, 2008). Early gray matter loss is likely mediated by NMDA receptors, while delayed loss of neurons and oligodendrocytes, as well as axonal and myelin injury, is thought to be predominantly mediated *via* AMPA over-activation (Stys, 2004). A valuable opportunity therefore exists after SCI for preventing cell injury and functional loss that occur during secondary degeneration. Importantly, secondary degeneration is a relevant therapeutic target given its relatively prolonged time window.

Glutamate is efficiently cleared from the synapse and other sites by transporters located on the plasma membrane (Maragakis and Rothstein, 2004). Astrocytes are supportive glial cells that play a host of crucial roles in CNS function (Pekny and Nilsson, 2005). Astrocytes express the major CNS glutamate transporter, GLT1, which is responsible for the vast majority of functional glutamate uptake and plays a central role in regulation of extracellular glutamate homeostasis in the spinal cord (Maragakis and Rothstein, 2006). Following SCI, astrocyte loss and/or altered GLT1 expression, function and localization can result in further susceptibility to excitotoxicity. For example, we previously found that in rodent models of unilateral mid-cervical (C4) contusion SCI, numbers of GLT1-expressing astrocytes, total intraspinal GLT1 protein expression and GLT1-mediated functional glutamate uptake in ventral horn are reduced soon after injury and this reduction persists chronically (Li et al., 2015). Astrocytes have traditionally been viewed in a negative light following CNS trauma because of their association with disease mechanisms such as glial scarring and pro-inflammatory cytokine release. However, their crucial neuroprotective/homeostatic roles, including GLT1-mediated glutamate uptake, have not been extensively targeted in SCI models using approaches such as NSC and NPC transplantation, despite obvious therapeutic implications (Maragakis and Rothstein, 2006).

Transplantation-based targeting of astrocytes provides a number of key benefits. Grafts can be anatomically delivered to precise locations for achieving neuroprotection of specific populations of cells (Lepore et al., 2008b). Alternative strategies such as gene therapy only target one/several specific genes (s), while astrocyte transplantation can participate in the restoration of a host of astrocyte functions. Transplantation also provides for long-term astrocyte integration and therapeutic replacement. For example, the lasting nature of dysregulation of extracellular glutamate homeostasis after SCI (Lepore et al., 2011a,2011c) calls for longer-term maintenance of therapeutic effects, both with respect to early cell loss occurring during secondary degeneration and outcomes of SCI associated with more persistent pathophysiology of glutamate signaling such as chronic neuropathic pain (Gwak et al., 2012; Hulsebosch, 2008).

To achieve translation of NSC/NPC-based interventions, clinically-relevant cell sources that address scientific, practical and ethical considerations must be extensively tested in relevant models of CNS disease. These cell types also need to be evaluated in the context of patient-relevant functional outcomes such as respiratory function. Induced pluripotent stem (iPS) cells are pluripotent cells generated from adult somatic cell types *via* expression of combinations of pluripotency-related factors, avoiding ethical issues of embryonic stem cells (Takahashi et al., 2007b). This technology allows for homogeneous derivation of cell types in large quantities for applications such as transplantation, potentially in an autologous fashion from the eventual recipient or from allogeneic sources (Das and Pal, 2010; Kiskinis and Eggan, 2010). Despite the promise of this approach, the iPS cell transplantation field is still in the early stages of evaluating therapeutic usefulness in relevant SCI models (Salewski et al., 2010).

Respiratory compromise is a major problem following cervical spinal cord trauma. Cervical SCI represents greater than half of all human cases, in addition to often resulting in the most severe physical and psychological debilitation (Lane et al., 2008). Respiratory compromise is the leading cause of morbidity and mortality following SCI. While a growing literature exists on respiratory function in animal models of SCI (Lane et al., 2008, 2009), few studies have examined cellular mechanisms involved in protection of this vital neural circuitry, and little work has been conducted to test therapies for targeting cervical spinal cord-related functional outcome measures such as breathing. Phrenic motor neuron (PhMN) loss plays a central role in respiratory compromise following cervical SCI. The diaphragm, a major inspiratory muscle, is innervated by PhMNs located at cervical levels 3–5 (Lane et al., 2009). PhMN output is driven by descending pre-motor bulbospinal neurons in the medullary rostral ventral respiratory group (rVRG) (Zimmer et al., 2007). Cervical SCI results in diaphragmatic respiratory compromise due to PhMN loss and/or injury to descending bulbospinal respiratory axons. The majority of these injuries affect mid-cervical levels (Shanmuganathan et al., 2008) (the location of the PhMN pool), and respiratory function following mid-cervical SCI is significantly determined by PhMN loss/sparing (Strakowski et al., 2007). Although use of thoracic models has predominated, cervical SCI animal models have recently been developed (Aguilar and Steward, 2010; Awad et al., 2013; Gensel et al., 2006; Lane et al., 2012; Lee et al., 2010; Sandrow-Feinberg et al., 2009, 2010; Sandrow et al., 2008; Stamegna et al., 2011), including our own (Nicaise et al., 2012). Because of the relevance of astrocyte and GLT1 dysfunction to PhMN loss/injury following cervical trauma, we targeted transplantation in the present study to cervical spinal cord ventral horn in a cervical contusion SCI model.

We previously investigated the therapeutic efficacy of transplanting rodent-derived glial-restricted precursors (GRP), a class of lineage-restricted astrocyte progenitor cell (Li et al., 2014). We transplanted either undifferentiated GRPs or GRP-derived astrocytes (pre-differentiated *in vitro* prior to injection) into our model of cervical contusion SCI, and found that both cell types survived, localized to the ventral horn and efficiently differentiated into mature astrocytes. However, animals injected with GRP-derived astrocytes had higher levels of intraspinal GLT1 expression than those injected with undifferentiated GRPs, suggesting that pre-differentiation enhanced the *in vivo* maturation of these cells. We also observed that modifying GRP-derived astrocytes to constitutively express GLT1 was more effective in achieving *in vivo* GLT1 expression and for protecting PhMNs.

Given the importance of astrocytes in SCI pathogenesis, the observations of GLT1 dysfunction following SCI, and our previous success targeting astrocyte GLT1 using rodent-derived glial progenitor cells, in the present study we evaluated intraspinal transplantation of hiPS cell-derived astrocytes (hIPSAs) into ventral horn following cervical contusion SCI as a novel therapeutic strategy for reconstituting GLT1 function. Specifically, we examined the *in vivo* fate of hIPSAs transplants in the injured spinal cord of both mouse and rat models of cervical contusion SCI, including long-term survival and integration, astrocyte differentiation, maturation into GLT1-expressing cells and safety. We also tested the therapeutic efficacy of hIPSA transplantation for protection of PhMNs and preservation of diaphragm function.

Derivation of cell types from iPS cells represents a relevant approach for clinical translation; therefore, it is critical to test both the safety and efficacy of these transplants in a patient-relevant SCI model. Importantly, previous work has shown that human- and rodent-derived versions of a given stem/progenitor type do not necessarily show similar *in vivo* fate or therapeutic properties in the disease nervous system. For example, we previously demonstrated that, following transplantation into the $SOD1^{G93A}$ rodent model of ALS, human glial progenitors cells show more persistent proliferation, greater migratory capacity, reduced efficiency of astrocyte differentiation, and decreased GLT1 expression

compared to their rodent counterparts, which resulted in a lack of therapeutic efficacy only with the human cells (Lepore et al., 2008b, 2011b). It is therefore important to extend our previous studies with rodent-derived glial progenitors in the cervical contusion SCI model to now test human iPS cells.

2. Materials and methods

2.1. Animals

2.1.1. Transplantation into rats and mice

Female Sprague–Dawley rats weighing 250–300 g were purchased from Taconic Farm (Rockville, MD). Female C57BL/6 wild-type mice weighing 20–30 g were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in a humidity-, temperature-, and light-controlled animal facility with *ad libitum* access to water and food. Experimental procedures were approved by the Thomas Jefferson University IACUC and conducted in compliance with ARRIVE (*Animal Research: Reporting of In Vivo Experiments*) guidelines.

2.2. Cervical contusion SCI

2.2.1. Rat SCI

Rats were anesthetized with ketamine (100 mg/kg), xylazine (5 mg/kg) and acepromazine (2 mg/kg). The cervical dorsal skin and underlying muscles were incised. The paravertebral muscles overlying C3–C5 were removed. Following unilateral laminectomy on the right side at C3, C4 and C5 levels, rats were subjected to a C4 spinal contusion injury with the Infinite Horizon impactor (Precision Systems and Instrumentation, Lexington, KY) using a 1.5 mm tip at a force of 395 kdyn. This injury paradigm is based on our previously published rat model that results in robust PhMN degeneration and chronic diaphragm dysfunction (Nicaise et al., 2012, 2013). Rats were transplanted in all studies immediately following injury. After surgical procedures, overlying muscles were closed in layers with sterile 4–0 silk sutures, and the skin incision was closed using wound clips. Animals were allowed to recover on a circulating warm water heating pad until awake and then returned to their home cages. They were monitored daily until sacrifice, and measures were taken to avoid dehydration and to minimize any pain or discomfort.

2.2.2. Mouse SCI

Mice were anesthetized with a cocktail of ketamine (120 mg/kg) and xylazine (5 mg/kg). The surgical procedure and post-surgical monitoring used for mice were the same as described above for rats. For the contusion injury, the 1 mm impactor tip was raised 1.25 mm above the dura prior to impact, and a force of 50 kdyn (kdyn) was used for impact.

2.3. Virus production

Lentiviral vector carrying the green fluorescent protein (GFP) gene or GLT1 gene was packaged in 293FT cells. Briefly, to produce control lentiviral-GFP vector, 293FT cells were transfected with pCDH-MSCV-MCS-EF1-GFP plasmid (System Biosciences, Mountain View, CA) and three other helper plasmids, pLP-1, pLP-2, and pLP/VSVG with Polyfect (Qiagen, Valencia, CA). To produce lentiviral-GLT1 vector, GLT1 gene CDS fragment was inserted into MCS of pCDH-MSCV-MCS-EF1-GFP plasmid, and the vector plasmid was then transfected into 293FT cells with three helper plasmids as described above. Supernatant was collected 72 h later, and lentiviral vector was concentrated with PEG-it Virus Precipitation Solution (System Biosciences, Mountain View, CA) and re-suspended with PBS to the final titer of 1×10^8 infectious units/ml.

2.4. Human induced pluripotent stem cell derived astrocytes

2.4.1. Human iPS cell derivation, culturing and astrocyte differentiation

iPS cells were derived from non-diseased healthy patient donors. Dermal fibroblasts were reprogrammed into iPS cells *via* retroviral transduction with KLF4, SOX2, OCT4, and c-MYC (Takahashi et al., 2007a). By immunohistochemistry and qRT-PCR, these putative iPS cells expressed proteins and transcripts associated with pluripotency, including Sox 2, and stem cell-associated antigens, including SSEA4, Nanog, alkaline phosphatase, and TRA 1–81, and capacity to differentiate into cells of three germ layers was established. Finally, the karyotype of these iPS cells was found to be normal. Once pluripotent iPS cells were generated, the stem cells were cultured in E8 medium (Life Technologies, Grand Island, NY). To maintain optimum pluripotency and limit spontaneous differentiation, the stem cell colonies were manually cleaned once every 6 days just before passage using dispase (Stem Cell Technologies, Vancouver, BC). To differentiate the iPS cells into astrocytes, a protocol previously described by Haidet-Phillips and colleagues (Haidet-Phillips et al., 2014) was used. To summarize, iPS cells were lifted with dispase, gently separated into single cells and plated as a monolayer. Using the smad dual inhibition pathway method to direct differentiation toward a neural phenotype, the cells were incubated in DMEM/F12 (Life Technologies, Grand Island, NY) enriched with 0.2 μM LDN (Stemgent, Cambridge, MA) and 10 μM SB431542 (Sigma, Saint Louis, MO). The cells were then exposed to 1 μM retinoic acid (Sigma, Saint Louis MO) and N2 (Life Technologies, Grand Island, NY) starting at day 5 and Sonic HedgeHog (Life Technologies, Grand Island, NY) starting at day 8. From day 15 to day 30 after starting the differentiation protocol, the medium was gradually changed to neurobasal medium. After day 30, to differentiate these iPS cell-derived glial progenitors into astrocytes, cells were maintained and expanded in DMEM/F12 supplemented with 1% Fetal Bovine Serum, B27, L-glutamine, non-essential amino acids, penicillin/streptomycin (all from Life Technologies, Grand Island, NY) and 2 μg/ml Heparin (Sigma-Aldrich, St. Louis, MO) for an additional 60 days. Astrocytes derived from human iPS were identified with immunostaining using GFAP antibody. For feeding and passaging of astrocyte progenitor cultures, cells were rinsed with PBS and incubated with 4 ml of 0.05% trypsin for 5 min. Cells were collected in trypsin and rinsed with 7 ml of culture medium and 1× trypsin inhibitor (Life Technologies, Grand Island, NY) to stop trypsinization. Cells were centrifuged at 1000 rpm for 5 min and re-suspended in fresh culture medium. Cells were counted and seeded onto poly-L-lysine coated dishes. Cells were fed twice a week and were passaged after they were 80%–90% confluent.

2.4.2. GLT1 overexpression

After differentiation for 90 days, hIPSAs (astrocytes derived from human iPS cells) were transduced with lentiviral-GFP vector or lentiviral-GLT1 vector, at the concentration of 1×10^6 infectious units/ml, one week before transplantation. On the second day of transduction, culture medium was changed and the cells were cultured for 5 more days.

2.5. Human dermal fibroblasts

Human dermal fibroblast cells (ATCC, Manassas, VA) were cultured with Fibroblast Growth Kit-low serum (ATCC, Manassas, VA). Fibroblasts were transduced with control lentiviral-GFP vector one week before transplantation. Transduced GFP was used to track transplanted cells *in vivo*.

2.6. Transplantation

2.6.1. Cell preparation for transplantation

On the day of transplantation, cells were rinsed with PBS and trypsinized with 0.05% trypsin, collected and rinsed with culture

medium and 1× trypsin inhibitor. The cells were washed with artificial cerebrospinal fluid twice. Cell viability was assessed using the trypan blue assay and was always found to be greater than 80%. The final cell concentration was adjusted to 1×10^8 cells/ml.

2.6.2. Intraspinal transplantation

Transplantation was conducted on deeply anesthetized rats and mice immediately post-injury. Following unilateral right-sided contusion injury at C4, cells were injected into the spinal cord at two locations. Each site contained 2 μl of cell suspension, which was administered into the spinal cord ventral horn using a Hamilton gas-tight syringe mounted on an electronic UMP3 micropump (World Precision International, Sarasota, FL) (Lepore and Maragakis, 2011; Lepore et al., 2011a). The sites of injections were located at the rostral and caudal edges of the contusion site. Ventral horns were targeted by lowering the 33-gauge 45-degree beveled needle 1.5 mm below the dorsal surface of the spinal cord. Each injection was delivered at a constant rate over 5 min. Upon completion of cell delivery, overlying muscles were then closed in layers with sterile 4–0 silk sutures, and the skin incision was closed using sterile wound clips. Animals were allowed to recover and monitored daily.

2.6.3. Immune suppression

All animals were immune suppressed. Rats received subcutaneous administration of cyclosporine A (10 mg/kg; Sandoz Pharmaceuticals, East Hanover, NJ) daily beginning three days before grafting and continuously until sacrifice. Mice were given both FK-506 and rapamycin (1 mg/kg each; LC Laboratories; Woburn, MA).

2.7. Tissue processing for histology

At the time of sacrifice, animals were anesthetized, and diaphragm muscle was freshly removed prior to perfusion and then further processed for neuromuscular junction (NMJ) labeling. Animals were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde infusion. Spinal cords were harvested, then cryoprotected in 30% sucrose for 3 days and embedded in freezing medium. Spinal cord tissue blocks were cut serially in the sagittal or transverse planes at a thickness of 30 μm. Sections were collected on glass slides and stored at −20 °C until analysis. Spinal cord sections were thawed, allowed to dry for 1 h at room temperature, and stained with 0.5% Cresyl violet acetate according to standard procedure (Nicaise et al., 2012).

2.8. Immunohistochemistry

Frozen spinal cord sections were air-dried, washed with PBS, permeabilized with 0.4% Triton X-100 in PBS for 5 min at room temperature, and then incubated in blocking solution (PBS containing 10% normal goat serum and 0.4% Triton X-100) for 1 h at room temperature. Sections were labeled overnight at 4 °C with the primary antibodies in blocking solution. Sections were then washed three times with PBS (5 min per wash) and incubated with secondary antibodies in blocking solution for 1 h at room temperature. After washing twice with PBS (10 min per wash), sections were cover-slipped. A number of primary antibodies were used. Mouse anti-GFAP antibody (EMD Millipore Corporation, Billerica, MA; 1:200) and rabbit anti-GFAP antibody (Dako North America, Carpinteria, CA; 1:200) were used to label astrocytes (Lepore et al., 2008a). Mouse anti-human GFAP antibody (StemCells, Inc, Newark, CA; 1:200) was used to label astrocytes of human origin in mice and rats. Rabbit anti-GLT1 (1:800) and mouse anti-GLT1 (1:200) were used to label GLT1 protein (both were provided by Jeffrey Rothstein's laboratory) (Lepore et al., 2008b). Rabbit anti-Ki67 (Thermo Fisher Scientific, Rockford, IL; 1:200) labeled proliferating cells (Lepore et al., 2008a). Mouse anti-human cytoplasmic marker antibody (StemCells, Inc, Newark, CA; 1:200) and mouse anti-HuNu antibody (EMD Millipore Corporation, Billerica, MA; 1:200) were used to label human cytoplasm and human nuclear antigen, respectively, for selectively identifying human-derived cells. Secondary antibodies included: FITC goat-anti-mouse IgG, FITC goat-anti-rabbit IgG, TRITC goat-anti-mouse IgG, TRITC goat-anti-rabbit IgG, Alexa Fluor 647 goat-anti-mouse IgG, Alexa Fluor 647 goat-anti-rabbit IgG. All secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were diluted at 1:200 to recognize the matched primary antibody. For fluorescence analysis, sections were cover-slipped with fluorescent-compatible mounting medium (ProLong Gold, Life Technologies, Grand Island, NY).

2.9. Quantification of in vitro cultured cell differentiation, proliferation and GLT1 expression

The proportions of $GFAP^+$ astrocytes and $Ki67^+$ proliferating cells were expressed as a percentage of the total number of cultured cells (labeled by DAPI). In order to quantify double-labeling of DAPI with GFAP or Ki67, images were taken at 10× magnification and analyzed using ImageJ software. In each image, cells with a $DAPI^+$ nucleus were assessed for expression of GFAP or Ki67.

2.10. Quantification of transplant differentiation

Rats and mice were sacrificed for quantification of astrocyte differentiation ($GFAP^+$) and proliferation ($Ki67^+$). The proportions $GFAP^+$ astrocytes and $Ki67^+$ proliferating cells were expressed as a percentage of the total number of transplanted human cells (labeled by anti-hCytoplasm or HuNu antibody). In order to quantify double-labeling of hCytoplasm or HuNA with GFAP and Ki67, double-labeled transverse sections were imaged at 10× magnification using MetaMorph software and were then analyzed using ImageJ software. In each image, cells expressing hCytoplasm or HuNu were assessed for co-expression of GFAP or Ki67.

2.11. Quantification of GLT1 expression by transplants

Rats and mice were sacrificed for quantification of GLT1 expression by hCyto-labeled cells in the ventral horn. $GLT1^+$ and $hCyto^+$ cells were identified in the ventral horn using ImageJ software, and the percentage of $hCyto^+$ cells (representing any transplant-derived cell) that co-expressed GLT1 were quantified.

2.12. Lesion imaging and quantification

Images were acquired with a Zeiss Imager M2 upright microscope and analyzed with ImageJ software. Lesion size was quantified in Cresyl violet stained sections (Li et al., 2015). Specifically, lesion area was determined in every 10th section by tracing both the total area of the hemi-spinal cord ipsilateral to the contusion site and the actual lesion area. Lesion was defined as areas including both lost tissue (cystic cavity formation) and surrounding damaged tissue in which the normal anatomical structure of the spinal cord was lost. The lesion epicenter was defined as the section with the largest percent lesioned tissue (relative to total tissue area in the same section).

2.13. Neuromuscular junction (NMJ) analysis

Fresh hemi-diaphragm muscle was dissected from each animal for whole-mount immunohistochemistry, as described previously (Wright et al., 2007). Hemi-diaphragm muscle was dissected, stretched, pinned down to Sylgard medium (Fisher Scientific, Pittsburgh, PA), and extensively cleaned to remove any connective tissue to allow for antibody penetration. Motor axons and their terminals were labeled with SMI-312R (Covance, Princeton, NJ; 1:1000) and

SV2-s (DSHB, Iowa City, IA; 1:10), respectively, and both primary antibodies were detected with FITC anti-mouse IgG secondary (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:100). Post-synaptic acetylcholine receptors were labeled with rhodamine-conjugated alpha-bungarotoxin (Life Technologies, Grand Island, NY; 1:400). Labeled muscles were analyzed for total numbers of NMJs and intact, denervated and multiply-innervated NMJs. Whole-mounted diaphragms were imaged on a FluoView FV1000 confocal microscope (Olympus, Center Valley, PA). We only conducted NMJ analysis in ipsilateral hemi-diaphragm because in our previously published work we did not observe denervation or sprouting in contralateral hemi-diaphragm after cervical hemi-contusion SCI (Nicaise et al., 2012).

2.14. Functional glutamate uptake assay

After transduction with lentiviral-GFP vector or lentiviral-GLT1 vector, hIPSAs were cultured for 10 days. Human fibroblasts transduced with lentiviral-GFP vector were used as control. Glutamate uptake activity was measured as previously described (Dowd and Robinson, 1996), with slight modification. Briefly, cells were washed and pre-incubated with either a sodium- or choline-containing uptake buffer (in mM: Tris, 5; HEPES, 10; NaCl or choline chloride, 140; KCl, 2.5; CaCl2, 1.2; $MgCl_2$, 1.2; $K2HPO_4$, 1.2; glucose, 10) for 20 min at 37 °C; and in DHK treatment groups, 100 μM of DHK was added to inhibit GLT1. The uptake buffer was then replaced with fresh uptake buffer containing 20 nM ^{3}H-glutamate (49 Ci/mmol; PerkinElmer, CA) and 20 μM unlabeled glutamate. The cells were incubated for 5 min at 37 °C. The reaction was terminated by washing cells three times with choline-containing uptake buffer containing 2 mM unlabeled glutamate, followed by immediate lysis in ice-cold 0.1 N NaOH. Cell extracts were then measured with a liquid scintillation counter (Beckman Instruments, Fullerton, CA). The protein content in each well was measured using the Bradford protein assay (Bio-Rad, Hercules, CA).

2.15. Diaphragm compound muscle action potentials (CMAPs)

Rats were anesthetized in the same manner described above. Phrenic nerve conduction studies were performed with single stimulation (0.5 ms duration; 6 mV amplitude) at the neck *via* near nerve needle electrodes placed along the phrenic nerve (Li et al., 2015; Nicaise et al., 2012). The ground needle electrode was placed in the tail, and the reference electrode was placed subcutaneously in the right abdominal region. Recording was obtained *via* a surface strip along the costal margin of the diaphragm, and CMAP amplitude was measured baseline to peak. Recordings were made using an ADI Powerlab 8/30 stimulator and BioAMP amplifier (ADInstruments, Colorado Springs, CO), followed by computer-assisted data analysis (Scope 3.5.6, ADInstruments). For each animal, 10–20 tracings were averaged to ensure reproducibility.

2.16 . Spontaneous EMG recordings

Prior to being euthanized, animals received a laparotomy. These EMG recordings were terminal experiments and were only conducted immediately prior to euthanasia. Bipolar electrodes spaced by 3 mm were inserted into specific sub-regions of the right hemi-diaphragm (*i.e.* dorsal, medial or ventral regions) (Li et al., 2015). Activity was recorded and averaged during spontaneous breathing at each of these 3 locations separately in each animal. The EMG signal was amplified, filtered through a band-pass filter (50–3000 Hz), and integrated using LabChart 7 software (ADInstruments). Parameters such as inspiratory bursts per minute, discharge duration and integrated peak amplitude were averaged over 2 min sample periods. No attempt was made to control or monitor the overall level of respiratory motor drive during the EMG recordings.

2.17. Statistics

Results were expressed as means ± standard error of the mean (SEM). A Kolmogorov–Smirnov test was conducted for all variables to assess normality. Unpaired *t* test or Mann–Whitney was used to assess statistical significance between two groups. With respect to multiple comparisons involving three groups or more, statistical significance was assessed by analysis of variance (one-way ANOVA) followed by *post-hoc* test (Bonferroni's method). Statistics were computed with Graphpad Prism 5 (GraphPad Software, Inc., La Jolla, CA). $p < 0.05$ was considered as statistically significant.

3. Results

3.1. In vitro characterization of human iPS cell-derived astrocytes (hIPSAs)

We differentiated human iPS cells into astrocytes by culturing them in differentiating medium containing FBS. We transduced cells with lentivirus (LV)-GFP or LV-GLT1-GFP to generate control cells (GFP–hIPSAs) and GLT1-overexpressing hIPSAs (GLT1–hIPSAs), respectively. The GFP–hIPSAs expressed little-to-no GLT1 protein (Fig. 1A, C), consistent with the limited expression of GLT1 by cultured astrocytes in the absence of neuronal co-culture (Li et al., 2014; Perego et al., 2000), while GLT1–hIPSAs expressed high levels of GLT1 protein *in vitro* (Fig. 1B, C). In addition, the vast majority of DAPI$^+$ GLT1–hIPSAs expressed GLT1 (Fig. 1B), which is expected given the high efficiency of transduction with our lentivirus (not shown). GLT1 overexpression did not alter hiPSA differentiation (Fig. 1D, E, H) or proliferation (Fig. 1F–H). In addition to significantly increased GLT1 protein expression levels, GLT1–hIPSAs showed a large increase in functional GLT1-mediated glutamate uptake compared to GFP–hIPSAs using an *in vitro* ^{3}H-glutamate uptake assay (Fig. 1J). In this ^{3}H-glutamate uptake assay and in the subsequent transplantation experiments, we used LV-GFP transduced human fibroblasts (GFP–hFibro) (Fig. 1I) as a non-glial cell control.

3.2. Human iPSA transplants robustly survived and differentiated into astrocytes following rat cervical contusion SCI

We characterized the fate of transplanted hIPSAs in both rats and mice following unilateral C4 contusion SCI, given the usefulness of both experimental models for studying nervous system diseases. Immediately following injury, we injected hIPSAs directly into the ventral horn at locations just rostral and caudal to the contusion site (Fig. 2A). We specifically delivered cells into the ventral horn to anatomically target the location of the PhMN pool (Fig. 2B).

We sacrificed rats at 2 days, 2 weeks and 4 weeks post-injury/transplantation. Double-labeling with panGFAP antibody and a human-specific GFAP antibody demonstrated that transplanted human-derived cells differentiated into astrocytes (Fig. 2C). Both transplanted GFP–hIPSAs (Fig. 2D, F, H) and GLT1–hIPSAs (Fig. 2E, G, I) robustly survived out to W4, and nearly all hCytoplasm$^+$ transplant-derived cells co-labeled with the astrocyte lineage marker, GFAP, at D2 (Fig. 2D–E), W2 (Fig. F–G) and W4 (Fig. 2H–I). There were no differences in the degree of astrocyte differentiation between GFP–hIPSAs and GLT1–hIPSAs at any of these time points (quantification shown in Fig. 2J). LV-GFP transduced human fibroblasts (GFP–hFibro) also survived in the injured spinal cord to at least W4 post-injury (Fig. 2K).

Despite efficient astrocyte differentiation, only a small percentage of GFP–hIPSA transplant-derived cells expressed GLT1 protein in the injury site at D2 (Fig. 3A), W2 (Fig. 3C) and W4 (Fig. 3E). On the contrary, the majority of GLT1–hIPSAs robustly expressed GLT1 at all times (Fig. 3B, D, and F) (quantification: Fig. 3G).

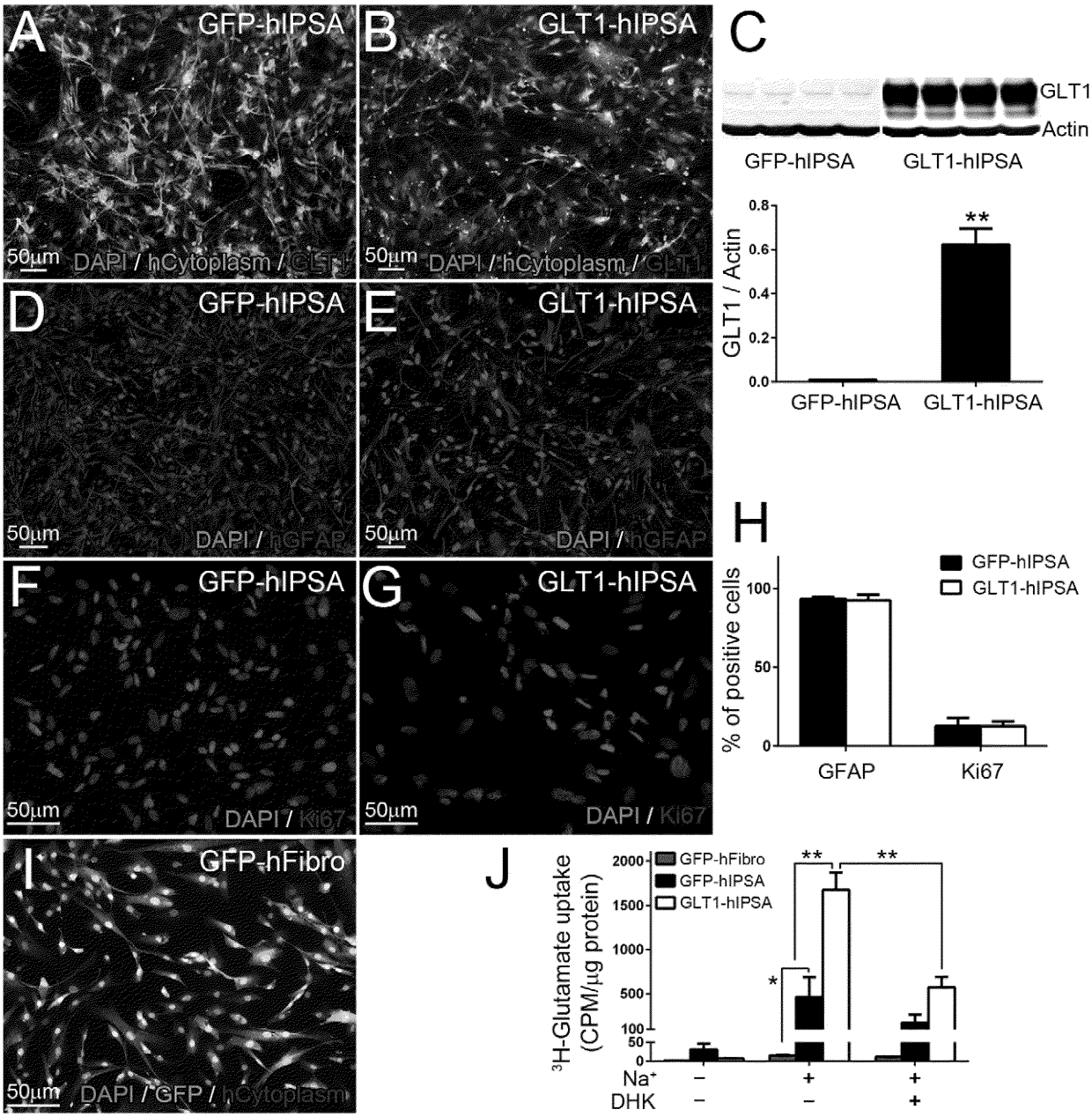

Fig. 1. *In vitro* characterization of human iPS cell-derived astrocytes (hIPSAs). Cells were transduced with lentivirus (LV)-GFP or LV-GLT1–GFP to generate control GFP–hIPSAs and GLT1-overexpressing hIPSAs (GLT1–hIPSAs), respectively. Human cytoplasm$^+$ GFP–hIPSAs expressed little-to-no GLT1 protein (A), while GLT1–hIPSAs expressed high levels of GLT1 protein *in vitro* (B), which was further confirmed with immunoblotting analysis (C, *lower:* quantification result). Following infection with either virus, astrocyte differentiation was determined by the percentage of cells expressing the astrocyte lineage marker, GFAP (D–E). Proliferation was determined by the percentage of cells expressing the proliferation marker, Ki67 (F–G). Quantification results of cell differentiation and proliferation are shown in (H). Human fibroblasts, which were transduced with LV-GFP vector (GFP–hFibro) (I), were used as non-glial control in the glutamate uptake assay and *in vivo* transplantation experiments. ^{3}H-glutamate uptake assay was performed to detect GLT1 function. GLT1–hIPSAs showed a large increase in Na^+ dependent glutamate uptake compared to GFP–hFibro and GFP–hIPSAs. This increased uptake was blocked with GLT1 specific inhibitor, DHK, at the concentration of 100 μmol/l (J). Results were expressed as means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$. n = 4 per group for GLT1 western blotting quantification analysis; n = 4 per group for cell differentiation and proliferation analysis; n = 4 per group for ^{3}H-glutamate uptake assay.

3.3 . Human iPSA transplants showed limited proliferation in vivo and did not form tumors

A major concern regarding NSC/NPC therapy (particularly with pluripotent cells such as iPS cells) is the potential for uncontrolled proliferation and even tumor formation. To address this concern, we immunostained for the proliferation marker, Ki67, and we examined transplant recipient rat spinal cords for overt tumor formation. With both GFP–hIPSAs (Fig. 4A, C, E) and GLT1–hIPSAs (Fig. 4B, D, F), less than 10% of HuNu$^+$ transplant-derived cells expressed Ki67 at D2 (Fig. 4A–B), W2 (Fig. 4C–D) and W4 (Fig. 4E–F) (quantification shown in Fig. 4G). In addition, we never observed tumor formation in any transplant-recipient animals.

3.4. Human iPSA transplants showed similar survival and differentiation in the injured mouse cervical spinal cord

Given the usefulness of the mouse model due to the availability of transgenic tools, we conducted similar characterization of hIPSA fate following transplantation into the mouse spinal cord immediately

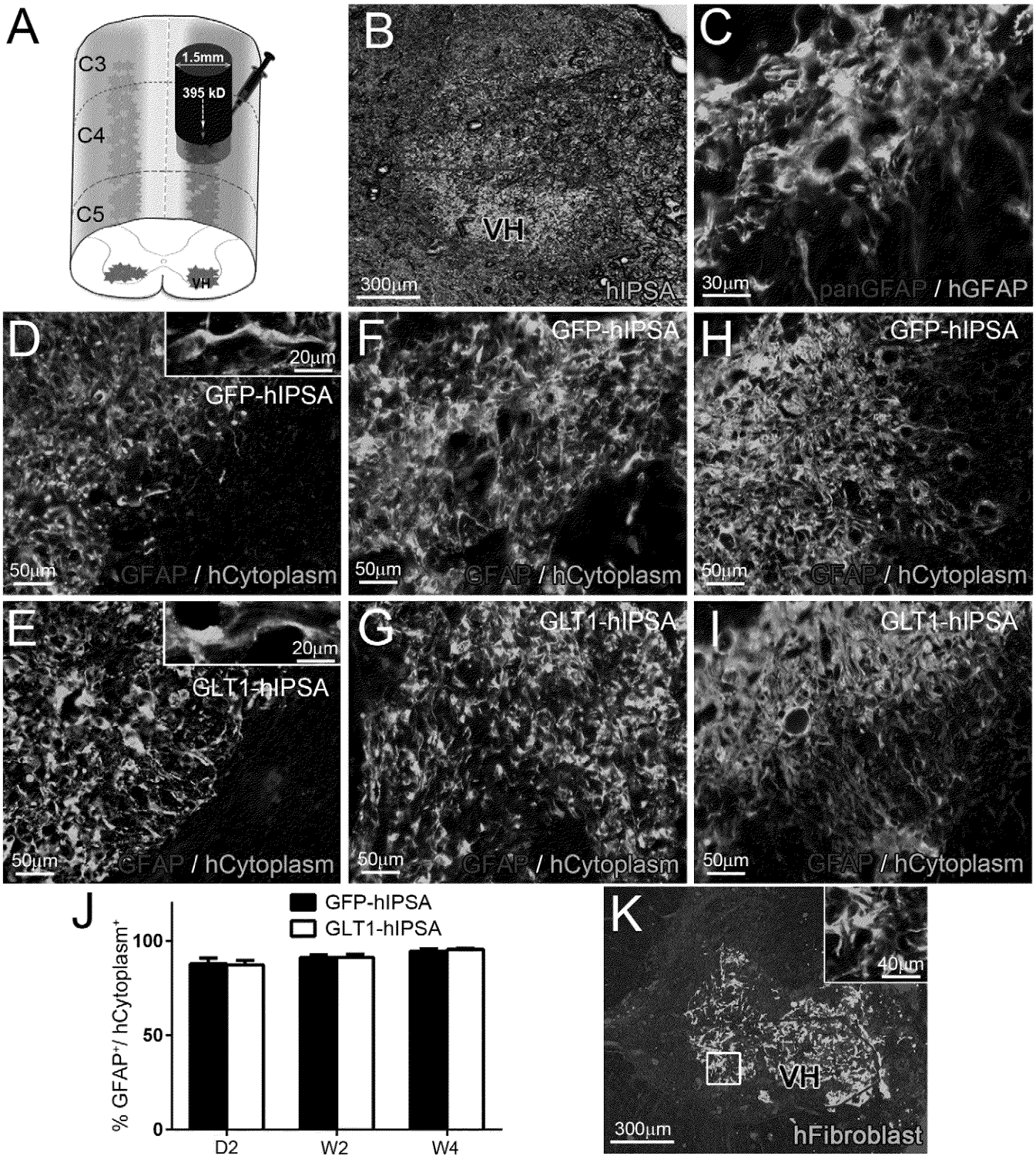

Fig. 2. Human iPSA transplants robustly survived, differentiated into astrocytes and localized to the ventral horn following rat cervical contusion SCI. Immediately following unilateral C4 contusion SCI, we injected GFP–hIPSAs, GLT1–hIPSAs or GFP–hFibro directly into the ventral horn (VH) at locations just rostral and caudal to the contusion site (A). GFP fluorescence indicated that the transplanted hIPSAs were delivered to the ventral horn (B). Double-labeling with pan-GFAP antibody and a human GFAP specific antibody confirmed that all human $GFAP^+$ cells were also pan-$GFAP^+$ (C). Double immunostaining for pan-GFAP and human cytoplasm marker was performed on spinal cord sections from the GFP–hIPSA (D, F, H) and GLT1–hIPSA (E, G, I) groups at day 2 (D–E), week 2 (F–G) and week 4 (H–I) post-injury/transplantation to quantify astrocyte differentiation by transplanted cells (J). We used LV-GFP transduced human fibroblasts (GFP–hFibro) as a non-glial cell control (K, *inset*: high magnification). Results were expressed as means ± SEM. n = 3 per group per time point for transplanted cell differentiation analysis. Red outlines in panels B and K denote the ventral horn.

following unilateral cervical contusion SCI. Similar to transplantation into the rat SCI model, hIPSAs robustly survived and integrated for at least 4 weeks post-injection. The majority of transplant-derived cells were differentiated $GFAP^+$ astrocytes (Fig. 4H). Control GFP–hIPSAs expressed little GLT1, while overexpression resulted in the majority of transplant-derived astrocytes expressing GLT1 (Fig. 4I). Less than 10%

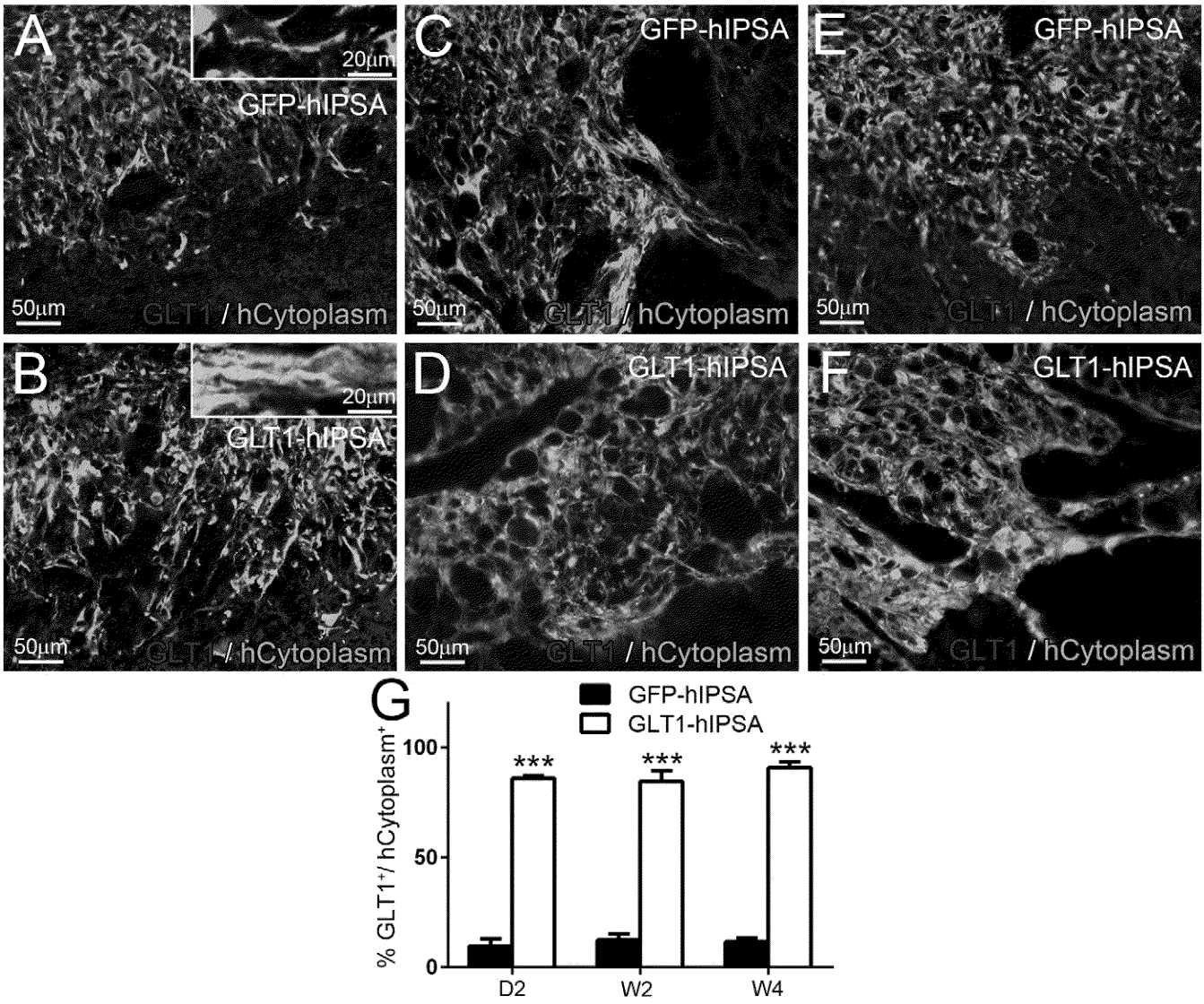

Fig. 3. GLT1–hIPSA transplants expresses GLT1 in the ventral horn following rat cervical contusion SCI. Double immunostaining for GLT1 and human cytoplasm was performed on spinal cord sections from the GFP–hIPSA (A, C, E) and GLT1–hIPSA (B, D, F) groups at day 2 (A–B), week 2 (C–D) and week 4 (E–F) post-injury/transplantation to assess GLT1 expression by transplanted cells *in vivo* (G). Results were expressed as means ± SEM. ***$p < 0.001$. n = 3 per group per time point for *in vivo* GLT1 expression analysis.

of transplant-derived cells continued to proliferate at D2, W2 and W4 (Fig. 4J), and again we never observed tumor formation in any mice.

3.5. GLT1 overexpressing hIPSA transplants reduced lesion size following cervical contusion SCI

To test the therapeutic efficacy of hIPSA transplants in the rat unilateral cervical contusion model, we first assessed lesion size. At 4 weeks post-injury, we quantified Cresyl-violet stained transverse sections of the cervical spinal cord surrounding the injury site for the degree of ipsilesional tissue sparing by calculating the percentage of total ipsilateral hemi-cord area comprised of damaged tissue (Fig. 5A). Lesion area (Fig. 5B) and total lesion volume (Fig. 5C) analysis (combined for both white and gray matter) revealed that GLT1–hIPSA transplants significantly reduced lesion size at multiple locations surrounding the epicenter compared to both GFP–hFibro and GFP–hIPSA control transplant groups. We observed this protective effect specifically within 1 mm rostral and caudal of the epicenter where the greatest tissue damage occurred.

3.6. GLT1 overexpressing hIPSA transplants preserved diaphragm innervation by phrenic motor neurons after SCI

We found that GLT1 overexpressing hIPSA transplants significantly preserved morphological innervation at the diaphragm neuromuscular junction (NMJ), the synapse which is critical for functional PMN–diaphragm connectivity. To examine pathological alterations at the diaphragm NMJ, we analyzed hemi-diaphragm muscle ipsilateral to the contusion in rats (Fig. 6A–B). We quantified the percentage of intact NMJs or partially denervated NMJs in the animals from the 3 injection groups at 4 weeks post-injury/transplantation (Wright et al., 2007, 2009; Wright and Son, 2007). For analysis, we divided the hemi-diaphragm into three anatomical regions (ventral, medial and dorsal) (Fig. 6C), as the rostral-caudal axis of the PMN pool within the cervical spinal cord topographically maps onto the ventral-dorsal axis of the diaphragm (Laskowski and Sanes, 1987). At the dorsal region of the hemi-diaphragm, the percentage of intact NMJs in the GLT1–hIPSA transplant group was significantly greater than both control groups, while at the ventral and medial regions of the diaphragm, there were no differences in the percentage of intact NMJs amongst the groups

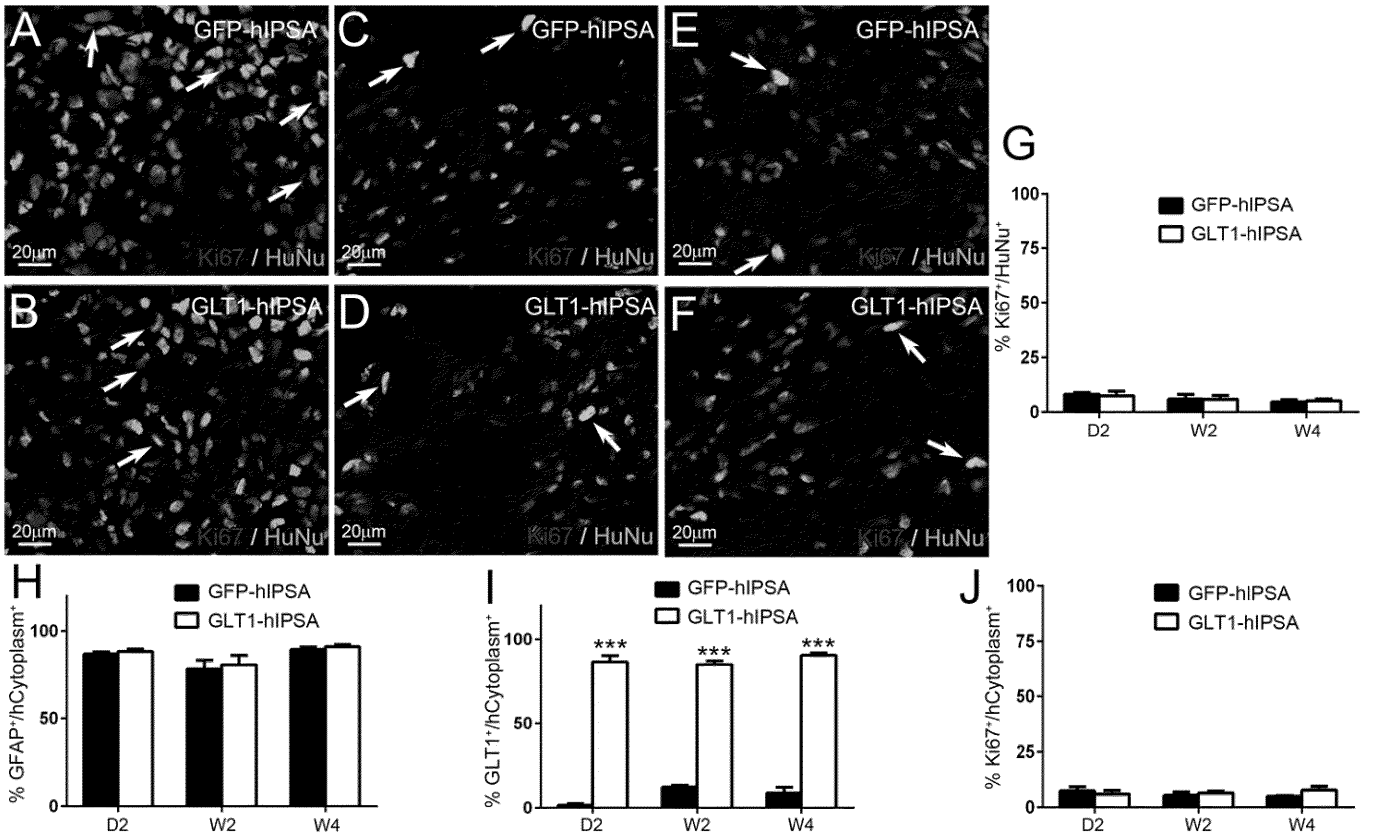

Fig. 4. Transplanted hiPSAs showed limited proliferation and did not form tumors. Double immunostaining for the proliferation marker Ki67 with human nuclei (HuNu) was performed on spinal cord sections from the GFP–hIPSA (A, C, E) and GLT1–hIPSA (B, D, F) groups at D2 (A–B), W2 (C–D) and W4 (E–F) post-transplantation, and quantification results are shown in (G). Tumor formation was never observed. We conducted similar *in vivo* characterization of hIPSA fate following transplantation into the mouse spinal cord immediately following unilateral cervical contusion SCI. The majority of transplant-derived cells were differentiated $GFAP^+$ astrocytes (H). Control GFP–hIPSAs did not express GLT1, while overexpression resulted in the majority of transplant-derived astrocytes expressing GLT1 (I). Less than 10% of transplant-derived cells continued to proliferate at D2, W2 and W4 (J). Results were expressed as means ± SEM. $^{***}p < 0.001$. n = 3 per group per time point in cell fate analysis.

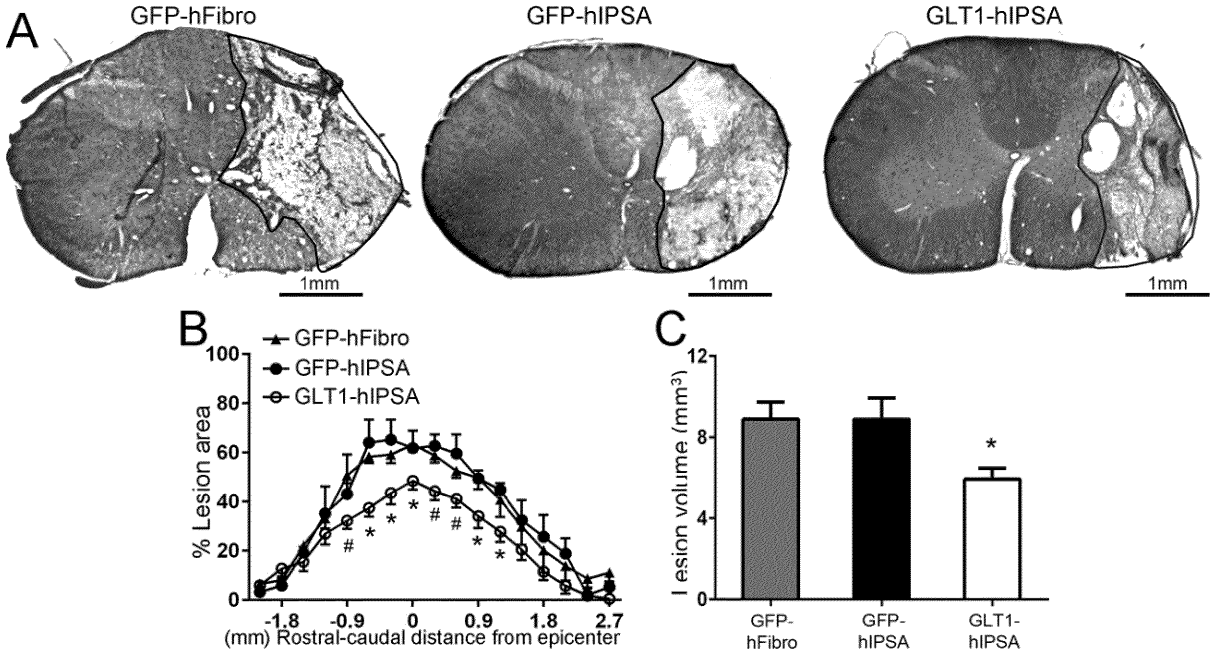

Fig. 5. GLT1 overexpressing hIPSA transplants reduced lesion size following cervical contusion SCI. At 4 weeks post-injury, we quantified Cresyl-violet stained transverse sections of the cervical spinal cord for the degree of ipsilesional tissue sparing by calculating the percentage of total ipsilateral hemi-cord area comprised of damaged tissue (A). Lesion area (B) and total lesion volume (C) analysis (combined for both white and gray matter) revealed that GLT1–hIPSA transplants significantly reduced lesion size at multiple locations surrounding the epicenter compared to both human fibroblast and control GFP–hIPSA transplant groups. Results were expressed as means ± SEM. $\#p < 0.05$, GLT1–hIPSA group *versus* GFP–hIPSA group only; $^*p < 0.05$, GLT1–hIPSA group *versus* both control groups. n = 6 per group for lesion area and volume analysis.

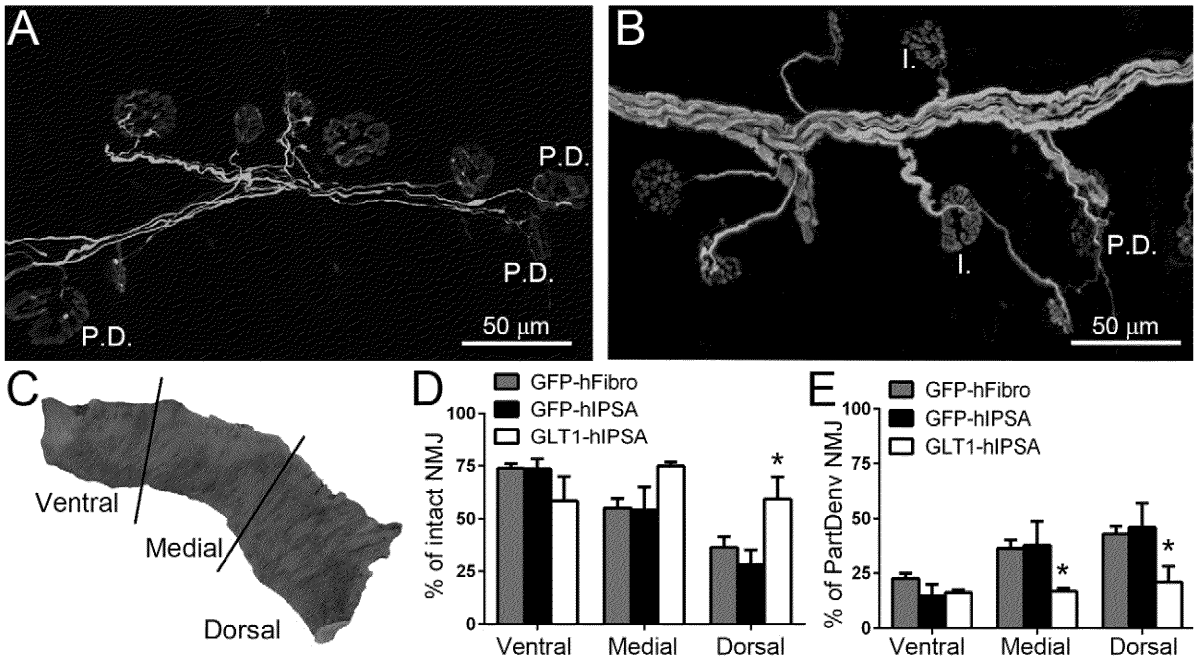

Fig. 6. GLT1 overexpressing hIPSA astrocyte transplants preserved diaphragm innervation by phrenic motor neurons following cervical contusion SCI. To examine pathological alterations at the diaphragm NMJ, hemi-diaphragm muscle ipsilateral to the contusion from the GFP–hFibro (A), GFP–hIPSA and GLT1–hIPSA (B) groups was examined at 4 weeks post-injury/transplantation. Individual NMJs were characterized as: intact (I.) and partially denervated (P.D.). For analysis, the hemi-diaphragm was divided into three anatomical regions (ventral, medial and dorsal) (C). At the dorsal region of the hemi-diaphragm, the percentage of intact NMJs in the GLT1–hIPSA group was significant greater than both control groups (D). GLT1–hIPSA transplants significantly reduced the percentage of partially denervated NMJs in the medial and dorsal hemi-diaphragm regions compared to both control groups (E). Results were expressed as means ± SEM. $^{*}p < 0.05$, GLT1–hIPSA group *versus* both control groups. n = 4–6 per group for NMJ analysis.

(Fig. 6D). GLT1–hIPSA transplants also significantly reduced the percentage of partially denervated NMJs in the medial and dorsal hemi-diaphragm regions compared to both control groups (Fig. 6E).

3.7. GLT1 overexpressing hIPSA transplants preserved diaphragm function following cervical contusion SCI

To determine the efficacy of preserving PMN-diaphragm innervation with respect to respiratory impairment, we characterized the *in vivo* functional effects of transplants on diaphragmatic function in cervical contusion rats. We recorded spontaneous EMG activity, which is indicative of PMN activation of diaphragm muscle due to central drive, at 4 weeks post-injury/transplantation (Fig. 7A). All groups showed reduced amplitude in rhythmic inspiratory EMG bursts associated with muscle contraction compared to uninjured animals (Nicaise et al., 2012). Integrated EMG analysis of this recording shows that the GLT1–hIPSA transplants significantly increased EMG amplitude in the dorsal region of the hemi-diaphragm compared to both control groups (Fig. 7B), again matching the anatomically-specific spinal cord and NMJ histological results. However, we observed no protective effects of GLT1–hIPSA transplants at either the medial or ventral regions, and the control GFP–hIPSA transplants showed no significant effects compared to control hFibroblast injection at all hemi-diaphragm locations (Fig. 7B). There were no significant differences in EMG burst frequency (Fig. 7C) or burst duration (Fig. 7D) amongst the three groups.

Following supramaximal phrenic nerve stimulus, we obtained compound muscle action potentials (CMAP) recordings from the ipsilateral hemi-diaphragm using a surface electrode (Fig. 7E). In all treatment groups, peak CMAP amplitude was significantly reduced compared to uninjured laminectomy only rats, whose CMAP amplitudes are approximately 7 mV (Nicaise et al., 2013). However, CMAP amplitudes in the GLT1–hIPSAtransplant group were significantly increased compared to the two control transplantation groups at weeks 2–4 post-injury (Fig. 7F). With the use of the surface electrode, we are recording from the entire hemi-diaphragm (or at least a significant portion of the muscle), yet we still observed this significant protective effect on overall muscle function, despite the fact that transplants only reduced central degeneration very near to the injury site and correspondingly preserved morphological innervation only in the dorsal hemi-diaphragm.

4. Discussion

The use of iPS cells as a source of mature cell types for therapeutic transplantation in CNS diseases represents an exciting direction in regenerative medicine. However, to date only a small number of studies have assessed the long-term fate and therapeutic efficacy of iPS cell-derived transplants in animal models of SCI.

A number of these studies reported significant therapeutic benefit when NSCs/NPCs derived from either mouse (Tsuji et al., 2010) or human (Fujimoto et al., 2012; Nori et al., 2011; Romanyuk et al., 2014) iPS cells were transplanted into contusion or cavity-type models of rodent SCI, as well as in non-human primate models (Kobayashi et al., 2012). Unlike our current work, these studies did not focus on, or achieve, targeted replacement of astrocytes in the injured spinal cord. In many cases, the cells were delivered in a multipotent NSC-like state and resulted in mixed differentiation into glial phenotypes, including astrocytes, and various neuronal subtypes. While these studies were able to achieve some functional benefit, future work may require more phenotypically targeted strategies, each of which depends on the nature of the SCI pathology (*e.g.* type of injury and anatomical locations affected) and the specific cell lineages being targeted for replacement. Nevertheless, these studies were able to nicely show promising properties of engrafted cells in the injured spinal cord environment, including synaptic integration into endogenous neuronal circuitry (Fujimoto et al., 2012; Nori et al., 2011). iPS cell-derived NSCs have also shown therapeutic promise in models of other spinal cord diseases such as spinal muscular atrophy (Simone et al., 2014).

A number of these studies with iPS cell transplantation reported a lack of beneficial outcomes in SCI models. Pomeshchik et al. (2014) did not observe functional improvement after transplantation of hIPS

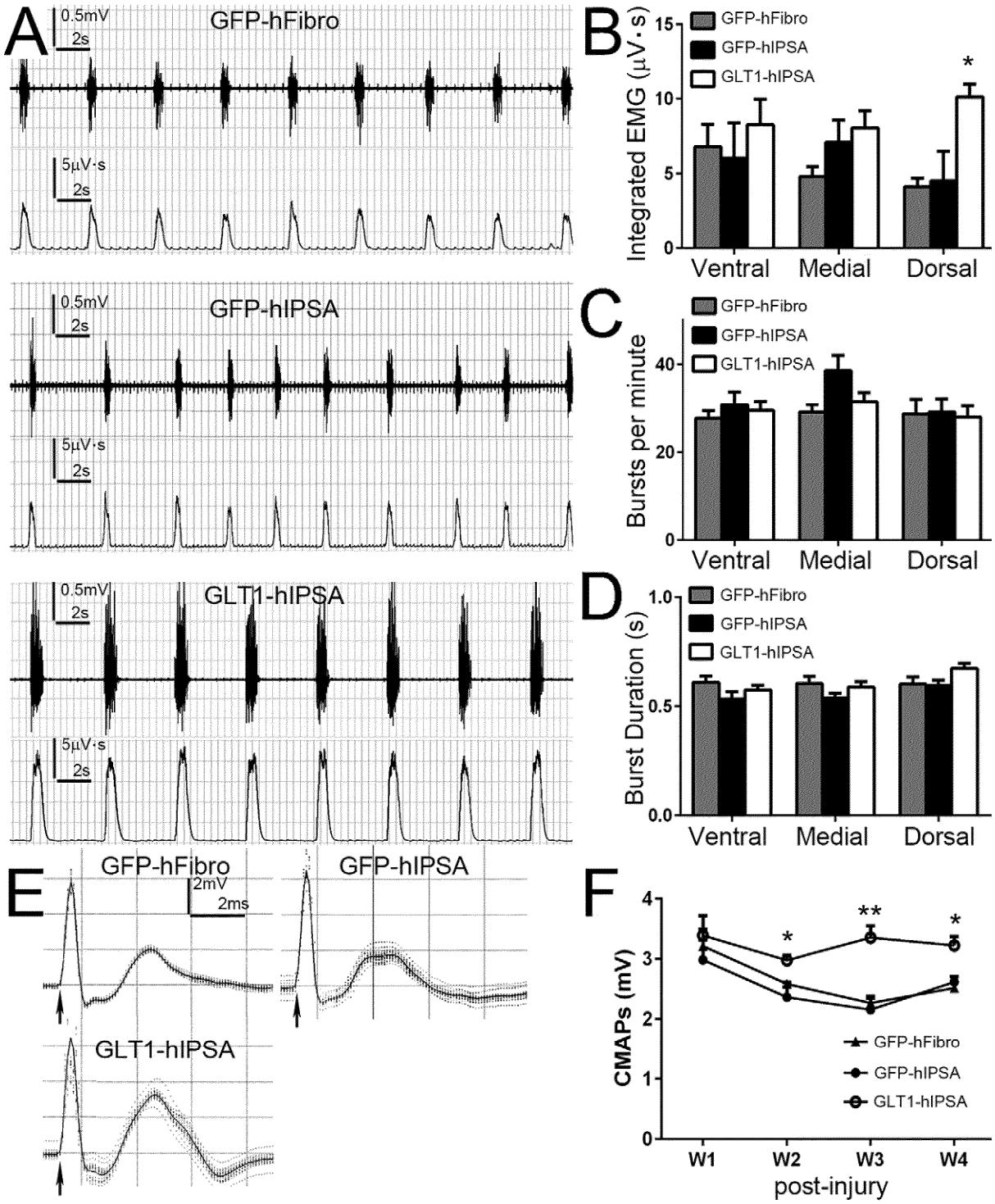

Fig. 7. GLT1 overexpressing hIPSA transplants preserved diaphragm function following cervical contusion SCI. Spontaneous EMG recordings from ipsilateral hemi-diaphram were obtained at 4 weeks post-injury/transplantation (A, *upper*: raw EMG; *lower*: integrated EMG). Integrated EMG amplitude (B), burst frequency (C), and burst duration (D) were analyzed. Following supramaximal phrenic nerve stimulation, we obtained compound muscle action potential (CMAP) recordings from the ipsilateral hemi-diaphragm using a surface electrode (E). CMAP amplitudes at different time points post-injury were analyzed (F). Results were expressed as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, GLT1–hIPSA group *versus* both control groups. n = 6 per group for EMG and CMAP analysis.

cell-derived NPCs in a contusion SCI model. However, they also did not find long term survival of grafted cells in these mice receiving a tacrolimus immune suppression regimen, unlike the robust and persistent integration that we observed in the present study using an immune suppression protocol consisting of both tacrolimus and rapamycin in mice or cyclosporine in rats. In addition to our work, other groups have reported impressive survival and differentiation of hIPS cells into mature CNS cell types after injection into adult spinal cord of similarly immunosuppressed rodents (Haidet-Phillips et al., 2014; Sareen et al., 2014).

An interesting study from the Horner group (Nutt et al., 2013) reported a lack of therapeutic improvement with transplantation of hIPS cell-derived NPCs in a SCI model, despite impressive graft integration. However, cells were delivered at a chronic time point, which may

represent an environment less amenable to transplant-induced plasticity, while we targeted early neuroprotection in this report.

A recent study from the Steward lab reported that transplantation of a mixed population of glial and neuronal progenitors into a transection model of SCI resulted in ectopic engraftment of large numbers of graft-derived cells in locations such as the central canal, ventricles and pial surface of the spinal cord (Steward et al., 2014), providing a note of caution when using transplantation of any class of NSC/NPC in SCI. This issue is particularly relevant to strategies employing cells derived from pluripotent sources such as ES and iPS cells given the possibility of incomplete and/or inefficient differentiation (Tsuji et al., 2010). In the current study and in our previous work (Lepore et al., 2004, 2005, 2006, 2008b, 2011b; Lepore and Fischer, 2005; Li et al., 2014), we never observed overt tumor formation or extensive migration away from injection sites beyond only a few spinal segments. In the current work, we did note the presence of a small residual population of proliferating transplant-derived cells even out to four weeks post-injection, though we never found any tumor formation. It will be important to assess very long-term time points post-transplantation in future experiments to establish the safety of these and similar types of cells before proceeding to the clinic. Unlike the Steward paper, we did not systematically assess distribution of transplant-derived cells throughout the neuraxis.

Mechanical allodynia (a form of neuropathic pain) was observed when mouse iPSAs were transplanted into a contusion SCI model (Hayashi et al., 2011). In addition to this work, other published studies have similarly reported sensory hypersensitivity in SCI models accompanying transplantation of progenitor-derived astrocytes (Davies et al., 2008; Hofstetter et al., 2005), possibly due to increased neuronal plasticity that is induced by transplantation of immature astrocyte populations (Smith et al., 1986). However, in a large body of work, we and others (Haas et al., 2012; Mitsui et al., 2005; Nutt et al., 2013) have not found such increased sensitivity, including following hIPSA transplantation (Nutt et al., 2013). The discrepancy amongst these studies may be due to heterogeneity in the subtypes of astrocytes being injected (Davies et al., 2008, 2011).

A number of practical issues that are beyond the scope of this discussion will need to be addressed before moving transplantation of iPS cells to the clinic in SCI and other diseases of the nervous system. Specifically with respect to targeting relative early events such as PhMN loss after cervical SCI, autologous derivation of cells will likely not be relevant given that PhMNs are lost within several days post-injury (Nicaise et al., 2013). Instead, cells to be used for transplantation will likely be obtained from banks of immune/HLA-matched cells (Zimmermann et al., 2012). Given the need to extensively test iPS cell lines prior to transplantation into a patient, as well as the costs and time that will be required for generating cells for each individual patient, this approach may actually be practically preferable to autologous derivation (Taylor et al., 2011). As human stem cell lines have shown donor variability in SCI models (Neuhuber et al., 2005), future studies will need to investigate *in vivo* properties and therapeutic efficacy of human iPS cells derived from multiple donors in an attempt to move this approach toward clinical translation.

Similar to our previous work using transplantation of astrocytes derived from rodent glial progenitors (Li et al., 2014), we find that GLT1-overexpresing hIPSAs promote significant preservation of diaphragm function and diaphragm innervation by PhMNs. In both studies, control unmodified transplant-derived astrocytes expressed relatively lower levels of GLT1 in the injured spinal cord, suggesting that the cells respond to the injured environment in a similar manner as host astrocytes that show extensive transporter downregulation. Interestingly, the unmodified hIPSA transplants, despite excellent survival and efficient differentiation, did not promote therapeutic benefit with respect to protection of diaphragmatic respiratory circuitry. These findings suggest that astrocyte replacement alone may insufficient when targeting certain pathological mechanisms (*e.g.* excitotoxocity) but that functional maturation of these astrocytes is necessary, which is not surprising given the diverse, complex and integral roles that astrocytes play in intact CNS function (Pekny and Nilsson, 2005).

We have made interesting observations over the course of a number of studies with respect to therapeutically targeting GLT1 following SCI. We have consistently observed significant GLT1 downregulation in endogenous reactive astrocyte populations in both contusion and crush, as well as both cervical and thoracic, models of SCI (Lepore et al., 2011a, 2011c; Li et al., 2015; Putatunda et al., 2014; Watson et al., 2014). When we selectively increased GLT1 expression in these endogenous astrocytes in the unilateral cervical contusion model using an AAV8 vector, we paradoxically found that secondary degeneration of PhMNs and diaphragm denervation were worsened (Li et al., 2015). This effect was due to compromise in the protective glial scar-forming properties of endogenous astrocytes, which resulted in unexpected expansion of the lesion. In the current study with hIPSAs and in our previous work with rodent-derived glial progenitors (Li et al., 2014), we found that delivery of an exogenous source of astrocytes that expresses high levels of functional GLT1 *via* transplantation (in the exact same cervical contusion model) results in significant preservation of PhMNs and diaphragm function. These findings, as well as other studies that tested the effects of pharmacologically elevating (Olsen et al., 2010) or genetically reducing (Lepore et al., 2011c) GLT1 in SCI, demonstrate that targeting GLT1 is a promising and powerful therapeutic strategy in SCI for targeting neuroprotection and possibly other outcomes of SCI such as neuronal hyperexcitability.

Despite the impressive therapeutic effect achieved in the present study, the degree of PhMN protection and diaphragm function preservation was only partial. In future work, we will need to optimize neuroprotective strategies such as hIPSA transplantation to enhance therapeutic effects, as well as combine these neuroprotective approaches with interventions aimed at promoting plasticity, axonal regrowth and targeted reconnection of the rVRG-PhMN-diaphragm circuit (Alilain et al., 2011). Preserving neural control of diaphragm function involves targeting a complex circuitry that extends beyond just protecting PhMNs (Lane et al., 2009). We focused on preservation of PhMNs centrally in the cervical spinal cord and NMJ innervation peripherally in the diaphragm. Nevertheless, our hIPSA intervention may have also exerted beneficial effects *via* protection of respiratory interneuron populations of the cervical spinal cord and/or descending bulbospinal input to PhMNs from the rVRG. hIPSA transplants may have also resulted in beneficial effects by promoting regrowth/regeneration and/or sprouting of rVRG axons and interneurons, which is possible given the growth-promoting properties of astrocyte transplants after SCI (Davies et al., 2006, 2008, 2011; Haas et al., 2012). However, we only observed therapeutic effects on diaphragm innervation and function with GLT1 overexpressing hIPSAs (but not with control unmodified hIPSAs), suggesting that neuroprotection mediated by increased GLT1 levels and consequent reduction in excitotoxicity was the likely mechanism, even if transplants also promoted some regrowth of respiratory axon populations. We also did not observe differences amongst groups in plasticity at the diaphragm NMJ such as sprouting or reinnervation, further supporting central neuroprotection as the responsible mechanism of therapeutic action.

In conclusion, we report exciting and novel results showing that targeted replacement of astrocyte GLT1 following cervical SCI using hIPSA transplantation significantly preserves diaphragmatic respiratory function. These findings are important for a number of reasons. We demonstrate the therapeutic efficacy and safety of hiPS transplantation in SCI, as well as the benefit of specifically addressing astrocyte dysfunction using this clinically-relevant source of cells. We also show mechanistically that targeting GLT1 using an astrocyte transplant-based approach has profound effects on functional and histopatholoigcal outcomes after SCI. Furthermore, we conducted these studies in a clinically-relevant SCI paradigm that models a large proportion of human disease cases. Excitingly, we find that this intervention results in therapeutic benefit on respiratory function, which has important implications for

SCI patients. Collectively, these studies lay the foundation for translating iPS cell transplantation to the treatment of SCI.

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KL: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing. EJ, TJH, SS, MCW: Collection and assembly of data, data analysis and interpretation. JPR, NJM: Provision of study materials. ACL: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. This work was supported by the Craig H. Neilsen Foundation (grant #190140 to A.C.L.) and the NINDS (grant #1R01NS079702 to A.C.L.).

References

Aguilar, R.M., Steward, O., 2010. A bilateral cervical contusion injury model in mice: assessment of gripping strength as a measure of forelimb motor function. Exp. Neurol. 221, 38–53.

Alilain, W.J., Horn, K.P., Hu, H., Dick, T.E., Silver, J., 2011. Functional regeneration of respiratory pathways after spinal cord injury. Nature 475, 196–200.

Awad, B.I., Warren, P.M., Steinmetz, M.P., Alilain, W.J., 2013. The role of the crossed phrenic pathway after cervical contusion injury and a new model to evaluate therapeutic interventions. Exp. Neurol. 248, 398–405.

Das, A.K., Pal, R., 2010. Induced pluripotent stem cells (iPSCs): the emergence of a new champion in stem cell technology-driven biomedical applications. J. Tissue Eng. Regen. Med. 4, 413–421.

Davies, J.E., Huang, C., Proschel, C., Noble, M., Mayer-Proschel, M., Davies, S.J., 2006. Astrocytes derived from glial-restricted precursors promote spinal cord repair. J. Biol. 5, 7.

Davies, J.E., Proschel, C., Zhang, N., Noble, M., Mayer-Proschel, M., Davies, S.J., 2008. Transplanted astrocytes derived from BMP- or CNTF-treated glial-restricted precursors have opposite effects on recovery and allodynia after spinal cord injury. J. Biol. 7, 24.

Davies, S.J., Shih, C.H., Noble, M., Mayer-Proschel, M., Davies, J.E., Proschel, C., 2011. Transplantation of specific human astrocytes promotes functional recovery after spinal cord injury. PLoS One 6, e17328.

Dowd, L.A., Robinson, M.B., 1996. Rapid stimulation of EAAC1-mediated Na+-dependent L-glutamate transport activity in C6 glioma cells by phorbol ester. J. Neurochem. 67, 508–516.

Fujimoto, Y., Abematsu, M., Falk, A., Tsujimura, K., Sanosaka, T., Juliandi, B., Semi, K., Namihira, M., Komiya, S., Smith, A., Nakashima, K., 2012. Treatment of a mouse model of spinal cord injury by transplantation of human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem cells. Stem Cells 30, 1163–1173.

Gage, F.H., 2000. Mammalian neural stem cells. Science 287, 1433–1438.

Gensel, J.C., Tovar, C.A., Hamers, F.P., Deibert, R.J., Beattie, M.S., Bresnahan, J.C., 2006. Behavioral and histological characterization of unilateral cervical spinal cord contusion injury in rats. J. Neurotrauma 23, 36–54.

Gwak, Y.S., Kang, J., Unabia, G.C., Hulsebosch, C.E., 2012. Spatial and temporal activation of spinal glial cells: role of gliopathy in central neuropathic pain following spinal cord injury in rats. Exp. Neurol. 234, 362–372.

Haas, C., Neuhuber, B., Yamagami, T., Rao, M., Fischer, I., 2012. Phenotypic analysis of astrocytes derived from glial restricted precursors and their impact on axon regeneration. Exp. Neurol. 233, 717–732.

Haidet-Phillips, A.M., Roybon, L., Gross, S.K., Tuteja, A., Donnelly, C.J., Richard, J.P., Ko, M., Sherman, A., Eggan, K., Henderson, C.E., Maragakis, N.J., 2014. Gene profiling of human induced pluripotent stem cell-derived astrocyte progenitors following spinal cord engraftment. Stem Cells Transl. Med. 3, 575–585.

Hayashi, K., Hashimoto, M., Koda, M., Naito, A.T., Murata, A., Okawa, A., Takahashi, K., Yamazaki, M., 2011. Increase of sensitivity to mechanical stimulus after transplantation of murine induced pluripotent stem cell-derived astrocytes in a rat spinal cord injury model. J. Neurosurg. Spine 15, 582–593.

Hofstetter, C.P., Holmstrom, N.A., Lilja, J.A., Schweinhardt, P., Hao, J., Spenger, C., Wiesenfeld-Hallin, Z., Kurpad, S.N., Frisen, J., Olson, L., 2005. Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. Nat. Neurosci. 8, 346–353.

Hulsebosch, C.E., 2008. Gliopathy ensures persistent inflammation and chronic pain after spinal cord injury. Exp. Neurol. 214, 6–9.

Kiskinis, E., Eggan, K., 2010. Progress toward the clinical application of patient-specific pluripotent stem cells. J. Clin. Invest. 120, 51–59.

Kobayashi, Y., Okada, Y., Itakura, G., Iwai, H., Nishimura, S., Yasuda, A., Nori, S., Hikishima, K., Konomi, T., Fujiyoshi, K., Tsuji, O., Toyama, Y., Yamanaka, S., Nakamura, M., Okano, H., 2012. Pre-evaluated safe human iPSC-derived neural stem cells promote functional recovery after spinal cord injury in common marmoset without tumorigenicity. PLoS One 7, e52787.

Lane, M.A., Fuller, D.D., White, T.E., Reier, P.J., 2008. Respiratory neuroplasticity and cervical spinal cord injury: translational perspectives. Trends Neurosci. 31, 538–547.

Lane, M.A., Lee, K.Z., Fuller, D.D., Reier, P.J., 2009. Spinal circuitry and respiratory recovery following spinal cord injury. Respir. Physiol. Neurobiol. 169, 123–132.

Lane, M.A., Lee, K.Z., Salazar, K., O'Steen, B.E., Bloom, D.C., Fuller, D.D., Reier, P.J., 2012. Respiratory function following bilateral mid-cervical contusion injury in the adult rat. Exp. Neurol. 235, 197–210.

Laskowski, M.B., Sanes, J.R., 1987. Topographic mapping of motor pools onto skeletal muscles. J. Neurosci. 7, 252–260.

Lee, J.H., Tigchelaar, S., Liu, J., Stammers, A.M., Streijger, F., Tetzlaff, W., Kwon, B.K., 2010. Lack of neuroprotective effects of simvastatin and minocycline in a model of cervical spinal cord injury. Exp. Neurol. 225, 219–230.

Lepore, A.C., Fischer, I., 2005. Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. Exp. Neurol. 194, 230–242.

Lepore, A.C., Maragakis, N.J., 2011. Stem cell transplantation for spinal cord neurodegeneration. Methods Mol. Biol. 793, 479–493.

Lepore, A.C., Han, S.S., Tyler-Polsz, C., Cai, J., Rao, M.S., Fischer, I., 2004. Differential fate of multipotent and lineage-restricted neural precursors following transplantation into the adult CNS. Neuron Glia Biol. 1, 113–126.

Lepore, A.C., Bakshi, A., Swanger, S.A., Rao, M.S., Fischer, I., 2005. Neural precursor cells can be delivered into the injured cervical spinal cord by intrathecal injection at the lumbar cord. Brain Res. 1045, 206–216.

Lepore, A.C., Neuhuber, B., Connors, T.M., Han, S.S., Liu, Y., Daniels, M.P., Rao, M.S., Fischer, I., 2006. Long-term fate of neural precursor cells following transplantation into developing and adult CNS. Neuroscience 139, 513–530.

Lepore, A.C., Dejea, C., Carmen, J., Rauck, B., Kerr, D.A., Sofroniew, M.V., Maragakis, N.J., 2008a. Selective ablation of proliferating astrocytes does not affect disease outcome in either acute or chronic models of motor neuron degeneration. Exp. Neurol. 211, 423–432.

Lepore, A.C., Rauck, B., Dejea, C., Pardo, A.C., Rao, M.S., Rothstein, J.D., Maragakis, N.J., 2008b. Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. Nat. Neurosci. 11, 1294–1301.

Lepore, A.C., O'Donnell, J., Bonner, J.F., Paul, C., Miller, M.E., Rauck, B., Kushner, R.A., Rothstein, J.D., Fischer, I., Maragakis, N.J., 2011a. Spatial and temporal changes in promoter activity of the astrocyte glutamate transporter GLT1 following traumatic spinal cord injury. J. Neurosci. Res. 89, 1001–1017.

Lepore, A.C., O'Donnell, J., Kim, A.S., Williams, T., Tuteja, A., Rao, M.S., Kelley, L.L., Campanelli, J.T., Maragakis, N.J., 2011b. Human glial-restricted progenitor transplantation into cervical spinal cord of the SOD1^{G93A} mouse model of ALS. PLoS One 6.

Lepore, A.C., O'Donnell, J., Kim, A.S., Yang, E.J., Tuteja, A., Haidet-Phillips, A., O'Banion, C.P., Maragakis, N.J., 2011c. Reduction in expression of the astrocyte glutamate transporter, GLT1, worsens functional and histological outcomes following traumatic spinal cord injury. Glia 59, 1996–2005.

Li, K., Nicaise, C., Sannie, D., Hala, T.J., Javed, E., Parker, J.L., Putatunda, R., Regan, K.A., Suain, V., Brion, J.P., Rhoderick, F., Wright, M.C., Poulsen, D.J., Lepore, A.C., 2014. Overexpression of the astrocyte glutamate transporter GLT1 exacerbates phrenic motor neuron degeneration, diaphragm compromise, and forelimb motor dysfunction following cervical contusion spinal cord injury. J. Neurosci. 34, 7622–7638.

Li, K., Javed, E., Hala, T.J., Sannie, D., Regan, K.A., Maragakis, N.J., Wright, M.C., Poulsen, D.J., Lepore, A.C., 2015. Transplantation of glial progenitors that overexpress glutamate transporter GLT1 preserves diaphragm function following cervical SCI. Mol. Ther. 23, 533–548.

Liu, D., Thangnipon, W., McAdoo, D.J., 1991. Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord. Brain Res. 547, 344–348.

Maragakis, N.J., Rothstein, J.D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiol. Dis. 15, 461–473.

Maragakis, N.J., Rothstein, J.D., 2006. Mechanisms of disease: astrocytes in neurodegenerative disease. Nat. Clin. Pract. Neurol. 2, 679–689.

McDonald, J.W., Becker, D., 2003. Spinal cord injury: promising interventions and realistic goals. Am. J. Phys. Med. Rehabil. 82, S38–S49.

Mitsui, T., Shumsky, J.S., Lepore, A.C., Murray, M., Fischer, I., 2005. Transplantation of neuronal and glial restricted precursors into contused spinal cord improves bladder and motor functions, decreases thermal hypersensitivity, and modifies intraspinal circuitry. J. Neurosci. 25, 9624–9636.

Neuhuber, B., Timothy Himes, B., Shumsky, J.S., Gallo, G., Fischer, I., 2005. Axon growth and recovery of function supported by human bone marrow stromal cells in the injured spinal cord exhibit donor variations. Brain Res. 1035, 73–85.

Nicaise, C., Hala, T.J., Frank, D.M., Parker, J.L., Authelet, M., Leroy, K., Brion, J.P., Wright, M.C., Lepore, A.C., 2012. Phrenic motor neuron degeneration compromises phrenic axonal circuitry and diaphragm activity in a unilateral cervical contusion model of spinal cord injury. Exp. Neurol. 235, 539–552.

Nicaise, C., Frank, D.M., Hala, T.J., Authelet, M., Pochet, R., Adriaens, D., Brion, J.P., Wright, M.C., Lepore, A.C., 2013. Early phrenic motor neuron loss and transient respiratory abnormalities after unilateral cervical spinal cord contusion. J. Neurotrauma 30, 1092–1099.

Nori, S., Okada, Y., Yasuda, A., Tsuji, O., Takahashi, Y., Kobayashi, Y., Fujiyoshi, K., Koike, M., Uchiyama, Y., Ikeda, E., Toyama, Y., Yamanaka, S., Nakamura, M., Okano, H., 2011. Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. Proc. Natl. Acad. Sci. U. S. A. 108, 16825–16830.

Nutt, S.E., Chang, E.A., Suhr, S.T., Schlosser, L.O., Mondello, S.E., Moritz, C.T., Cibelli, J.B., Horner, P.J., 2013. Caudalized human iPSC-derived neural progenitor cells produce neurons and glia but fail to restore function in an early chronic spinal cord injury model. Exp. Neurol. 248, 491–503.

Olsen, M.L., Campbell, S.C., McFerrin, M.B., Floyd, C.L., Sontheimer, H., 2010. Spinal cord injury causes a wide-spread, persistent loss of Kir4.1 and glutamate transporter 1: benefit of 17 beta-oestradiol treatment. Brain 133, 1013–1025.

Panter, S.S., Yum, S.W., Faden, A.I., 1990. Alteration in extracellular amino acids after traumatic spinal cord injury. Ann. Neurol. 27, 96–99.

Park, E., Velumian, A.A., Fehlings, M.G., 2004. The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. J. Neurotrauma 21, 754–774.
Pekny, M., Nilsson, M., 2005. Astrocyte activation and reactive gliosis. Glia 50, 427–434.
Perego, C., Vanoni, C., Bossi, M., Massari, S., Basudev, H., Longhi, R., Pietrini, G., 2000. The GLT-1 and GLAST glutamate transporters are expressed on morphologically distinct astrocytes and regulated by neuronal activity in primary hippocampal cocultures. J. Neurochem. 75, 1076–1084.
Pomeshchik, Y., Puttonen, K.A., Kidin, I., Ruponen, M., Lehtonen, S., Malm, T., Akesson, E., Hovatta, O., Koistinaho, J., 2014. Transplanted human iPSC-derived neural progenitor cells do not promote functional recovery of pharmacologically immunosuppressed mice with contusion spinal cord injury. Cell Transplant.
Putatunda, R., Hala, T.J., Chin, J., Lepore, A.C., 2014. Chronic at-level thermal hyperalgesia following rat cervical contusion spinal cord injury is accompanied by neuronal and astrocyte activation and loss of the astrocyte glutamate transporter, GLT1, in superficial dorsal horn. Brain Res. 18, 64–79.
Romanyuk, N., Amemori, T., Turnovcova, K., Prochazka, P., Onteniente, B., Sykova, E., Jendelova, P., 2014. Beneficial effect of human induced pluripotent stem cell-derived neural precursors in spinal cord injury repair. Cell Transplant.
Salewski, R.P., Eftekharpour, E., Fehlings, M.G., 2010. Are induced pluripotent stem cells the future of cell-based regenerative therapies for spinal cord injury? J. Cell. Physiol. 222, 515–521.
Sandrow, H.R., Shumsky, J.S., Amin, A., Houle, J.D., 2008. Aspiration of a cervical spinal contusion injury in preparation for delayed peripheral nerve grafting does not impair forelimb behavior or axon regeneration. Exp. Neurol. 210, 489–500.
Sandrow-Feinberg, H.R., Izzi, J., Shumsky, J.S., Zhukareva, V., Houle, J.D., 2009. Forced exercise as a rehabilitation strategy after unilateral cervical spinal cord contusion injury. J. Neurotrauma 26, 721–731.
Sandrow-Feinberg, H.R., Zhukareva, V., Santi, L., Miller, K., Shumsky, J.S., Baker, D.P., Houle, J.D., 2010. PEGylated interferon-beta modulates the acute inflammatory response and recovery when combined with forced exercise following cervical spinal contusion injury. Exp. Neurol. 223, 439–451.
Sareen, D., Gowing, G., Sahabian, A., Staggenborg, K., Paradis, R., Avalos, P., Latter, J., Ornelas, L., Garcia, L., Svendsen, C.N., 2014. Human induced pluripotent stem cells are a novel source of neural progenitor cells (iNPCs) that migrate and integrate in the rodent spinal cord. J. Comp. Neurol. 522, 2707–2728.
Shanmuganathan, K., Gullapalli, R.P., Zhuo, J., Mirvis, S.E., 2008. Diffusion tensor MR imaging in cervical spine trauma. AJNR Am. J. Neuroradiol. 29, 655–659.
Simone, C., Nizzardo, M., Rizzo, F., Ruggieri, M., Riboldi, G., Salani, S., Bucchia, M., Bresolin, N., Comi, G.P., Corti, S., 2014. iPSC-derived neural stem cells act via kinase inhibition to exert neuroprotective effects in spinal muscular atrophy with respiratory distress type 1. Stem Cell Rep. 3, 297–311.
Smith, G.M., Miller, R.H., Silver, J., 1986. Changing role of forebrain astrocytes during development, regenerative failure, and induced regeneration upon transplantation. J. Comp. Neurol. 251, 23–43.
Stamegna, J.C., Felix, M.S., Roux-Peyronnet, J., Rossi, V., Feron, F., Gauthier, P., Matarazzo, V., 2011. Nasal OEC transplantation promotes respiratory recovery in a subchronic rat model of cervical spinal cord contusion. Exp. Neurol. 229, 120–131.
Steward, O., Sharp, K.G., Yee, K.M., Hatch, M.N., Bonner, J.F., 2014. Characterization of ectopic colonies that form in widespread areas of the nervous system with neural stem cell transplants into the site of a severe spinal cord injury. J. Neurosci. 34, 14013–14021.
Strakowski, J.A., Pease, W.S., Johnson, E.W., 2007. Phrenic nerve stimulation in the evaluation of ventilator-dependent individuals with C4- and C5-level spinal cord injury. Am. J. Phys. Med. Rehabil. 86, 153–157.
Stys, P.K., 2004. White matter injury mechanisms. Curr. Mol. Med. 4, 113–130.
Takahashi, K., Okita, K., Nakagawa, M., Yamanaka, S., 2007a. Induction of pluripotent stem cells from fibroblast cultures. Nat. Protoc. 2, 3081–3089.
Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007b. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872.
Taylor, C.J., Bolton, E.M., Bradley, J.A., 2011. Immunological considerations for embryonic and induced pluripotent stem cell banking. Philos. Trans. R. Soc. Lond. B Biol. Sci. 366, 2312–2322.
Tsuji, O., Miura, K., Okada, Y., Fujiyoshi, K., Mukaino, M., Nagoshi, N., Kitamura, K., Kumagai, G., Nishino, M., Tomisato, S., Higashi, H., Nagai, T., Katoh, H., Kohda, K., Matsuzaki, Y., Yuzaki, M., Ikeda, E., Toyama, Y., Nakamura, M., Yamanaka, S., Okano, H., 2010. Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. Proc. Natl. Acad. Sci. U. S. A. 107, 12704–12709.
Watson, J.L., Hala, T.J., Putatunda, R., Sannie, D., Lepore, A.C., 2014. Persistent at-level thermal hyperalgesia and tactile allodynia accompany chronic neuronal and astrocyte activation in superficial dorsal horn following mouse cervical contusion spinal cord injury. PLoS One 9, e109099.
Wright, M.C., Son, Y.J., 2007. Ciliary neurotrophic factor is not required for terminal sprouting and compensatory reinnervation of neuromuscular synapses: re-evaluation of CNTF null mice. Exp. Neurol. 205, 437–448.
Wright, M.C., Cho, W.J., Son, Y.J., 2007. Distinct patterns of motor nerve terminal sprouting induced by ciliary neurotrophic factor vs. botulinum toxin. J. Comp. Neurol. 504, 1–16.
Wright, M.C., Potluri, S., Wang, X., Dentcheva, E., Gautam, D., Tessler, A., Wess, J., Rich, M.M., Son, Y.J., 2009. Distinct muscarinic acetylcholine receptor subtypes contribute to stability and growth, but not compensatory plasticity, of neuromuscular synapses. J. Neurosci. 29, 14942–14955.
Xu, G.Y., Hughes, M.G., Ye, Z., Hulsebosch, C.E., McAdoo, D.J., 2004. Concentrations of glutamate released following spinal cord injury kill oligodendrocytes in the spinal cord. Exp. Neurol. 187, 329–336.
Xu, G.Y., Hughes, M.G., Zhang, L., Cain, L., McAdoo, D.J., 2005. Administration of glutamate into the spinal cord at extracellular concentrations reached post-injury causes functional impairments. Neurosci. Lett. 384, 271–276.
Zimmer, M.B., Nantwi, K., Goshgarian, H.G., 2007. Effect of spinal cord injury on the respiratory system: basic research and current clinical treatment options. J. Spinal Cord Med. 30, 319–330.
Zimmermann, A., Preynat-Seauve, O., Tiercy, J.M., Krause, K.H., Villard, J., 2012. Haplotype-based banking of human pluripotent stem cells for transplantation: potential and limitations. Stem Cells Dev. 21, 2364–2373.

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Thursday, June 11, 2020 5:31 PM
To: Daniel Eldridge; Jourdan E. Brune; Kristin Zabrecky
Cc: Leandra Mosca
Subject: assignment for first protocol review on 6/18/20
Attachments: Print_ PROTO202000003 - 4174-03_ Magnesium Stem Cell.pdf; ZhangH_cited_paper.pdf

Hi all,

Here is the first assignment. It's a pdf I saved of the "print version" of a new protocol that is currently being reviewed. *Please do not look this protocol up in Hoverboard*, as my first round of questions are in there and the protocol has probably changed since then (edited), so that would defeat the purpose of our exercise. Just use this pdf. I also attached a pdf of a paper that the lab attached for your reference. You're welcome to look at whatever you want in Pubmed/online, just sending this paper to you to save you a step. You can also look at other protocols in Hoverboard if you want to, just not this particular protocol.

I don't love the print version format, but right now it is the only way to "freeze" a protocol in a certain state, and I wanted you to see it as I was seeing it on my first review, but I didn't want to delay it moving through the review process.

Please write up your review questions in a Word doc and email them to me as an attachment no later than 8pm on Wednes 6/17 (earlier is fine!), so I have some time to look over what you came up with before we meet via Zoom on Thurs AM. Remember to address your questions/comments to the group (not to me). And please be prepared to discuss, it will be small and informal and I don't want to do all of the talking. If you can use video I think it would be nice.

Let me know if you have questions and have fun ☺
Molly

HoverBoard

Date: Tuesday, June 2, 2020 3:37:32 PM Print Close

View: SF: Basic Information

Basic Information

1. *** Select research team:**
   Zhang, H
2. *** Title of protocol:**
   Effect of magnesium, I50 and iPSC on rat extraction socket preservation
3. *** Short title:**
   4174-03: Magnesium Stem Cell
4. *** Summary of research:**
   This research uses rat molar extraction model to determine the effects of magnesium ion, induced pluripotent stem cells (iPSCs) and Tie2 super agonist I50 on socket preservation. The general approach is as followed: magnesium ion, osteogenic pre-induced rat derived iPSCs (abbreviated as riPOBs, which will be generated from rat peripheral blood mononuclear cells) and I50 will be mixed with deproteinized bovine bone mineralized matrix (BMM) and applied in the maxillary first molar extraction sockets of 12-week-old SD rats individually and in combination. Bone formation in the sockets and the dimension of alveolar ridge in height and width will be evaluated by high-resolution micro-CT at 2 weeks (live animals) and 6 weeks (sacrificed animals). At 6 weeks, the alveolar bone samples will be harvested and submitted for histology. New blood vessel formation will be evaluated by H&E staining and immunohistochemical staining.
5. *** Principal investigator:**
   Hai Zhang
6. *** What is the intention of the animal protocol?**
   Experimental Research

View: SF: Experimental Research Protocol Addition

Experimental Research Protocol Addition

1. * **Will the protocol include breeding?**
   ○ Yes ● **No**

View: Custom SF: Protocol Team Members

Protocol Team Members

1. Identify each additional person involved in the design, conduct, or reporting of the research:

Name	Role	Involved in Animal Handling	Authorized To Order Animals	E-mail	Phone	FERPA RCW 42.56.070(1)
[redacted]	Graduate Student	yes	yes	[redacted]		

2. If veterinary care will be provided by individuals outside of DCM or WaNPRC, provide the name, credentials and contact information below:

N/A

View: Custom SF: Funding Sources

Funding Sources

1. Identify each organization supplying funding for the protocol:

	Funding Organization	eGC1 Number(s)
View	Restorative Dentistry	N/A

Scientific Aims

1. * Scientific aims of the research:

One aim is to establish a rat derived induced pluripotent stem cell (riPSC) cell line from rat peripheral blood mononuclear cell (rPBMC) and induce its initial differentiation towards riPSCs derived pre-osteoblasts (riPOBs). Another aim is to determine the effects of magnesium ion, riPOBs and I50 in a socket preservation model *in vivo*.

We hypothesize that the riPSC cell line will be successfully established, and magnesium ion can promote the proliferation and osteogenic differentiation of riPOBs in the rat socket preservation model. In addition, I50 can accelerate new blood vessel ingrowth in socket, thus prompting bone formation to achieve better socket preservation outcome.

2. * Using language understandable to non-scientists, describe the goals and significance of the protocol to humans, animals and science:

This research addresses a critical clinical problem (bone loss after tooth extraction resulting in deficient foundation tissue for implant or prosthetic treatment) that has a significant impact in the field of restorative dentistry. Many patients still need an operation to acquire enough bone for implant placement. The entire treatment is lengthy, costly and accompanied with morbidity. This has significantly affected patient's acceptance of implant therapy and quality of life during the treatment.

In recent years, magnesium, induced pluripotent stem cells (iPSCs, which is a new kind of stem cell that can differentiate to different body cells) and agent promoting vessel growth showed promising potential in bone regeneration. This research will generate iPSCs, and evaluate the effect of magnesium, iPSCs and an agent promoting vessel growth (which is called I50 in this study) on bone growth in a rat extraction socket. The results of this research will provide insights for new approaches either by applying them individually or combined. The outcome of the bone formation in the socket will be much improved and the need of additional surgical procedure will be significantly reduced. In the meantime, patient's satisfaction will be significantly improved due to the reduced surgical procedures and treatment time.

3. * Provide a statement to address the potential harm to the animals on this study (e.g., pain, distress, morbidity, mortality) relative to the benefits to be gained by performing the proposed work:

The animals in this study will lose one maxillary first molar, and experience post-operation local pain which can be controlled by analgesics, just as the human patients who undergo tooth extraction do. The chewing efficiency will decrease at the beginning, but will be gradually compensated by other teeth. The results of this research will provide insights for new approaches of socket preservation by evaluating the effect of magnesium, riPSCs and I50 on bone formation in rat extraction socket as well as the ridge dimension.

View: Custom SF: Experiments

Experiments

Note: If you will be administering cells, cell lines, sera or other biologicals to rodents, contact the Rodent Health Monitoring Program (RHMP, rhmp@uw.edu). Testing may be required prior to administration to rodents.

1. * Define the experiments to be used in this protocol:

Name	Species	USDA	Count	Count by Pain Category	Procedures	Husbandry Exception Types
01. Blood Collection for riPSC Cell Line Generation	Rats	no	2	B: 0 C: 0 D: 2 E: 0	▪ Other: Body Condition Score (Standard) ▪ Substance Administration: Anesthesia, Terminal, Ketamine and Xylazine (Standard) ▪ Tissue/Blood Collection: Zhang: Intracardiac Blood Collection Under Anesthesia (Team)	Rats - No husbandry or enrichment exceptions.

Name	Species	USDA	Count	Count by Pain Category	Procedures	Husbandry Exception Types
02. Socket Preservation - Pilot Study	Rats	no	6	B: 0 C: 0 D: 6 E: 0	▪ Euthanasia: CO2 followed by Secondary Method (>10 days of age) (Standard) ▪ Imaging: Zhang: Bioluminescence Imaging (Team) ▪ Other: Body Condition Score (Standard) ▪ Substance Administration: Analgesia, Meloxicam (SC, 72 hours) (Standard) ▪ Substance Administration: Zhang: Administration of Ampicillin (Team) ▪ Substance Administration: Zhang: Administration of Cyclosporine (Team) ▪ Substance Administration: Anesthesia, Ketamine and Xylazine (Standard) ▪ Substance Administration: Zhang: Filling Reagents in Tooth Socket (Team) ▪ Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team)	Rats - No husbandry or enrichment exceptions.

Name	Species	USDA	Count	Count by Pain Category	Procedures	Husbandry Exception Types
03. Socket Preservation - Full Study	Rats	no	72	B: 0 C: 0 D: 72 E: 0	▪ Euthanasia: CO2 followed by Secondary Method (>10 days of age) (Standard) ▪ Imaging: Zhang: Micro CT Imaging (Team) ▪ Other: Body Condition Score (Standard) ▪ Substance Administration: Anesthesia, Ketamine and Xylazine (Standard) ▪ Substance Administration: Zhang: Filling Reagents in Tooth Socket (Team) ▪ Substance Administration: Zhang: Administration of Ampicillin (Team) ▪ Substance Administration: Zhang: Administration of Cyclosporine (Team) ▪ Substance Administration: Analgesia, Meloxicam (SC, 72 hours) (Standard) ▪ Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team)	Rats - No husbandry or enrichment exceptions.

2. Will any single animal undergo more than one survival surgery? (include any animal that underwent surgery prior to use on this protocol) ○ Yes ● **No**

View: SF: Procedure Personnel Assignment

Procedure Personnel Assignment

1. * Select the team members who will be performing each procedure:

Procedure	Species	Is USDA Species	Team Members
Euthanasia: CO2 followed by Secondary Method (>10 days of age), ver. 2 (Standard)	Rats	no	
Imaging: Zhang: Bioluminescence Imaging, ver. 1 (Team)	Rats	no	
Imaging: Zhang: Micro CT Imaging, ver. 1 (Team)	Rats	no	
Other: Body Condition Score , ver. 1 (Standard)	Rats	no	
Substance Administration: Analgesia, Meloxicam (SC, 72 hours), ver. 1 (Standard)	Rats	no	
Substance Administration: Anesthesia, Ketamine and Xylazine, ver. 1 (Standard)	Rats	no	
Substance Administration: Anesthesia, Terminal, Ketamine and Xylazine, ver. 2 (Standard)	Rats	no	
Substance Administration: Zhang: Administration of Ampicillin, ver. 1 (Team)	Rats	no	
Substance Administration: Zhang: Administration of Cyclosporine, ver. 1 (Team)	Rats	no	
Substance Administration: Zhang: Filling Reagents in Tooth Socket, ver. 1 (Team)	Rats	no	
Survival Surgery: Zhang: Rat Tooth Extraction and Implantation, ver. 1 (Team)	Rats	no	
Tissue/Blood Collection: Zhang: Intracardiac Blood Collection Under Anesthesia, ver. 1 (Team)	Rats	no	

FERPA

RCW 42.56.070(1)

2. Team member training:

First Name Last Name Training

Course	Category	Source	Stage	Stage Number	Completion Date	Expiration Date
No experience data to display						

Course	Category	Source	Stage	Stage Number	Completion Date	Expiration Date
Annual DCM Facility Access Training (Rodent)	General	Online	Basic Course	Stage 1	2/13/2020	2/28/2021
Animal Use Medical Screening	General	Online	Basic Course	Stage 1	2/19/2020	2/28/2023
Rat Hands-On Laboratory	Animal Handling	In Person	Basic Course	Stage 1	3/5/2020	
Surgery Laboratory Part 2	Surgery	In Person	Basic Course	Stage 1	3/6/2020	
Surgery Laboratory Part 1A	Surgery	In Person	Basic Course	Stage 1	3/2/2020	
Animal Use Laws & Regulations	General	Online	Basic Course	Stage 1	10/8/2019	10/8/2024

Hai Zhang

Course	Category	Source	Stage	Stage Number	Completion Date	Expiration Date
Animal Use Laws & Regulations	General	Online	Basic Course	Stage 1	3/8/2017	3/8/2022
Foege Facility Orientation	Orientation	In Person	Basic Course	Stage 1	7/18/2014	
Annual DCM Facility Access Training (Rodent)	General	Online	Basic Course	Stage 1	3/1/2020	3/31/2021
Rat Online Course: Working with Rats at UW	Animal Handling	Online	Basic Course	Stage 1	3/5/2020	
Cervical Dislocation, Mouse Anesthetized	Procedure	In Person	Basic Course	Stage 1	1/19/2011	
Cervical Dislocation, Mouse Unanesthetized	Procedure	In Person	Basic Course	Stage 1	1/19/2011	
Mouse Hands-On Laboratory	Animal Handling	In Person	Basic Course	Stage 1	1/19/2011	
Animal Use Medical Screening	General	Online	Basic Course	Stage 1	1/17/2019	1/31/2022

No experience data to display

View: Custom SF: Animal Details

Animal Details

1. *** How are animals acquired?**
   Purchased
2. **Describe the acquisition for:**
   a. **Not purchasing through DCM or WaNPRC:**
      N/A
3. **Identification of individual animals (other than cage cards):**
   a. **Method(s) (e.g., ear punch/tag, tattoo, tagging/banding, radio collar, etc.) (Note: If method is implantation (e.g. PIT tag), create or select an Implant procedure to describe the details. If method is surgical (e.g., satellite tag), create or select Survival Surgery procedure to describe the details):**
      Ear tag
   b. **Will external identification be replaced if it falls off/out? If yes, describe the plan for replacement:**
      No
   c. **Will external identification be removed as part of the protocol (e.g., radio collars on field animals)? If yes, describe the plan for removal:**
      No
4. **Identify strain/stock for rodents and genetically modified animals:**

	Species	Is USDA Species	Strain	Genetically Modified Strain	Phenotype Description
View	Rats	no	Crl:CD(SD); Hsd:SD; NTac:SD (Sprague-Dawley)	no	No anticipated deleterious phenotypes.

View: Custom SF: Animal Number Adjustments

Animal Number Adjustments

"Animals Identified in Experiments" is the total number of animals per pain category listed in all experiments on this protocol. If more or fewer animals will be used on the protocol (see Help Text for examples), click Update to enter this new number in the corresponding "Adjusted Animal Count" column. **Only input numeric values in this field; 0 is acceptable.**
If no adjustment is required, the values in the "Animals Identified in Experiments" and "Adjusted Animal Count" columns must match. Click Update in each Pain Category row to input the matching value.
For questions about adjusting animal numbers, contact OAW.

1. * Click Update to adjust the number of animals to be used or produced for this protocol:

	Species	USDA Covered Species	Pain Category	Animals Identified in Experiments	Adjusted Animal Count
View	Rats	no	Pain Category B	0	0
View	Rats	no	Pain Category C	0	0
View	Rats	no	Pain Category D	80	80
View	Rats	no	Pain Category E	0	0

2. If you adjusted the number of animals for this protocol, explain why:

N/A

3. If you will be using animals to train personnel or to practice procedures included in this protocol, describe below:

N/A

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Alternatives and Duplication Searches

Alternatives and Duplication Searches

Display Procedures that cause pain or distress:

- Survival Surgery: Zhang: Rat Tooth Extraction and Implantation, ver. 1 (Team)

1. Record all searches for any previous research that this protocol might duplicate:

	Search Date	Searched Databases	Other
View	5/11/2020	EMBASE (searches multiple databases) Web of Science (searches multiple databases)	N/A
View	5/11/2020	EMBASE (searches multiple databases) Web of Science (searches multiple databases)	N/A

2. Briefly describe the results of your searches and why you can or cannot incorporate the findings. Or, if a literature search was not performed, describe the methods used to determine that alternatives are not available or feasible:

I50 used in this protocol is a newly synthesized compound, which hasn't been reported by any former articles. The effect of magnesium on riPSC hasn't been investigated, and riPSCs haven't been applied in animal ridge preservation model in combination with magnesium. So there's no duplicate of this protocol. The results of searches for alternatives don't yield any practical methods for this protocol. The results include culturing cells in various kinds of scaffolds. However, the in vitro models cannot totally mimic in vivo microenvironment of tooth socket, and cannot simulate bone maturation and angiogenesis at the same time. Thus, the animal experiments in this protocol cannot be replaced by in vitro studies.

3. Confirm that you have made every effort to ensure that this protocol is not unnecessary duplication of previous research: ☑

View: Custom SF: Housing and Use

Housing and Use

Housing and use outside of the vivarium is not allowed without strong scientific justification.

1. Identify each location where animals will be housed:

	Facility	Species	Justification for Housing Outside Vivarium
View	ARCF ABSL1	Rats	N/A

2. Identify each location where animals will be used:

	Facility Use	Species	Justification for Use Outside Vivarium
View	ARCF ABSL1 All procedures will be performed here. A cleared place will be used for operation and a power source will be needed for portal dental drill motor.	Rats	N/A

View: Custom SF: Disposition

Disposition

1. **Disposition plans for the animals when this research is complete:**
   (check all that apply)
   Euthanasia
2. **If other, provide an animal disposition description:**
   N/A
3. **If protocol involves fixing tissues, list agents (e.g., paraformaldehyde, formalin):**
   4% paraformaldehyde for fixing the bone tissue that is collected after euthanasia.

View: Custom SF: Refinement, Replacement and Reduction

Refinement, Replacement and Reduction

1. Describe below how the three R's (refinement, replacement and reduction) have been employed on this project. Include alternatives that were considered for the procedures above that cause pain or distress:

* Refinement (use of methods to decrease animals' sensitivity to pain)

Surgical refinements to reduce animal discomfort and stress will always be considered. Analgesia will begin before the surgery, and anesthesia will be given properly during tooth extraction to reduce pain to the largest extent.

* Replacement (include in vitro tests, use of less sentient animals)

Established iPSC cell line will be used prior to the animal experiments, and in vitro cell culture constitutes the bulk of the research done for our project. We will use in vitro experiment to determine the optimal concentrations of the MgCl2 solution for iPSCs osteogenic differentiation, instead of testing the concentrations in animal model. When strong and positive results are observed in vitro, these results must then will be confirmed in animal models- the living system. In vitro bone cultures have not been successful in our lab and others, so animal model is still irreplaceable to date.

* Reduction (use of fewer animals to attain statistical significance)

Larger sample size will provide higher statistical power for the study, however, use of power analysis (based on pilot work) helped us identify the minimal sample size needed to draw a valid conclusion. Whenever a reduction is possible without compromising the findings, it is undertaken.

2. Describe the rationale for using animals and the appropriateness of the species proposed:

The in vitro cell experiments can't totally mimic the environment of live animal. In this study, bone formation is influenced not only by the reagents filled in the bony defect, but also by the cytokines circulating with the blood and microenvironment in 3D live tissue. So the animal model is non-substitutable. Rat and mouse are most commonly used animals in dentistry experiment. In our research, rats are more suitable because the larger size of molar makes it easier to perform the operation.

View: SF: Supporting Documents

Supporting Documents

1. Attach supporting files:

Document Name	Date Modified
flow chart (1).pptx	5/11/2020 1:04 PM

Procedures Appendix:

HoverBoard

View: Custom SF: Procedure Identification

Procedure Identification: Body Condition Score

1. * Name of the procedure or surgery:
Body Condition Score

2. * Select procedure type:
Other

3. * Species:
Rats

4. * Will administering this procedure cause any more than momentary pain or distress? Yes **No**

If yes,

i. Identify expected symptoms from administering this procedure:
N/A

ii. Identify criteria under which animals will be removed from research:
N/A

View: Custom SF: Other

Other

1. Description of Procedure:

Rats are handled gently during palpation of bony prominences over the shoulders, spinal column, and pelvis. This is usually performed with rats standing comfortably on the wiretop of the cage with minimal tail-base restraint.

A numerical Body Condition Score (BCS) (see attached diagram from Hickman and Swan, 2010) is assigned for each individual animal. Frequency of BCS assessment is described in the experiment.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
Hickman and Swan, 2010.pdf	10/6/2016 5:25 PM

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Bioluminescence Imaging

1. *** Name of the procedure or surgery:**
   Zhang: Bioluminescence Imaging
2. *** Select procedure type:**
   Imaging
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Imaging

Imaging

1. **Imaging types:**
   Other
2. **If Other, specify:**
   Bioluminescence imaging
3. **Select the anesthesia and analgesia procedures to be used:**

Anesthesia, Ketamine and Xylazine	Substance Administration	1 Standard

4. **Frequency, including minimum time between imaging sessions and the maximum number of sessions (enter specific, detailed procedure timing in the Experiment):**
   See experiment for timing and frequency.
5. **Duration of imaging session:**
   Approximately 15-30 minutes
6. **Purpose:**
   To detect transplanted cell survival with bioluminescence imaging
7. **Will supportive care of animals be necessary during the imaging session?**
   Yes No
8. **If yes, describe:**
   Upon removal from the scanner, rats are placed in a recovery cage until they resume normal ambulation. The recovery cage is warmed to 37°C with a small animal heating pad.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Administration of Cyclosporine

1. *** Name of the procedure or surgery:**
   Zhang: Administration of Cyclosporine
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)	Standard	Subcutaneous	10mg/kg	N/A	250-500 uL	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Cyclosporine A will be diluted to the appropriate concentration and then administered via SC injection. The daily administration of cyclosporine A (10mg/kg) begins at three days before grafting and continue daily until sacrifice.

3. Describe the intended effects of administering the substance(s):

To suppress transplant rejection.

4. Describe any potential adverse reactions to administering the substance(s):

High doses cause renal and hepatic toxicity.

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

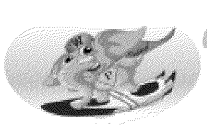
1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)
2. **Route:**
   Subcutaneous

   If you indicated Other, specify the route:
3. **Dose:**
   10mg/kg
4. **Frequency and duration of dosages:**
   Once per day; see experiment for duration
5. **Volume (for rodents or intracranial injections):**
   250-500 uL
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Cyclosporine A will be pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A



View: Custom SF: Procedure Identification

Procedure Identification: Anesthesia, Ketamine and Xylazine

1. *** Name of the procedure or surgery:**
   Anesthesia, Ketamine and Xylazine
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Ketamine (Ketaset, Ketaflo, Vetalar)	Standard	Intraperitoneal	45 – 68.2 mg/kg	N/A	Up to 10 microliters per gram of body weight	N/A
View	Xylazine	Standard	Intraperitoneal	1.1 – 4.4 mg/kg	N/A	Up to 10 microliters per gram of body weight	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Ketamine/Xylazine is mixed together and diluted in sterile pharmaceutical grade saline. The cocktail is administered IP to induce 25-30 minutes of general anesthesia. Appropriate depth of anesthesia is monitored by respiratory rate, corneal reflex, and response to front toe pinch. Heat support and eye lubrication will be provided.

3. Describe the intended effects of administering the substance(s):

General anesthesia

4. Describe any potential adverse reactions to administering the substance(s):

Respiratory and cardiac depression, including bradycardia and hypotension

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Needles must not be recapped unless a recapping device is used.

Gloves must be worn when handling these agents.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Ketamine (Ketaset, Ketaflo, Vetalar)
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   45 – 68.2 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Up to 10 microliters per gram of body weight
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Ketamine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   Xylazine
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   1.1 – 4.4 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Up to 10 microliters per gram of body weight
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Xylazine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Rat Tooth Extraction and Implantation

1. *** Name of the procedure or surgery:**
   Zhang: Rat Tooth Extraction and Implantation
2. *** Select procedure type:**
   Survival Surgery
3. *** Species:**
   Rats

4. * Will administering this procedure cause any more than momentary pain or distress? **Yes** No

If yes,

i. **Identify expected symptoms from administering this procedure:**
This procedure is expected to cause discomfort that should be relieved by anesthesia and/or analgesia. Please see procedure description and/or experimental description for monitoring plan, including specific behavioral and clinical signs to be monitored.

ii. **Identify criteria under which animals will be removed from research:**
Please see experimental description for end point criteria.

View: Custom SF: Survival Surgery

Survival Surgery

1. *** Surgery Type:**
   Major
2. *** Describe how the animal, surgeon, and instruments will be prepared for surgery:**
   Surgical instruments including dental explorer and dental bur will be autoclaved prior to the initial surgery. Several sets of sterile instruments that are sufficient for one day use will be prepared.

   Sterile surgical gloves and face mask will be used. Surgical gloves will be changed between animals. No food/water restriction will be needed. Animal will be weighed prior to surgery.

   Extraction site will be scrubbed by sterile gauze soaked with 1% chlorhexidine and dried using sterile gauze prior to tooth extraction. Rats will receive eye lubricant in each eye to prevent corneal drying. Rats will be anesthetized through injection of ketamine and xylazine cocktail mixed with sterile 0.9% saline and mounted on a jaw retraction board.
3. *** Describe the surgical procedure, including any deficits expected as a result of the surgery:**

   The rats will be kept warm on a warm-water pad during the surgical procedure. After anesthetized by intraperitoneal injection with ketamine and xylazine, 1 mg/kg meloxicam will be injected subcutaneously, and sterile eye lubricant will be placed in each eye.

   The left maxillary first molars will be extracted, and a standardized bone defect (approximately 3 mm in length, 2.6 mm in width and 2 mm in depth) will be created in the extraction area with approximately 0.5 mm to the mesial of maxillary second molar using a sterilized round bur and copious amount of sterilized saline for cooling. Depending on the experiment/group, the defects will be (1) left unfilled, or (2) filled by bone mineralized matrix (Bio-Oss®) solely, or (3) filled by bone mineralized matrix (Bio-Oss®) mixed with different reagents ($MgCl_2$ solution, Tie2 super agonist, osteogenic pre-induced riPSCs (riPOBs) solely or in combination). See related experiments for filling reagents in each group. Collagen membrane (Bio-Gide®, approximately 3×3 mm^2) will be sutured to gingival margin by 5-0 absorbable suture to seal the wound.

   Duration of procedure: 40 minutes.

   Expected Deficits: difficulty with eating during 24-48 hours post-surgery. Soft food will be provided during this period.
4. *** Select associated substance administration procedures, including anesthesia and analgesia procedures to be used:**

Analgesia, Meloxicam (SC, 72 hours)	Substance Administration	1 Standard
Anesthesia, Ketamine and Xylazine	Substance Administration	1 Standard
Zhang: Administration of Ampicillin	Substance Administration	1 Team

5. **Describe how animals will be monitored during the procedure:**
   Front toe pinch will be used to monitor depth of Ketamine/Xylazine anesthesia. Heart

rate, respiration, temperature, movement, relaxed jaw, corneal reflex will be monitored until the rats recover from anesthesia.

6. Describe the routine for postoperative care: (including removal of sutures, if applicable)

The rats will be placed in the recovery cage and monitored every 5 minutes for breathing rate and body temperature. After righting reflex has been regained, rats will be placed into a clean cage with gel on the cage floor and immediately placed back into the rack in the housing room.

We anticipate that subcutaneous injections of meloxicam will last for 20-24 hours, so we will administer meloxicam every 20-24 hours for 72 hours post-surgery. If rats are exhibiting signs of pain or distress, we will consult with Vet Services.

All animals will also be under penicillin/ampicillin treatment (8WU for intraperitoneal injection daily) for 5 days, starting on the day of the operation to prevent infection. If prominent reduction of iPOB longevity is proved in the pilot study, immunosuppressant (10mg/kg cyclosporine A daily) will be administered in iPOB-transplanted groups in formal experiment beginning three days before grafting and continuously until sacrifice. See related procedures for details.

Animals will be monitored daily for 7 days post-surgery for body weight, signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. The surgical site will be monitored daily for 3 days post-surgery for bleeding, dislodgement of suture and any signs of infection such as redness, swelling and pus. Because the wound will be closed by absorbable sutures, the removal of sutures will not be needed.

7. Describe how postoperative pain and distress will be assessed:

(including need for further care)

The analgesic (subcutaneous injections of meloxicam) will be administered for 72 hours as described in the related procedure. Animals will be monitored daily for 7 days post-surgery for body weight, signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. If rats are exhibiting signs of pain or distress, we will consult with Vet Services.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
diagram for rat socket preservation model.docx	2/18/2020 3:40 PM

View: Custom SF: Procedure Identification

Procedure Identification: Anesthesia, Terminal, Ketamine and Xylazine

1. *** Name of the procedure or surgery:**
   Anesthesia, Terminal, Ketamine and Xylazine
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Ketamine (Ketaset, Ketaflo, Vetalar)	Standard	Intraperitoneal	≥68.2 mg/kg	N/A	Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.	N/A
View	Xylazine	Standard	Intraperitoneal	≥4.4 mg/kg	N/A	Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Ketamine/Xylazine is mixed together and diluted in sterile pharmaceutical grade saline or water. The cocktail is administered IP to induce anesthesia appropriate for a short (<20 minutes) terminal procedure such as perfusion.

Deep anesthesia is confirmed by lack of response to toe pinch, change in respiratory character and decreased respiratory rate.

3. Describe the intended effects of administering the substance(s):

Anesthesia for short (<20 minutes) terminal procedure

4. Describe any potential adverse reactions to administering the substance(s):

N/A

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Needles must not be recapped unless a recapping device is used.

Gloves must be worn when handling this agent.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA)**

paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Ketamine (Ketaset, Ketaflo, Vetalar)
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   ≥68.2 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Ketamine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   Xylazine
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   ≥4.4 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Xylazine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: CO2 followed by Secondary Method (>10 days of age)

1. *** Name of the procedure or surgery:**
   CO2 followed by Secondary Method (>10 days of age)
2. *** Select procedure type:**
   Euthanasia
3. *** Species:**
   Rats

4. * Will administering this procedure cause any more than momentary pain or distress? Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

View: Custom SF: Euthanasia

Euthanasia

1. *** Method of euthanasia:**
   CO2 Overdose
2. **Describe procedure:**
   CO2 will be administered from a compressed commercial cylinder utilizing a flow meter to deliver 30-70% of the chamber volume per minute. Total gas exposure will be at least 10 minutes, with gas flow being maintained for at least 1 minute after apparent clinical death. A timer will be used to ensure adequate length of exposure.

   Secondary method will be one of the following: placed in a bag filled with CO2, decapitation, exsanguination, thoracotomy/tissue collection.
3. *** Will anesthesia be used?** Yes **No**
4. **Describe how death will be confirmed:**
   Death will be confirmed by lack of respirations and heartbeat.
5. **Is this method approved by the AVMA Guidelines on Euthanasia (2013)?**
   Yes No

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Intracardiac Blood Collection Under Anesthesia

1. *** Name of the procedure or surgery:**
   Zhang: Intracardiac Blood Collection Under Anesthesia
2. *** Select procedure type:**
   Tissue/Blood Collection
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Live Tissue/Blood Collection

Live Tissue/Blood Collection

1. *** Identify tissues to be collected:**
   Blood from heart
2. **Describe timing and frequency of collection and amount to be collected:**
   Volume: 2-4 ml. Once during terminal procedure under anesthesia.
3. **Select the anesthesia and analgesia procedures to be used:**

Anesthesia, Terminal, Ketamine and Xylazine	Substance Administration	2 Standard

4. **If withholding anesthesia/analgesia when normally required, provide scientific justification:**
   N/A
5. **Describe any potential complications from collection:**
   None anticipated.
6. *** Describe the collection procedure:**
   The rat is anesthetized. Before thorax opening, front toe pinch will be used to monitor depth of ketamine/xylazine anesthesia. The thorax is then opened to expose the heart. A 5-ml syringe with a 15G needle are primed with EDTA to prevent clotting. The needle is introduced into a heart ventricle and a terminal blood sample is collected.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Administration of Ampicillin

1. *** Name of the procedure or surgery:**
   Zhang: Administration of Ampicillin
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Penicillin (Ampicillin)	Standard	Intraperitoneal	80,000 IU	N/A	0.2 ml	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Penicillin G sodium will be injected intraperitoneally 1 hour before surgery and daily for 4 days after surgery.

Restrain the rats appropriately in the head-down position. Injections are performed with a small gauge needles (22 to 27 gauge), which is inserted to the depth in which the entire bevel is within the abdominal cavity. The barrel of the syringe is drawn back to make certain that the needle is not in a blood vessel. The injection is delivered slowly but steadily, checking for leakage around needle.

3. Describe the intended effects of administering the substance(s):

Broad-spectrum antibiotic to reduce the risk of infection as a result of biomaterials and reagents implantation.

4. Describe any potential adverse reactions to administering the substance(s):

No side effects have been seen with this antibiotic.

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Penicillin (Ampicillin)
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
3. **Dose:**
   80,000 IU
4. **Frequency and duration of dosages:**
   Daily for 5 days
5. **Volume (for rodents or intracranial injections):**
   0.2 ml
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Pharmaceutical grade will be obtained
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Filling Reagents in Tooth Socket

1. *** Name of the procedure or surgery:**
   Zhang: Filling Reagents in Tooth Socket
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Bone mineralized matrix (Bio-Oss®)	Team	Other	N/A	N/A	Approximately 16 cubic milliliter	N/A
View	I53-50 (abbreviated as I50)	Team	Other	N/A	1000ng/ml of F-domains	20µl	N/A
View	magnesium chloride	Team	Other	N/A	0.8, 1.8, 5, 10 or 20 mM (decided by in vitro experiments)	20µl	N/A
View	riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)	Team	Other	20,000 cells per site in media	N/A	N/A	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Bone mineralized matrix (Bio-Oss®) alone or together with one or more of the below reagents will be implanted into the tooth socket of rats (prepared into a box-like defect) during a survival surgery (see related survival surgery for details):

- riPOBs (riPOBs used in pilot study will be labelled with luciferase prior to implantation)
- magnesium chloride solution
- I50

See the treatment of each group in experiment protocol for combination of the reagents in each group.

Note: The optimal concentration of magnesium chloride will be verified by cell proliferation and osteogenic differentiation experiments on riPOBs (riPSCs derived pre-osteoblasts) in vitro, and will be applied in grafting procedure. The concentration will be chosen from 0.8, 1.8, 5, 10 and 20 mM.

3. Describe the intended effects of administering the substance(s):

riPOBs labelled with luciferase: To test the longevity of transplanted riPOBs in rat tooth socket with or without immunosuppressive therapy.

riPOBs used in full study: To promote bone growth in rat tooth socket.

I50 is a Tie2 super agonist that can activate Ang-1/Tie2 pathway, thus to accelerate angiogenesis and promote bone growth in rat tooth socket.

Magnesium chloride: To reduce bone resorption after tooth extraction, and promote bone growth in tooth socket.

4. **Describe any potential adverse reactions to administering the substance(s):**
   No
5. **If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.**
6. *** Does this procedure include the use of a paralytic agent?**
   Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Bone mineralized matrix (Bio-Oss®)
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket
3. **Dose:**
   N/A
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   Approximately 16 cubic milliliter
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Bone mineralized matrix (Bio-Oss®) is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   I53-50 (abbreviated as I50)
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket together with bone matrix
3. **Dose:**
   N/A
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   20µl
6. **Concentration:**
   1000ng/ml of F-domains
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   I50 is an investigational new compound, not available in pharmaceutical grade. It will be dissovled in Dulbecco's Modified Eagle Medium (or similar media) and sterile filtered prior to use.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   magnesium chloride
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket together with bone matrix
3. **Dose:**
   N/A
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   20µl
6. **Concentration:**
   0.8, 1.8, 5, 10 or 20 mM (decided by in vitro experiments)
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Magnesium chloride is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket together with bone matrix
3. **Dose:**
   20,000 cells per site in media
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   N/A
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Not available pharmaceutical grade; cell mixture will be prepared in sterile culture hood prior to administration.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Micro CT Imaging

1. *** Name of the procedure or surgery:**
   Zhang: Micro CT Imaging
2. *** Select procedure type:**
   Imaging
3. *** Species:**
   Rats

4. * Will administering this procedure cause any more than momentary pain or distress? Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

View: Custom SF: Imaging

Imaging

1. **Imaging types:**
   Computed Tomography (CT)
2. **If Other, specify:**
3. **Select the anesthesia and analgesia procedures to be used:**

Anesthesia, Ketamine and Xylazine	Substance Administration	1 Standard

4. **Frequency, including minimum time between imaging sessions and the maximum number of sessions (enter specific, detailed procedure timing in the Experiment):**
   See experiment for timing and frequency.
5. **Duration of imaging session:**
   20 minutes
6. **Purpose:**
   Within experiment assessment of bone formation in mandible defect.
7. **Will supportive care of animals be necessary during the imaging session?**
   Yes No
8. **If yes, describe:**
   Upon removal from microCT scanner, rats are placed in a recovery cage until they resume normal ambulation. The recovery cage is warmed to 37°C with a small animal heating pad.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Procedure Identification

Procedure Identification: Analgesia, Meloxicam (SC, 72 hours)

1. *** Name of the procedure or surgery:**
   Analgesia, Meloxicam (SC, 72 hours)
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

View: Custom SF: Administration of Substances

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Meloxicam (Metacam)	Standard	Subcutaneous	1 mg/kg	N/A	Total volume will not exceed 5 mL/kg of body weight.	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

One dose of meloxicam will be injected subcutaneously (SC) at least 30 minutes prior to recovery from the procedure, and then administered every 20-24 hours for 72 hours.

If signs of pain are noted despite meloxicam administration or following this period, Veterinary Services will be consulted.

If dilution is necessary, it will be done with sterile water or saline for injection (pharmaceutical grade).

Note: Many category 2 and 3 procedures require multimodal analgesia and more than one type of analgesic is ideally administered. Please consult with Veterinary Services if questions. Please refer to the IACUC policy on "Analgesia in Research Animals," for more information on what types of procedures fall into this category.

3. Describe the intended effects of administering the substance(s):

Provide analgesia for 72 hours

4. Describe any potential adverse reactions to administering the substance(s):

Gastrointestinal ulceration or bleeding, renal toxicity

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Needles must not be recapped unless a recapping device is used.

Gloves must be worn when handling this agent.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA)**

paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or ehsbio@uw.edu**.**

View: SF: Procedure Documents

Procedure Documents

1. Supporting documents:

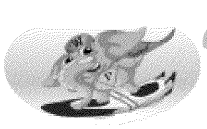
Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Meloxicam (Metacam)
2. **Route:**
   Subcutaneous

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   1 mg/kg
4. **Frequency and duration of dosages:**
   Once at the time of the procedure, then every 20-24 hours for 72 hours
5. **Volume (for rodents or intracranial injections):**
   Total volume will not exceed 5 mL/kg of body weight.
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Meloxicam is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

Substances Appendix:



View: Custom SF: Substance Information

Substance Information: riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)

1. *** Name:**
   riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)
2. *** Substance types:** (select all that apply)
   Cell, Cell Line, or Tissue - Other

3. *** Is this a hazardous agent:** Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: I53-50 (abbreviated as I50)

1. *** Name:**
   I53-50 (abbreviated as I50)
2. *** Substance types:** (select all that apply)
   Chemical Agent
3. *** Is this a hazardous agent:** Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: magnesium chloride

1. *** Name:**
   magnesium chloride
2. *** Substance types:** (select all that apply)
   Chemical Agent
3. *** Is this a hazardous agent:** Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Bone mineralized matrix (Bio-Oss®)

1. * Name:

Bone mineralized matrix (Bio-Oss®)

2. * Substance types: (select all that apply)

Other

3. * Is this a hazardous agent: Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Ketamine (Ketaset, Ketaflo, Vetalar)

1. * Name:

Ketamine (Ketaset, Ketaflo, Vetalar)

2. * Substance types: (select all that apply)

Anesthetic
Reproductive Hazard/Teratogen

3. * Is this a hazardous agent: **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Xylazine

1. * Name:

Xylazine

2. * Substance types: (select all that apply)

Anesthetic

3. * Is this a hazardous agent: **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Meloxicam (Metacam)

1. * Name:

Meloxicam (Metacam)

2. * Substance types: (select all that apply)

Analgesic
Reproductive Hazard/Teratogen
Other

3. * Is this a hazardous agent: **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions,**

contact EH&S Research and Occupational Safety at 206-221-7770 or ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Penicillin (Ampicillin)

1. * Name:

Penicillin (Ampicillin)

2. * Substance types: (select all that apply)

Antibiotic
Reproductive Hazard/Teratogen

3. * Is this a hazardous agent: **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)

1. * Name:

Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)

2. * Substance types: (select all that apply)

Analgesic
Carcinogen
Immunosuppressant
Reproductive Hazard/Teratogen

3. * Is this a hazardous agent: **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create and Edit

1. *** Select the funding organization:**
   Restorative Dentistry

   If Other was selected in question 1, provide Funding Organization:

2. *** All animal use projects must be reviewed for scientific merit prior to initiating animal use. Choose the required reviews for this project:**
   Has already been conducted and approved by a funding agency

3. **Provide name of the committee or the department reviewer** (Required if "Has been conducted by my department or school and has been found to be scientifically meritorious" was selected):
   Dr. Marty Anderson, Margaret Spencer Fund Committee Chair

4. **eGC1 Number(s):**(assigned internally)
   N/A

View: Custom: Create and Edit

Experiments Appendix:

01. Blood Collection for riPSC Cell Line Generation

1. *** Experiment name:**
   01. Blood Collection for riPSC Cell Line Generation
2. *** Species:**
   Rats
3. **If other was selected, provide a species:**
4. **What is the scientific goal of this experiment:**
   To establish a rat induced pluripotent stem cell (riPSC) cell line with rat peripheral blood mononuclear cells (rPBMC).
5. *** Describe the animal experience in the experiment, from enrollment in the study to the final endpoint, including all procedures in chronological order and the minimum time between procedures. We encourage using bullet points, timeline, table, or a flow chart as appropriate:**

   1. Rats will be anesthetized by ketamine/xylazine cocktail overdose before blood collection.

   2. **Thorax will be opened, and 2-4 milliliter blood will be collected from heart ventricle. Rat will be euthanized by exsanguination.**

   Animal Sex:
   Female

   Animal Ages:
   3 months

   Animal Size:
   250-300g
6. **Select experimental procedures:**

Name	Type	Version	Scope
Body Condition Score	Other	1	Standard
Anesthesia, Terminal, Ketamine and Xylazine	Substance Administration	2	Standard
Zhang: Intracardiac Blood Collection Under Anesthesia	Tissue/Blood Collection	1	Team

7. **Monitoring protocol, including frequency and specific behavioral and clinical signs to be monitored. Include humane endpoints (criteria for euthanasia):**
   The rats will be monitored once every week for weight, body condition score and

other general condition before euthanasia.

Rats will be euthanized before blood collection according to the following criteria:
1. More than 20% weight loss;
2. Body condition score of 2 or less. See Body Condition Score Procedure for detailed evaluation method.
3. Inability or reluctance to move when stimulated, or moribund condition.
4. Impairment of ability to eat, drink, or ambulate normally.
5. Labored breathing.
6. Hypothermia.
7. Ulcerated tumors.

8. If there is expected mortality (spontaneous death) in this experiment:

a. Procedure/condition associated with mortality:
N/A

b. Estimated mortality rate, i.e. percentage of animals expected to die spontaneously (not via euthanasia) or need to be euthanized as a result of the procedure. (Be sure to account for this in your animal number calculations):
N/A

c. Explain why euthanasia is not possible or appropriate:
N/A

9. Will some animals live out their natural lifespan as part of this experiment? If so, indicate their use and describe the monitoring plan for aged animals (e.g., rodents >18 months of age), including frequency, behavioral and clinical signs to be monitored and criteria for euthanasia.
No

10. * Total number of animals used in this experiment:(including all the animals to be produced)
2

a. Justify total number of animals used in this experiment:

Two milliliter blood is needed for isolation of the monocytes at one time according to manufacturer's instruction (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/General_Information/1/ge-isolation-of-mononuclear-cells.pdf). It is difficult to collect this volume of blood from other sites, and blood sample is easy to get contaminated by other survival blood collection techniques. So exsanguination by this open method is more suitable for our cell line generation purpose. One rat is needed for single blood collection procedure. One more rat will be prepared for additional blood collection in case the iPSCs generation fails.

11. Number of animals by pain and distress category:(include each animal only once in the highest pain category)
B: 0
C: 0
D: 2
E: 0

a. Justify the need for any animals in pain category E:

N/A

12. * Identify husbandry exceptions:

	Exception Type	Description and Justification
View	Rats - No husbandry or enrichment exceptions.	N/A

13. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create and Edit

1. * Exception type:

Rats - No husbandry or enrichment exceptions.

2. Description and justification:

N/A

View: Custom: Create and Edit

02. Socket Preservation - Pilot Study

1. *** Experiment name:**
   02. Socket Preservation - Pilot Study
2. *** Species:**
   Rats
3. **If other was selected, provide a species:**
4. **What is the scientific goal of this experiment:**
   To evaluate the longevity of transplanted riPSCs derived pre-osteoblasts (riPOBs) in rat tooth socket, and the efficacy of immunosuppressive therapy on survival of riPOBs.
5. *** Describe the animal experience in the experiment, from enrollment in the study to the final endpoint, including all procedures in chronological order and the minimum time between procedures. We encourage using bullet points, timeline, table, or a flow chart as appropriate:**
   1. **Three-month-old SD rats will be enrolled in this study. Female rats will be selected because the size is smaller and easier to handle compared to male ones.**
   2. **Animals will be divided into 2 groups (n=3 in each group). All rats will receive left maxillary first molar extraction and bony defect creation, with defect filled by riPOBs and** Bio-oss**. The immunosuppressive therapy group will receive immunosuppressant administration, while control group will not. See procedure** Zhang: Rat Tooth Extraction and Implantation **for detailed process of surgery, and** Zhang: Administration of Cyclosporine **for details of** immunosuppressant administration**.**
   3. **A**nalgesic **and antibiotic will be administered as described in the related procedures. Immunosuppressant will be administrated in** immunosuppressive therapy group**.** See procedures Analgesia, Meloxicam (SC, 72 hours), Zhang: Administration of Ampicillin, and Zhang: Administration of Cyclosporine for details of drug administration.
   4. **Transplanted cell survival will be monitored at 2 weeks and 6 weeks post-operative via bioluminescent imaging (BLI)**. See procedure Zhang: bioluminescence imaging for details of BLI.
   5. Euthanasia will be conducted a**t 6 weeks post-operation.**

Animal Sex:
Female

Animal Ages:
3 months

Animal Size:
250-300g

6. Select experimental procedures:

Name	Type	Version	Scope
CO2 followed by Secondary Method (>10 days of age)	Euthanasia	2	Standard
Zhang: Bioluminescence Imaging	Imaging	1	Team
Body Condition Score	Other	1	Standard
Analgesia, Meloxicam (SC, 72 hours)	Substance Administration	1	Standard
Anesthesia, Ketamine and Xylazine	Substance Administration	1	Standard
Zhang: Administration of Ampicillin	Substance Administration	1	Team
Zhang: Administration of Cyclosporine	Substance Administration	1	Team
Zhang: Filling Reagents in Tooth Socket	Substance Administration	1	Team
Zhang: Rat Tooth Extraction and Implantation	Survival Surgery	1	Team

7. Monitoring protocol, including frequency and specific behavioral and clinical signs to be monitored. Include humane endpoints (criteria for euthanasia):
Animals will be monitored daily for 7 days post-surgery for body weight, signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. The surgical site will be monitored daily for 3 days post-surgery for bleeding, dislodgement of suture and any signs of infection such as redness, swelling and pus. Then, the rats will be monitored 3 times a week for appetite, body weight, signs of pain/distress and signs of infection or tumor generation in surgical site throughout to the endpoint .

Criteria for euthanasia:
1. More than 20% weight loss;
2. Body condition score of 2 or less. See Body Condition Score Procedure for detailed evaluation method.
3. Inability or reluctance to move when stimulated, or moribund condition.
4. Impairment of ability to eat, drink, or ambulate normally.

5. Labored breathing.
6. Ulcerated tumors.
7. Severe infection in surgical site.

8. If there is expected mortality (spontaneous death) in this experiment:

a. Procedure/condition associated with mortality:
N/A

b. Estimated mortality rate, i.e. percentage of animals expected to die spontaneously (not via euthanasia) or need to be euthanized as a result of the procedure. (Be sure to account for this in your animal number calculations):
N/A

c. Explain why euthanasia is not possible or appropriate:
N/A

9. Will some animals live out their natural lifespan as part of this experiment? If so, indicate their use and describe the monitoring plan for aged animals (e.g., rodents >18 months of age), including frequency, behavioral and clinical signs to be monitored and criteria for euthanasia.
N/A

10. * Total number of animals used in this experiment:(including all the animals to be produced)
6

a. Justify total number of animals used in this experiment:

This is only a pilot study, and we only want to observe the trend of cell survival, and want to include the least number of animals possible while still drawing a valid conclusion. We will use 3 rats per group, and have 2 groups (control, immunosuppressive therapy).

We based our immunosuppressive therapy and choice in group number on similar work performed by Li et al (Li K, Javed E, Scura D, Hala TJ, Seetharam S, Falnikar A, et al. Human iPS cell-derived astrocyte transplants preserve respiratory function after spinal cord injury. Experimental Neurology 2015;271:479-92), which included 3 animals in each group for each time point, and allowed them to see positive results. We will start with this sample size. If it doesn't work, we will amend the protocol to expand the sample size (based on the acquired pilot data) and/or amend the immunosuppressive therapy as needed.

11. Number of animals by pain and distress category:(include each animal only once in the highest pain category)

B: 0
C: 0
D: 6
E: 0

a. Justify the need for any animals in pain category E:
N/A

12. * Identify husbandry exceptions:

Exception Type	Description and Justification

	Exception Type	Description and Justification
View	Rats - No husbandry or enrichment exceptions.	N/A

13. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create and Edit

1. * Exception type:

Rats - No husbandry or enrichment exceptions.

2. Description and justification:

N/A

View: Custom: Create and Edit

03. Socket Preservation - Full Study

1. *** Experiment name:**
   03. Socket Preservation - Full Study
2. *** Species:**
   Rats
3. **If other was selected, provide a species:**
4. **What is the scientific goal of this experiment:**
   To determine the effects of magnesium ion, riPOBs and I50 in a socket preservation model in vivo.
5. *** Describe the animal experience in the experiment, from enrollment in the study to the final endpoint, including all procedures in chronological order and the minimum time between procedures. We encourage using bullet points, timeline, table, or a flow chart as appropriate:**

   a. **Three-month-old SD rats will be enrolled in this study. Female rats will be selected because the size is smaller and easier to handle compared to male ones.**

   b. **Animals will be divided into 9 groups (n=8 in each group). All rats will receive left maxillary first molar extraction and bony defect creation, with defect filled by different combination of Bio-oss, magnesium chloride solution, I50 and riPOBs or left untreated. Treatment design for each group is listed in the following table. See procedure** Zhang: Rat Tooth Extraction and Implantation **for detailed process of surgery.**

Table: Groups in Animal Study

Group Number	Bone Mineralized Matrix (BMM)	riPOBs	$MgCl_2$	I50
1	Applied			
2	Applied	Applied		
3	Applied		Applied	
4	Applied			Applied
5	Applied	Applied	Applied	
6	Applied	Applied		Applied

7	Applied		Applied	Applied
8	Applied	Applied	Applied	Applied
9				

3. Analgesic and antibiotic will be given as described in the related procedures. Immunosuppressant will be administrated if immune rejection is proven to be prominent and immunosuppressant is verified to be necessary in pilot study (see Expt 02). See procedures Analgesia, Meloxicam (SC, 72 hours), Zhang: Administration of Ampicillin, and Zhang: Administration of Cyclosporine for details of drug administration.

4. We will evaluate animals at 2 weeks post-operation by micro CT under anesthesia.

5. Euthanasia will be conducted at 6 weeks post-operation.

Animal Sex:
Female

Animal Ages:
3 months

Animal Size:
250-300g

6. Select experimental procedures:

Name	Type	Version	Scope
CO2 followed by Secondary Method (>10 days of age)	Euthanasia	2	Standard
Zhang: Micro CT Imaging	Imaging	1	Team
Body Condition Score	Other	1	Standard
Analgesia, Meloxicam (SC, 72 hours)	Substance Administration	1	Standard
Anesthesia, Ketamine and Xylazine	Substance Administration	1	Standard
Zhang: Administration of Ampicillin	Substance Administration	1	Team
Zhang: Administration of Cyclosporine	Substance Administration	1	Team
Zhang: Filling Reagents in Tooth Socket	Substance Administration	1	Team
Zhang: Rat Tooth Extraction and Implantation	Survival Surgery	1	Team

7. **Monitoring protocol, including frequency and specific behavioral and clinical signs to be monitored. Include humane endpoints (criteria for euthanasia):**
Animals will be monitored daily for 7 days post-surgery for body weight, signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. The surgical site will be monitored daily for 3 days post-surgery for bleeding, dislodgement of suture and any signs of infection such as redness, swelling and pus. Then, the rats will be monitored 3 times a week for appetite, body weight, signs of pain/distress and signs of infection or tumor generation in surgical site throughout to the endpoint.

Criteria for euthanasia:
1. More than 20% weight loss;
2. Body condition score of 2 or less. See Body Condition Score Procedure for detailed evaluation method.
3. Inability or reluctance to move when stimulated, or moribund condition.
4. Impairment of ability to eat, drink, or ambulate normally.
5. Labored breathing.
6. Ulcerated tumors.
7. Severe infection in surgical site.

8. **If there is expected mortality (spontaneous death) in this experiment:**
   a. Procedure/condition associated with mortality:
   N/A
   b. Estimated mortality rate, i.e. percentage of animals expected to die spontaneously (not via euthanasia) or need to be euthanized as a result of the procedure. (Be sure to account for this in your animal number calculations):
   N/A
   c. Explain why euthanasia is not possible or appropriate:
   N/A

9. **Will some animals live out their natural lifespan as part of this experiment? If so, indicate their use and describe the monitoring plan for aged animals (e.g., rodents >18 months of age), including frequency, behavioral and clinical signs to be monitored and criteria for euthanasia.**
N/A

10. *** Total number of animals used in this experiment:**(including all the animals to be produced)
72
    a. **Justify total number of animals used in this experiment:**

    We used an effect size of 0.75 which was the effect size found in the pilot study. Using ANOVA with a significance level of 0.05, there will be 90% power to detect an effect size of 0.75. This applies to the effects of each of the 3 factors (riPOBs, $MgCl_2$ and I50) being tested. Therefore we concluded that 8 animals in each group is an appropriate sample size for our study. We have 9 groups in total, so 72 is the total sample size for this study.

11. Number of animals by pain and distress category:(include each animal only once in the highest pain category)

B: 0
C: 0
D: 72
E: 0

a. Justify the need for any animals in pain category E:

N/A

12. * Identify husbandry exceptions:

	Exception Type	Description and Justification
View	Rats - No husbandry or enrichment exceptions.	N/A

13. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create and Edit

1. * Exception type:

Rats - No husbandry or enrichment exceptions.

2. Description and justification:

N/A

View: Custom: Add Vivarium Location

1. * Identify the location where animals will be used:

ARCF ABSL1

a. For locations that are lab managed, provide justification for housing outside of the vivarium:

N/A

2. * What species will be housed in this location?

Common Name	Scientific Name
Rats	Rattus

View: UW IACUC Select Room Level

1. Campus:

Vivarium

2. Vivarium:

ARCF (Animal Research & Care Facility)

3. * BSL Level:

ARCF ABSL1

View: Custom: Add Animal Use Location

1. * Identify the location where animals will be used:

ARCF ABSL1

a. For locations that are outside of the vivarium, provide justification for the use of this space:

N/A

2. * What species will be used in this location?

Common Name	Scientific Name
Rats	Rattus

3. Describe how this location will be used:

All procedures will be performed here.

A cleared place will be used for operation and a power source will be needed for portal dental drill motor.

4. * If animals are left unattended in this location, provide an explanation and include maximum duration:

The researcher maybe leave to get equipment essential for surgery. The animals won't be under unattended longer than half an hour.

5. Describe how animals will be transported to and from this location, including container and route. (Note: use of private vehicles requires IACUC approval):

The animal will be purchased through AOps/DCM, and the animals will be transported by Vendor's vehicle in cage. When transported in buildings, the animals will be in draped cages.

View: UW IACUC Select Room Level

1. Campus:

Vivarium

2. Vivarium:

ARCF (Animal Research & Care Facility)

3. * BSL Level:

ARCF ABSL1

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Research Paper

Human iPS cell-derived astrocyte transplants preserve respiratory function after spinal cord injury

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ABSTRACT

Transplantation-based replacement of lost and/or dysfunctional astrocytes is a promising therapy for spinal cord injury (SCI) that has not been extensively explored, despite the integral roles played by astrocytes in the central nervous system (CNS). Induced pluripotent stem (iPS) cells are a clinically-relevant source of pluripotent cells that both avoid ethical issues of embryonic stem cells and allow for homogeneous derivation of mature cell types in large quantities, potentially in an autologous fashion. Despite their promise, the iPS cell field is in its infancy with respect to evaluating *in vivo* graft integration and therapeutic efficacy in SCI models. Astrocytes express the major glutamate transporter, GLT1, which is responsible for the vast majority of glutamate uptake in spinal cord. Following SCI, compromised GLT1 expression/function can increase susceptibility to excitotoxicity. We therefore evaluated intraspinal transplantation of human iPS cell-derived astrocytes (hIPSAs) following cervical contusion SCI as a novel strategy for reconstituting GLT1 expression and for protecting diaphragmatic respiratory neural circuitry. Transplant-derived cells showed robust long-term survival post-injection and efficiently differentiated into astrocytes in injured spinal cord of both immunesuppressed mice and rats. However, the majority of transplant-derived astrocytes did not express high levels of GLT1, particularly at early times post-injection. To enhance their ability to modulate extracellular glutamate levels, we engineered hIPSAs with lentivirus to constitutively express GLT1. Overexpression significantly increased GLT1 protein and functional GLT1-mediated glutamate uptake levels in hIPSAs both *in vitro* and *in vivo* post-transplantation. Compared to human fibroblast control and unmodified hIPSA transplantation, GLT1-overexpressing hIPSAs reduced (1) lesion size within the injured cervical spinal cord, (2) morphological denervation by respiratory phrenic motor neurons at the diaphragm neuromuscular junction, and (3) functional diaphragm denervation as measured by recording of spontaneous EMGs and evoked compound muscle action potentials. Our findings demonstrate that hiPSA transplantation is a therapeutically-powerful approach for SCI.

1. Introduction

Transplantation of neural stem cells (NSCs) and neural progenitor cells (NPCs) is a promising therapeutic strategy for both neurodegenerative diseases of the central nervous system (CNS) and traumatic CNS injury, including spinal cord injury (SCI), because of the ability to replace lost and/or dysfunctional nervous system cell types, promote neuroprotection, deliver gene factors of interest and provide other benefits (Gage, 2000).

Initial trauma following SCI results in immediate cell death and axotomy of passing fibers. Contusion- and compression-type injuries, the predominant forms of traumatic SCI observed in the clinical population, are followed by an extended period of secondary cell death and consequent exacerbation of functional deficits (McDonald and Becker,

Abbreviations: SCI, spinal cord injury; iPS cells, induced pluripotent stem cells; hIPSAs, human induced pluripotent stem cell-derived astrocytes; GLT1, glutamate transporter 1; PhMN, phrenic motor neuron; C3 (4 5, *etc.*), cervical spinal cord level 3 (4, 5, *etc.*); GRP, glial-restricted precursor; CMAP, compound muscle action potential; NMJ, neuromuscular junction; GFP–hIPSA, lentivirus-GFP transduced hIPSA; GLT1–hIPSA, lentivirus-GLT1 transduced hIPSA; GFP–hFibro, lentivirus-GFP transduced human fibroblast; LV-GFP, lentivirus-GFP; LV-GLT1, lentivirus-GLT1.

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2003). One of the major causes of secondary degeneration following SCI is excitotoxic cell death due to dysregulation of extracellular glutamate homeostasis (Park et al., 2004; Stys, 2004). Exogenous parenchymal administration of glutamate to uninjured spinal cord results in tissue and function loss similar to SCI (Xu et al., 2005). While large increases in glutamate can occur shortly after SCI, elevation can also persist depending on injury severity (Liu et al., 1991; Panter et al., 1990; Xu et al., 2004). In addition to focal increases, levels can also rise in regions removed from the lesion site, possibly *via* a spreading mechanism involving activated glia (Hulsebosch, 2008). Early gray matter loss is likely mediated by NMDA receptors, while delayed loss of neurons and oligodendrocytes, as well as axonal and myelin injury, is thought to be predominantly mediated *via* AMPA over-activation (Stys, 2004). A valuable opportunity therefore exists after SCI for preventing cell injury and functional loss that occur during secondary degeneration. Importantly, secondary degeneration is a relevant therapeutic target given its relatively prolonged time window.

Glutamate is efficiently cleared from the synapse and other sites by transporters located on the plasma membrane (Maragakis and Rothstein, 2004). Astrocytes are supportive glial cells that play a host of crucial roles in CNS function (Pekny and Nilsson, 2005). Astrocytes express the major CNS glutamate transporter, GLT1, which is responsible for the vast majority of functional glutamate uptake and plays a central role in regulation of extracellular glutamate homeostasis in the spinal cord (Maragakis and Rothstein, 2006). Following SCI, astrocyte loss and/or altered GLT1 expression, function and localization can result in further susceptibility to excitotoxicity. For example, we previously found that in rodent models of unilateral mid-cervical (C4) contusion SCI, numbers of GLT1-expressing astrocytes, total intraspinal GLT1 protein expression and GLT1-mediated functional glutamate uptake in ventral horn are reduced soon after injury and this reduction persists chronically (Li et al., 2015). Astrocytes have traditionally been viewed in a negative light following CNS trauma because of their association with disease mechanisms such as glial scarring and pro-inflammatory cytokine release. However, their crucial neuroprotective/homeostatic roles, including GLT1-mediated glutamate uptake, have not been extensively targeted in SCI models using approaches such as NSC and NPC transplantation, despite obvious therapeutic implications (Maragakis and Rothstein, 2006).

Transplantation-based targeting of astrocytes provides a number of key benefits. Grafts can be anatomically delivered to precise locations for achieving neuroprotection of specific populations of cells (Lepore et al., 2008b). Alternative strategies such as gene therapy only target one/several specific genes (s), while astrocyte transplantation can participate in the restoration of a host of astrocyte functions. Transplantation also provides for long-term astrocyte integration and therapeutic replacement. For example, the lasting nature of dysregulation of extracellular glutamate homeostasis after SCI (Lepore et al., 2011a,2011c) calls for longer-term maintenance of therapeutic effects, both with respect to early cell loss occurring during secondary degeneration and outcomes of SCI associated with more persistent pathophysiology of glutamate signaling such as chronic neuropathic pain (Gwak et al., 2012; Hulsebosch, 2008).

To achieve translation of NSC/NPC-based interventions, clinically-relevant cell sources that address scientific, practical and ethical considerations must be extensively tested in relevant models of CNS disease. These cell types also need to be evaluated in the context of patient-relevant functional outcomes such as respiratory function. Induced pluripotent stem (iPS) cells are pluripotent cells generated from adult somatic cell types *via* expression of combinations of pluripotency-related factors, avoiding ethical issues of embryonic stem cells (Takahashi et al., 2007b). This technology allows for homogeneous derivation of cell types in large quantities for applications such as transplantation, potentially in an autologous fashion from the eventual recipient or from allogeneic sources (Das and Pal, 2010; Kiskinis and Eggan, 2010). Despite the promise of this approach, the iPS cell transplantation field is still in the early stages of evaluating therapeutic usefulness in relevant SCI models (Salewski et al., 2010).

Respiratory compromise is a major problem following cervical spinal cord trauma. Cervical SCI represents greater than half of all human cases, in addition to often resulting in the most severe physical and psychological debilitation (Lane et al., 2008). Respiratory compromise is the leading cause of morbidity and mortality following SCI. While a growing literature exists on respiratory function in animal models of SCI (Lane et al., 2008, 2009), few studies have examined cellular mechanisms involved in protection of this vital neural circuitry, and little work has been conducted to test therapies for targeting cervical spinal cord-related functional outcome measures such as breathing. Phrenic motor neuron (PhMN) loss plays a central role in respiratory compromise following cervical SCI. The diaphragm, a major inspiratory muscle, is innervated by PhMNs located at cervical levels 3–5 (Lane et al., 2009). PhMN output is driven by descending pre-motor bulbospinal neurons in the medullary rostral ventral respiratory group (rVRG) (Zimmer et al., 2007). Cervical SCI results in diaphragmatic respiratory compromise due to PhMN loss and/or injury to descending bulbospinal respiratory axons. The majority of these injuries affect mid-cervical levels (Shanmuganathan et al., 2008) (the location of the PhMN pool), and respiratory function following mid-cervical SCI is significantly determined by PhMN loss/sparing (Strakowski et al., 2007). Although use of thoracic models has predominated, cervical SCI animal models have recently been developed (Aguilar and Steward, 2010; Awad et al., 2013; Gensel et al., 2006; Lane et al., 2012; Lee et al., 2010; Sandrow-Feinberg et al., 2009, 2010; Sandrow et al., 2008; Stamegna et al., 2011), including our own (Nicaise et al., 2012). Because of the relevance of astrocyte and GLT1 dysfunction to PhMN loss/injury following cervical trauma, we targeted transplantation in the present study to cervical spinal cord ventral horn in a cervical contusion SCI model.

We previously investigated the therapeutic efficacy of transplanting rodent-derived glial-restricted precursors (GRP), a class of lineage-restricted astrocyte progenitor cell (Li et al., 2014). We transplanted either undifferentiated GRPs or GRP-derived astrocytes (pre-differentiated *in vitro* prior to injection) into our model of cervical contusion SCI, and found that both cell types survived, localized to the ventral horn and efficiently differentiated into mature astrocytes. However, animals injected with GRP-derived astrocytes had higher levels of intraspinal GLT1 expression than those injected with undifferentiated GRPs, suggesting that pre-differentiation enhanced the *in vivo* maturation of these cells. We also observed that modifying GRP-derived astrocytes to constitutively express GLT1 was more effective in achieving *in vivo* GLT1 expression and for protecting PhMNs.

Given the importance of astrocytes in SCI pathogenesis, the observations of GLT1 dysfunction following SCI, and our previous success targeting astrocyte GLT1 using rodent-derived glial progenitor cells, in the present study we evaluated intraspinal transplantation of hiPS cell-derived astrocytes (hIPSAs) into ventral horn following cervical contusion SCI as a novel therapeutic strategy for reconstituting GLT1 function. Specifically, we examined the *in vivo* fate of hIPSAs transplants in the injured spinal cord of both mouse and rat models of cervical contusion SCI, including long-term survival and integration, astrocyte differentiation, maturation into GLT1-expressing cells and safety. We also tested the therapeutic efficacy of hIPSA transplantation for protection of PhMNs and preservation of diaphragm function.

Derivation of cell types from iPS cells represents a relevant approach for clinical translation; therefore, it is critical to test both the safety and efficacy of these transplants in a patient-relevant SCI model. Importantly, previous work has shown that human- and rodent-derived versions of a given stem/progenitor type do not necessarily show similar *in vivo* fate or therapeutic properties in the disease nervous system. For example, we previously demonstrated that, following transplantation into the $SOD1^{G93A}$ rodent model of ALS, human glial progenitors cells show more persistent proliferation, greater migratory capacity, reduced efficiency of astrocyte differentiation, and decreased GLT1 expression

compared to their rodent counterparts, which resulted in a lack of therapeutic efficacy only with the human cells (Lepore et al., 2008b, 2011b). It is therefore important to extend our previous studies with rodent-derived glial progenitors in the cervical contusion SCI model to now test human iPS cells.

2. Materials and methods

2.1. Animals

2.1.1. Transplantation into rats and mice

Female Sprague–Dawley rats weighing 250–300 g were purchased from Taconic Farm (Rockville, MD). Female C57BL/6 wild-type mice weighing 20–30 g were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in a humidity-, temperature-, and light-controlled animal facility with *ad libitum* access to water and food. Experimental procedures were approved by the Thomas Jefferson University IACUC and conducted in compliance with ARRIVE (*Animal Research: Reporting of In Vivo Experiments*) guidelines.

2.2. Cervical contusion SCI

2.2.1. Rat SCI

Rats were anesthetized with ketamine (100 mg/kg), xylazine (5 mg/kg) and acepromazine (2 mg/kg). The cervical dorsal skin and underlying muscles were incised. The paravertebral muscles overlying C3–C5 were removed. Following unilateral laminectomy on the right side at C3, C4 and C5 levels, rats were subjected to a C4 spinal contusion injury with the Infinite Horizon impactor (Precision Systems and Instrumentation, Lexington, KY) using a 1.5 mm tip at a force of 395 kdyn. This injury paradigm is based on our previously published rat model that results in robust PhMN degeneration and chronic diaphragm dysfunction (Nicaise et al., 2012, 2013). Rats were transplanted in all studies immediately following injury. After surgical procedures, overlying muscles were closed in layers with sterile 4–0 silk sutures, and the skin incision was closed using wound clips. Animals were allowed to recover on a circulating warm water heating pad until awake and then returned to their home cages. They were monitored daily until sacrifice, and measures were taken to avoid dehydration and to minimize any pain or discomfort.

2.2.2. Mouse SCI

Mice were anesthetized with a cocktail of ketamine (120 mg/kg) and xylazine (5 mg/kg). The surgical procedure and post-surgical monitoring used for mice were the same as described above for rats. For the contusion injury, the 1 mm impactor tip was raised 1.25 mm above the dura prior to impact, and a force of 50 kdyn (kdyn) was used for impact.

2.3. Virus production

Lentiviral vector carrying the green fluorescent protein (GFP) gene or GLT1 gene was packaged in 293FT cells. Briefly, to produce control lentiviral-GFP vector, 293FT cells were transfected with pCDH-MSCV-MCS-EF1-GFP plasmid (System Biosciences, Mountain View, CA) and three other helper plasmids, pLP-1, pLP-2, and pLP/VSVG with Polyfect (Qiagen, Valencia, CA). To produce lentiviral-GLT1 vector, GLT1 gene CDS fragment was inserted into MCS of pCDH-MSCV-MCS-EF1-GFP plasmid, and the vector plasmid was then transfected into 293FT cells with three helper plasmids as described above. Supernatant was collected 72 h later, and lentiviral vector was concentrated with PEG-it Virus Precipitation Solution (System Biosciences, Mountain View, CA) and re-suspended with PBS to the final titer of 1×10^8 infectious units/ml.

2.4. Human induced pluripotent stem cell derived astrocytes

2.4.1. Human iPS cell derivation, culturing and astrocyte differentiation

iPS cells were derived from non-diseased healthy patient donors. Dermal fibroblasts were reprogrammed into iPS cells *via* retroviral transduction with KLF4, SOX2, OCT4, and c-MYC (Takahashi et al., 2007a). By immunohistochemistry and qRT-PCR, these putative iPS cells expressed proteins and transcripts associated with pluripotency, including Sox 2, and stem cell-associated antigens, including SSEA4, Nanog, alkaline phosphatase, and TRA 1–81, and capacity to differentiate into cells of three germ layers was established. Finally, the karyotype of these iPS cells was found to be normal. Once pluripotent iPS cells were generated, the stem cells were cultured in E8 medium (Life Technologies, Grand Island, NY). To maintain optimum pluripotency and limit spontaneous differentiation, the stem cell colonies were manually cleaned once every 6 days just before passage using dispase (Stem Cell Technologies, Vancouver, BC). To differentiate the iPS cells into astrocytes, a protocol previously described by Haidet-Phillips and colleagues (Haidet-Phillips et al., 2014) was used. To summarize, iPS cells were lifted with dispase, gently separated into single cells and plated as a monolayer. Using the smad dual inhibition pathway method to direct differentiation toward a neural phenotype, the cells were incubated in DMEM/F12 (Life Technologies, Grand Island, NY) enriched with 0.2 μM LDN (Stemgent, Cambridge, MA) and 10 μM SB431542 (Sigma, Saint Louis, MO). The cells were then exposed to 1 μM retinoic acid (Sigma, Saint Louis MO) and N2 (Life Technologies, Grand Island, NY) starting at day 5 and Sonic HedgeHog (Life Technologies, Grand Island, NY) starting at day 8. From day 15 to day 30 after starting the differentiation protocol, the medium was gradually changed to neurobasal medium. After day 30, to differentiate these iPS cell-derived glial progenitors into astrocytes, cells were maintained and expanded in DMEM/F12 supplemented with 1% Fetal Bovine Serum, B27, L-glutamine, non-essential amino acids, penicillin/streptomycin (all from Life Technologies, Grand Island, NY) and 2 μg/ml Heparin (Sigma-Aldrich, St. Louis, MO) for an additional 60 days. Astrocytes derived from human iPS were identified with immunostaining using GFAP antibody. For feeding and passaging of astrocyte progenitor cultures, cells were rinsed with PBS and incubated with 4 ml of 0.05% trypsin for 5 min. Cells were collected in trypsin and rinsed with 7 ml of culture medium and 1× trypsin inhibitor (Life Technologies, Grand Island, NY) to stop trypsinization. Cells were centrifuged at 1000 rpm for 5 min and re-suspended in fresh culture medium. Cells were counted and seeded onto poly-L-lysine coated dishes. Cells were fed twice a week and were passaged after they were 80%–90% confluent.

2.4.2. GLT1 overexpression

After differentiation for 90 days, hIPSAs (astrocytes derived from human iPS cells) were transduced with lentiviral-GFP vector or lentiviral-GLT1 vector, at the concentration of 1×10^6 infectious units/ml, one week before transplantation. On the second day of transduction, culture medium was changed and the cells were cultured for 5 more days.

2.5. Human dermal fibroblasts

Human dermal fibroblast cells (ATCC, Manassas, VA) were cultured with Fibroblast Growth Kit-low serum (ATCC, Manassas, VA). Fibroblasts were transduced with control lentiviral-GFP vector one week before transplantation. Transduced GFP was used to track transplanted cells *in vivo*.

2.6. Transplantation

2.6.1. Cell preparation for transplantation

On the day of transplantation, cells were rinsed with PBS and trypsinized with 0.05% trypsin, collected and rinsed with culture

medium and 1× trypsin inhibitor. The cells were washed with artificial cerebrospinal fluid twice. Cell viability was assessed using the trypan blue assay and was always found to be greater than 80%. The final cell concentration was adjusted to 1×10^8 cells/ml.

2.6.2. Intraspinal transplantation

Transplantation was conducted on deeply anesthetized rats and mice immediately post-injury. Following unilateral right-sided contusion injury at C4, cells were injected into the spinal cord at two locations. Each site contained 2 μl of cell suspension, which was administered into the spinal cord ventral horn using a Hamilton gas-tight syringe mounted on an electronic UMP3 micropump (World Precision International, Sarasota, FL) (Lepore and Maragakis, 2011; Lepore et al., 2011a). The sites of injections were located at the rostral and caudal edges of the contusion site. Ventral horns were targeted by lowering the 33-gauge 45-degree beveled needle 1.5 mm below the dorsal surface of the spinal cord. Each injection was delivered at a constant rate over 5 min. Upon completion of cell delivery, overlying muscles were then closed in layers with sterile 4–0 silk sutures, and the skin incision was closed using sterile wound clips. Animals were allowed to recover and monitored daily.

2.6.3. Immune suppression

All animals were immune suppressed. Rats received subcutaneous administration of cyclosporine A (10 mg/kg; Sandoz Pharmaceuticals, East Hanover, NJ) daily beginning three days before grafting and continuously until sacrifice. Mice were given both FK-506 and rapamycin (1 mg/kg each; LC Laboratories; Woburn, MA).

2.7. Tissue processing for histology

At the time of sacrifice, animals were anesthetized, and diaphragm muscle was freshly removed prior to perfusion and then further processed for neuromuscular junction (NMJ) labeling. Animals were transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde infusion. Spinal cords were harvested, then cryoprotected in 30% sucrose for 3 days and embedded in freezing medium. Spinal cord tissue blocks were cut serially in the sagittal or transverse planes at a thickness of 30 μm. Sections were collected on glass slides and stored at −20 °C until analysis. Spinal cord sections were thawed, allowed to dry for 1 h at room temperature, and stained with 0.5% Cresyl violet acetate according to standard procedure (Nicaise et al., 2012).

2.8. Immunohistochemistry

Frozen spinal cord sections were air-dried, washed with PBS, permeabilized with 0.4% Triton X-100 in PBS for 5 min at room temperature, and then incubated in blocking solution (PBS containing 10% normal goat serum and 0.4% Triton X-100) for 1 h at room temperature. Sections were labeled overnight at 4 °C with the primary antibodies in blocking solution. Sections were then washed three times with PBS (5 min per wash) and incubated with secondary antibodies in blocking solution for 1 h at room temperature. After washing twice with PBS (10 min per wash), sections were cover-slipped. A number of primary antibodies were used. Mouse anti-GFAP antibody (EMD Millipore Corporation, Billerica, MA; 1:200) and rabbit anti-GFAP antibody (Dako North America, Carpinteria, CA; 1:200) were used to label astrocytes (Lepore et al., 2008a). Mouse anti-human GFAP antibody (StemCells, Inc, Newark, CA; 1:200) was used to label astrocytes of human origin in mice and rats. Rabbit anti-GLT1 (1:800) and mouse anti-GLT1 (1:200) were used to label GLT1 protein (both were provided by Jeffrey Rothstein's laboratory) (Lepore et al., 2008b). Rabbit anti-Ki67 (Thermo Fisher Scientific, Rockford, IL; 1:200) labeled proliferating cells (Lepore et al., 2008a). Mouse anti-human cytoplasmic marker antibody (StemCells, Inc, Newark, CA; 1:200) and mouse anti-HuNu antibody (EMD Millipore Corporation, Billerica, MA; 1:200) were used to label human cytoplasm and human nuclear antigen, respectively, for selectively identifying human-derived cells. Secondary antibodies included: FITC goat-anti-mouse IgG, FITC goat-anti-rabbit IgG, TRITC goat-anti-mouse IgG, TRITC goat-anti-rabbit IgG, Alexa Fluor 647 goat-anti-mouse IgG, Alexa Fluor 647 goat-anti-rabbit IgG. All secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were diluted at 1:200 to recognize the matched primary antibody. For fluorescence analysis, sections were cover-slipped with fluorescent-compatible mounting medium (ProLong Gold, Life Technologies, Grand Island, NY).

2.9. Quantification of in vitro cultured cell differentiation, proliferation and GLT1 expression

The proportions of $GFAP^+$ astrocytes and $Ki67^+$ proliferating cells were expressed as a percentage of the total number of cultured cells (labeled by DAPI). In order to quantify double-labeling of DAPI with GFAP or Ki67, images were taken at 10× magnification and analyzed using ImageJ software. In each image, cells with a $DAPI^+$ nucleus were assessed for expression of GFAP or Ki67.

2.10. Quantification of transplant differentiation

Rats and mice were sacrificed for quantification of astrocyte differentiation ($GFAP^+$) and proliferation ($Ki67^+$). The proportions $GFAP^+$ astrocytes and $Ki67^+$ proliferating cells were expressed as a percentage of the total number of transplanted human cells (labeled by anti-hCytoplasm or HuNu antibody). In order to quantify double-labeling of hCytoplasm or HuNA with GFAP and Ki67, double-labeled transverse sections were imaged at 10× magnification using MetaMorph software and were then analyzed using ImageJ software. In each image, cells expressing hCytoplasm or HuNu were assessed for co-expression of GFAP or Ki67.

2.11. Quantification of GLT1 expression by transplants

Rats and mice were sacrificed for quantification of GLT1 expression by hCyto-labeled cells in the ventral horn. $GLT1^+$ and $hCyto^+$ cells were identified in the ventral horn using ImageJ software, and the percentage of $hCyto^+$ cells (representing any transplant-derived cell) that co-expressed GLT1 were quantified.

2.12. Lesion imaging and quantification

Images were acquired with a Zeiss Imager M2 upright microscope and analyzed with ImageJ software. Lesion size was quantified in Cresyl violet stained sections (Li et al., 2015). Specifically, lesion area was determined in every 10th section by tracing both the total area of the hemi-spinal cord ipsilateral to the contusion site and the actual lesion area. Lesion was defined as areas including both lost tissue (cystic cavity formation) and surrounding damaged tissue in which the normal anatomical structure of the spinal cord was lost. The lesion epicenter was defined as the section with the largest percent lesioned tissue (relative to total tissue area in the same section).

2.13. Neuromuscular junction (NMJ) analysis

Fresh hemi-diaphragm muscle was dissected from each animal for whole-mount immunohistochemistry, as described previously (Wright et al., 2007). Hemi-diaphragm muscle was dissected, stretched, pinned down to Sylgard medium (Fisher Scientific, Pittsburgh, PA), and extensively cleaned to remove any connective tissue to allow for antibody penetration. Motor axons and their terminals were labeled with SMI-312R (Covance, Princeton, NJ; 1:1000) and

SV2-s (DSHB, Iowa City, IA; 1:10), respectively, and both primary antibodies were detected with FITC anti-mouse IgG secondary (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:100). Post-synaptic acetylcholine receptors were labeled with rhodamine-conjugated alpha-bungarotoxin (Life Technologies, Grand Island, NY; 1:400). Labeled muscles were analyzed for total numbers of NMJs and intact, denervated and multiply-innervated NMJs. Whole-mounted diaphragms were imaged on a FluoView FV1000 confocal microscope (Olympus, Center Valley, PA). We only conducted NMJ analysis in ipsilateral hemi-diaphragm because in our previously published work we did not observe denervation or sprouting in contralateral hemi-diaphragm after cervical hemi-contusion SCI (Nicaise et al., 2012).

2.14. Functional glutamate uptake assay

After transduction with lentiviral-GFP vector or lentiviral-GLT1 vector, hIPSAs were cultured for 10 days. Human fibroblasts transduced with lentiviral-GFP vector were used as control. Glutamate uptake activity was measured as previously described (Dowd and Robinson, 1996), with slight modification. Briefly, cells were washed and pre-incubated with either a sodium- or choline-containing uptake buffer (in mM: Tris, 5; HEPES, 10; NaCl or choline chloride, 140; KCl, 2.5; CaCl2, 1.2; $MgCl_2$, 1.2; $K2HPO_4$, 1.2; glucose, 10) for 20 min at 37 °C; and in DHK treatment groups, 100 μM of DHK was added to inhibit GLT1. The uptake buffer was then replaced with fresh uptake buffer containing 20 nM ^{3}H-glutamate (49 Ci/mmol; PerkinElmer, CA) and 20 μM unlabeled glutamate. The cells were incubated for 5 min at 37 °C. The reaction was terminated by washing cells three times with choline-containing uptake buffer containing 2 mM unlabeled glutamate, followed by immediate lysis in ice-cold 0.1 N NaOH. Cell extracts were then measured with a liquid scintillation counter (Beckman Instruments, Fullerton, CA). The protein content in each well was measured using the Bradford protein assay (Bio-Rad, Hercules, CA).

2.15. Diaphragm compound muscle action potentials (CMAPs)

Rats were anesthetized in the same manner described above. Phrenic nerve conduction studies were performed with single stimulation (0.5 ms duration; 6 mV amplitude) at the neck *via* near nerve needle electrodes placed along the phrenic nerve (Li et al., 2015; Nicaise et al., 2012). The ground needle electrode was placed in the tail, and the reference electrode was placed subcutaneously in the right abdominal region. Recording was obtained *via* a surface strip along the costal margin of the diaphragm, and CMAP amplitude was measured baseline to peak. Recordings were made using an ADI Powerlab 8/30 stimulator and BioAMP amplifier (ADInstruments, Colorado Springs, CO), followed by computer-assisted data analysis (Scope 3.5.6, ADInstruments). For each animal, 10–20 tracings were averaged to ensure reproducibility.

2.16 . Spontaneous EMG recordings

Prior to being euthanized, animals received a laparotomy. These EMG recordings were terminal experiments and were only conducted immediately prior to euthanasia. Bipolar electrodes spaced by 3 mm were inserted into specific sub-regions of the right hemi-diaphragm (*i.e.* dorsal, medial or ventral regions) (Li et al., 2015). Activity was recorded and averaged during spontaneous breathing at each of these 3 locations separately in each animal. The EMG signal was amplified, filtered through a band-pass filter (50–3000 Hz), and integrated using LabChart 7 software (ADInstruments). Parameters such as inspiratory bursts per minute, discharge duration and integrated peak amplitude were averaged over 2 min sample periods. No attempt was made to control or monitor the overall level of respiratory motor drive during the EMG recordings.

2.17. Statistics

Results were expressed as means ± standard error of the mean (SEM). A Kolmogorov–Smirnov test was conducted for all variables to assess normality. Unpaired *t* test or Mann–Whitney was used to assess statistical significance between two groups. With respect to multiple comparisons involving three groups or more, statistical significance was assessed by analysis of variance (one-way ANOVA) followed by *post-hoc* test (Bonferroni's method). Statistics were computed with Graphpad Prism 5 (GraphPad Software, Inc., La Jolla, CA). $p < 0.05$ was considered as statistically significant.

3. Results

3.1. In vitro characterization of human iPS cell-derived astrocytes (hIPSAs)

We differentiated human iPS cells into astrocytes by culturing them in differentiating medium containing FBS. We transduced cells with lentivirus (LV)-GFP or LV-GLT1-GFP to generate control cells (GFP–hIPSAs) and GLT1-overexpressing hIPSAs (GLT1–hIPSAs), respectively. The GFP–hIPSAs expressed little-to-no GLT1 protein (Fig. 1A, C), consistent with the limited expression of GLT1 by cultured astrocytes in the absence of neuronal co-culture (Li et al., 2014; Perego et al., 2000), while GLT1–hIPSAs expressed high levels of GLT1 protein *in vitro* (Fig. 1B, C). In addition, the vast majority of DAPI$^+$ GLT1–hIPSAs expressed GLT1 (Fig. 1B), which is expected given the high efficiency of transduction with our lentivirus (not shown). GLT1 overexpression did not alter hiPSA differentiation (Fig. 1D, E, H) or proliferation (Fig. 1F–H). In addition to significantly increased GLT1 protein expression levels, GLT1–hIPSAs showed a large increase in functional GLT1-mediated glutamate uptake compared to GFP–hIPSAs using an *in vitro* ^{3}H-glutamate uptake assay (Fig. 1J). In this ^{3}H-glutamate uptake assay and in the subsequent transplantation experiments, we used LV-GFP transduced human fibroblasts (GFP–hFibro) (Fig. 1I) as a non-glial cell control.

3.2. Human iPSA transplants robustly survived and differentiated into astrocytes following rat cervical contusion SCI

We characterized the fate of transplanted hIPSAs in both rats and mice following unilateral C4 contusion SCI, given the usefulness of both experimental models for studying nervous system diseases. Immediately following injury, we injected hIPSAs directly into the ventral horn at locations just rostral and caudal to the contusion site (Fig. 2A). We specifically delivered cells into the ventral horn to anatomically target the location of the PhMN pool (Fig. 2B).

We sacrificed rats at 2 days, 2 weeks and 4 weeks post-injury/transplantation. Double-labeling with panGFAP antibody and a human-specific GFAP antibody demonstrated that transplanted human-derived cells differentiated into astrocytes (Fig. 2C). Both transplanted GFP–hIPSAs (Fig. 2D, F, H) and GLT1–hIPSAs (Fig. 2E, G, I) robustly survived out to W4, and nearly all hCytoplasm$^+$ transplant-derived cells co-labeled with the astrocyte lineage marker, GFAP, at D2 (Fig. 2D–E), W2 (Fig. F–G) and W4 (Fig. 2H–I). There were no differences in the degree of astrocyte differentiation between GFP–hIPSAs and GLT1–hIPSAs at any of these time points (quantification shown in Fig. 2J). LV-GFP transduced human fibroblasts (GFP–hFibro) also survived in the injured spinal cord to at least W4 post-injury (Fig. 2K).

Despite efficient astrocyte differentiation, only a small percentage of GFP–hIPSA transplant-derived cells expressed GLT1 protein in the injury site at D2 (Fig. 3A), W2 (Fig. 3C) and W4 (Fig. 3E). On the contrary, the majority of GLT1–hIPSAs robustly expressed GLT1 at all times (Fig. 3B, D, and F) (quantification: Fig. 3G).

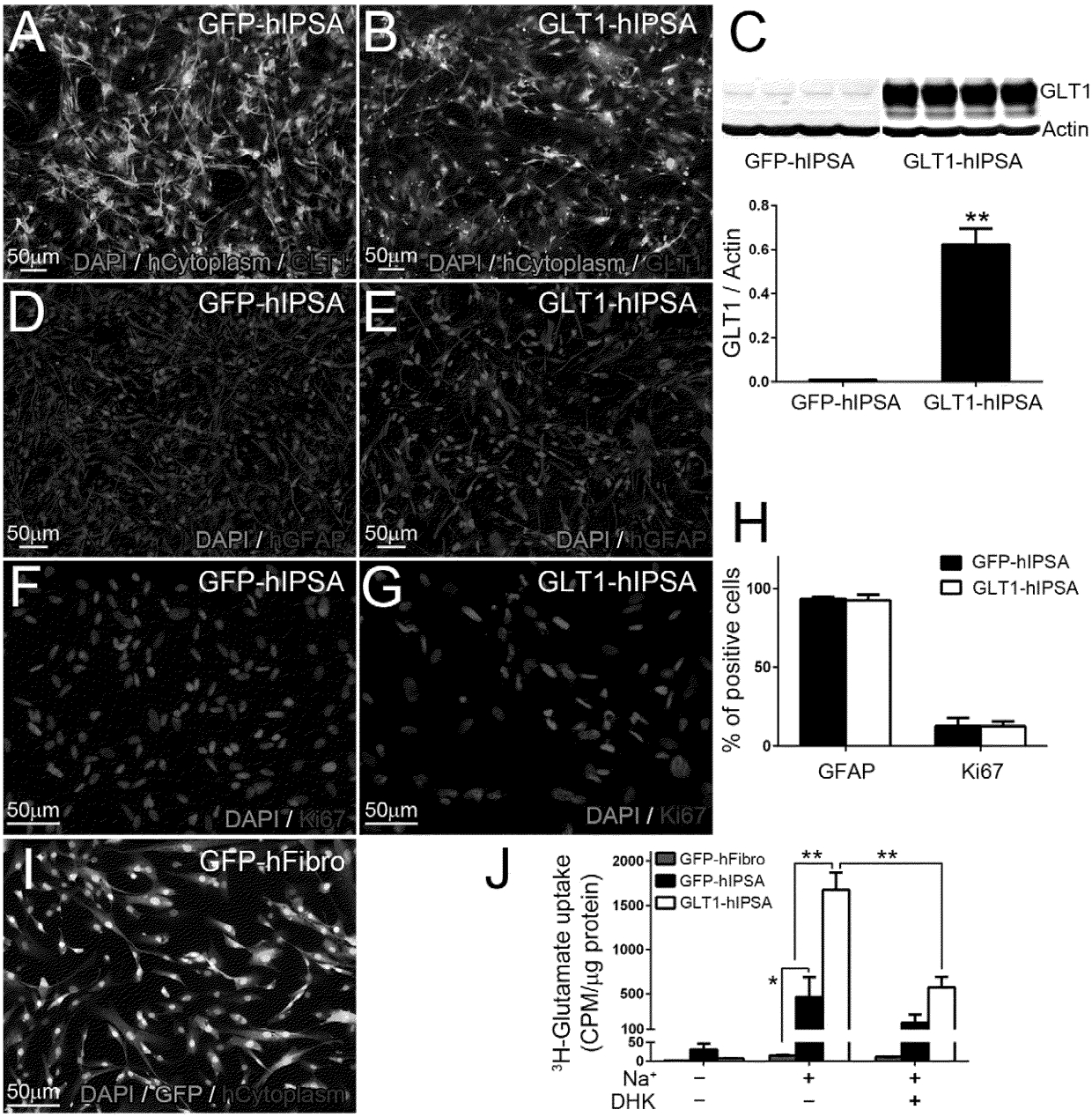

Fig. 1. *In vitro* characterization of human iPS cell-derived astrocytes (hIPSAs). Cells were transduced with lentivirus (LV)-GFP or LV-GLT1–GFP to generate control GFP–hIPSAs and GLT1-overexpressing hIPSAs (GLT1–hIPSAs), respectively. Human cytoplasm$^+$ GFP–hIPSAs expressed little-to-no GLT1 protein (A), while GLT1–hIPSAs expressed high levels of GLT1 protein *in vitro* (B), which was further confirmed with immunoblotting analysis (C, *lower:* quantification result). Following infection with either virus, astrocyte differentiation was determined by the percentage of cells expressing the astrocyte lineage marker, GFAP (D–E). Proliferation was determined by the percentage of cells expressing the proliferation marker, Ki67 (F–G). Quantification results of cell differentiation and proliferation are shown in (H). Human fibroblasts, which were transduced with LV-GFP vector (GFP–hFibro) (I), were used as non-glial control in the glutamate uptake assay and *in vivo* transplantation experiments. ^{3}H-glutamate uptake assay was performed to detect GLT1 function. GLT1–hIPSAs showed a large increase in Na^+ dependent glutamate uptake compared to GFP–hFibro and GFP–hIPSAs. This increased uptake was blocked with GLT1 specific inhibitor, DHK, at the concentration of 100 μmol/l (J). Results were expressed as means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$. n = 4 per group for GLT1 western blotting quantification analysis; n = 4 per group for cell differentiation and proliferation analysis; n = 4 per group for ^{3}H-glutamate uptake assay.

3.3 . Human iPSA transplants showed limited proliferation in vivo and did not form tumors

A major concern regarding NSC/NPC therapy (particularly with pluripotent cells such as iPS cells) is the potential for uncontrolled proliferation and even tumor formation. To address this concern, we immunostained for the proliferation marker, Ki67, and we examined transplant recipient rat spinal cords for overt tumor formation. With both GFP–hIPSAs (Fig. 4A, C, E) and GLT1–hIPSAs (Fig. 4B, D, F), less than 10% of HuNu$^+$ transplant-derived cells expressed Ki67 at D2 (Fig. 4A–B), W2 (Fig. 4C–D) and W4 (Fig. 4E–F) (quantification shown in Fig. 4G). In addition, we never observed tumor formation in any transplant-recipient animals.

3.4. Human iPSA transplants showed similar survival and differentiation in the injured mouse cervical spinal cord

Given the usefulness of the mouse model due to the availability of transgenic tools, we conducted similar characterization of hIPSA fate following transplantation into the mouse spinal cord immediately

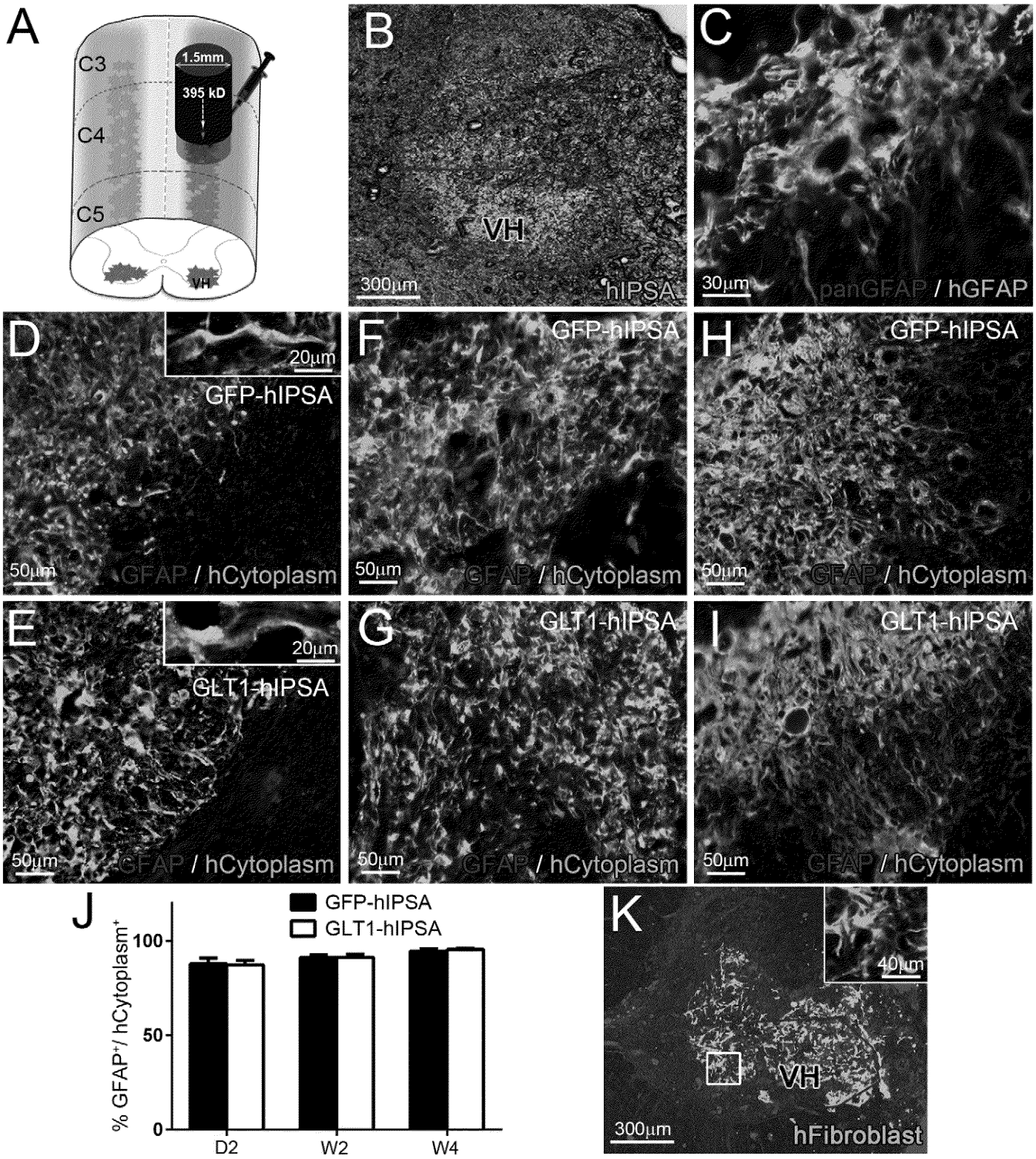

Fig. 2. Human iPSA transplants robustly survived, differentiated into astrocytes and localized to the ventral horn following rat cervical contusion SCI. Immediately following unilateral C4 contusion SCI, we injected GFP–hIPSAs, GLT1–hIPSAs or GFP–hFibro directly into the ventral horn (VH) at locations just rostral and caudal to the contusion site (A). GFP fluorescence indicated that the transplanted hIPSAs were delivered to the ventral horn (B). Double-labeling with pan-GFAP antibody and a human GFAP specific antibody confirmed that all human $GFAP^+$ cells were also pan-$GFAP^+$ (C). Double immunostaining for pan-GFAP and human cytoplasm marker was performed on spinal cord sections from the GFP–hIPSA (D, F, H) and GLT1–hIPSA (E, G, I) groups at day 2 (D–E), week 2 (F–G) and week 4 (H–I) post-injury/transplantation to quantify astrocyte differentiation by transplanted cells (J). We used LV-GFP transduced human fibroblasts (GFP–hFibro) as a non-glial cell control (K, *inset*: high magnification). Results were expressed as means ± SEM. n = 3 per group per time point for transplanted cell differentiation analysis. Red outlines in panels B and K denote the ventral horn.

following unilateral cervical contusion SCI. Similar to transplantation into the rat SCI model, hIPSAs robustly survived and integrated for at least 4 weeks post-injection. The majority of transplant-derived cells were differentiated $GFAP^+$ astrocytes (Fig. 4H). Control GFP–hIPSAs expressed little GLT1, while overexpression resulted in the majority of transplant-derived astrocytes expressing GLT1 (Fig. 4I). Less than 10%

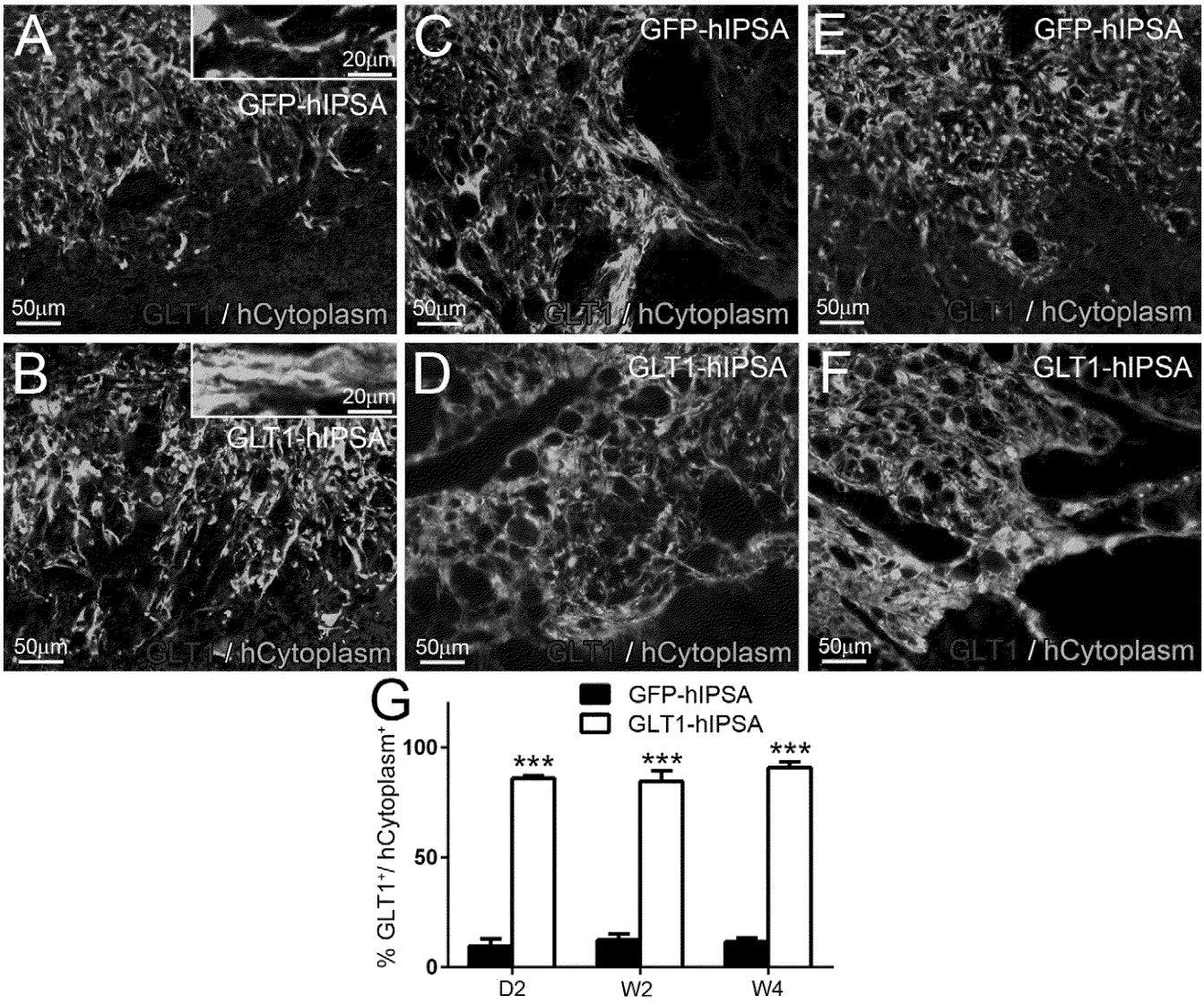

Fig. 3. GLT1–hIPSA transplants expresses GLT1 in the ventral horn following rat cervical contusion SCI. Double immunostaining for GLT1 and human cytoplasm was performed on spinal cord sections from the GFP–hIPSA (A, C, E) and GLT1–hIPSA (B, D, F) groups at day 2 (A–B), week 2 (C–D) and week 4 (E–F) post-injury/transplantation to assess GLT1 expression by transplanted cells *in vivo* (G). Results were expressed as means ± SEM. ***$p < 0.001$. n = 3 per group per time point for *in vivo* GLT1 expression analysis.

of transplant-derived cells continued to proliferate at D2, W2 and W4 (Fig. 4J), and again we never observed tumor formation in any mice.

3.5. GLT1 overexpressing hIPSA transplants reduced lesion size following cervical contusion SCI

To test the therapeutic efficacy of hIPSA transplants in the rat unilateral cervical contusion model, we first assessed lesion size. At 4 weeks post-injury, we quantified Cresyl-violet stained transverse sections of the cervical spinal cord surrounding the injury site for the degree of ipsilesional tissue sparing by calculating the percentage of total ipsilateral hemi-cord area comprised of damaged tissue (Fig. 5A). Lesion area (Fig. 5B) and total lesion volume (Fig. 5C) analysis (combined for both white and gray matter) revealed that GLT1–hIPSA transplants significantly reduced lesion size at multiple locations surrounding the epicenter compared to both GFP–hFibro and GFP–hIPSA control transplant groups. We observed this protective effect specifically within 1 mm rostral and caudal of the epicenter where the greatest tissue damage occurred.

3.6. GLT1 overexpressing hIPSA transplants preserved diaphragm innervation by phrenic motor neurons after SCI

We found that GLT1 overexpressing hIPSA transplants significantly preserved morphological innervation at the diaphragm neuromuscular junction (NMJ), the synapse which is critical for functional PMN–diaphragm connectivity. To examine pathological alterations at the diaphragm NMJ, we analyzed hemi-diaphragm muscle ipsilateral to the contusion in rats (Fig. 6A–B). We quantified the percentage of intact NMJs or partially denervated NMJs in the animals from the 3 injection groups at 4 weeks post-injury/transplantation (Wright et al., 2007, 2009; Wright and Son, 2007). For analysis, we divided the hemi-diaphragm into three anatomical regions (ventral, medial and dorsal) (Fig. 6C), as the rostral-caudal axis of the PMN pool within the cervical spinal cord topographically maps onto the ventral-dorsal axis of the diaphragm (Laskowski and Sanes, 1987). At the dorsal region of the hemi-diaphragm, the percentage of intact NMJs in the GLT1–hIPSA transplant group was significantly greater than both control groups, while at the ventral and medial regions of the diaphragm, there were no differences in the percentage of intact NMJs amongst the groups

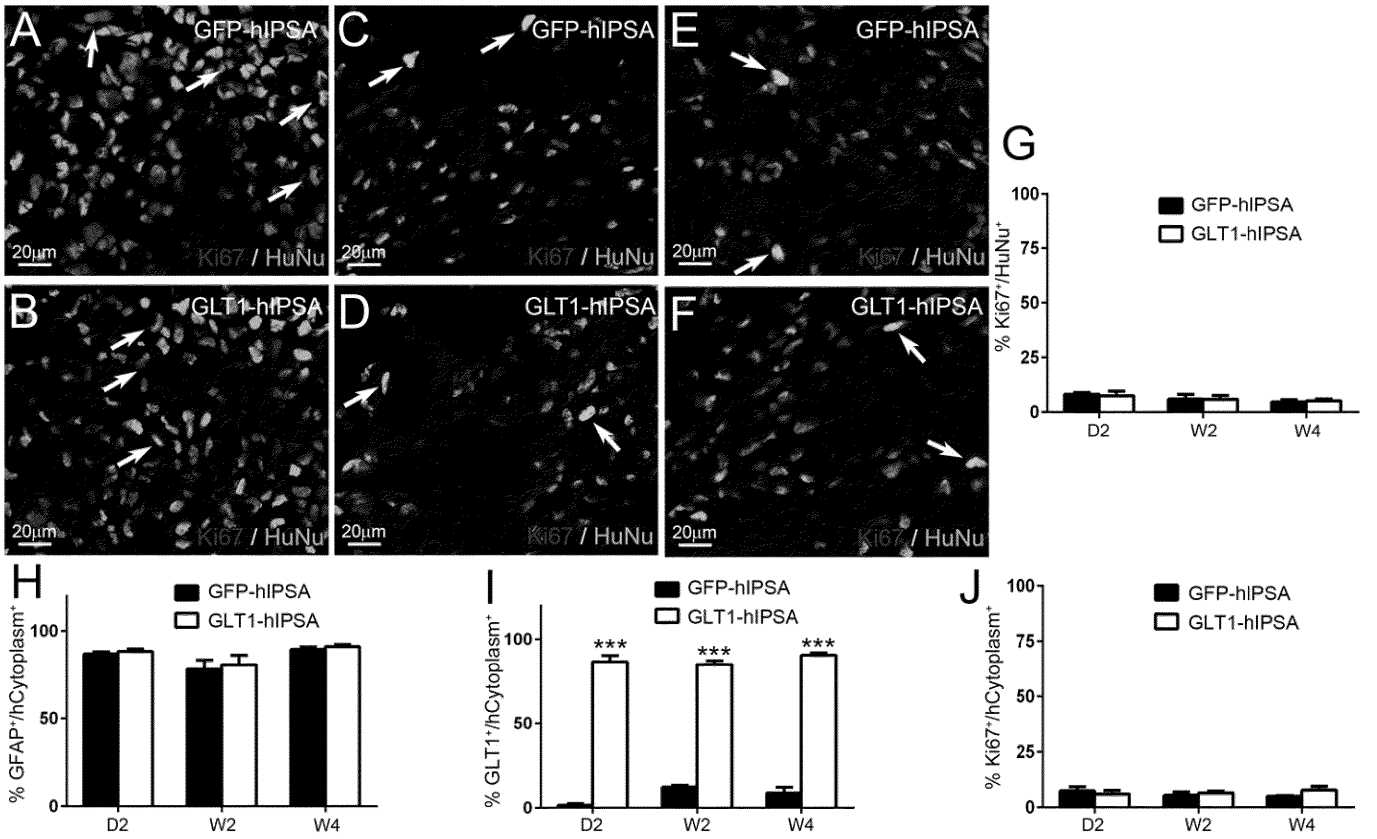

Fig. 4. Transplanted hiPSAs showed limited proliferation and did not form tumors. Double immunostaining for the proliferation marker Ki67 with human nuclei (HuNu) was performed on spinal cord sections from the GFP–hIPSA (A, C, E) and GLT1–hIPSA (B, D, F) groups at D2 (A–B), W2 (C–D) and W4 (E–F) post-transplantation, and quantification results are shown in (G). Tumor formation was never observed. We conducted similar *in vivo* characterization of hIPSA fate following transplantation into the mouse spinal cord immediately following unilateral cervical contusion SCI. The majority of transplant-derived cells were differentiated $GFAP^+$ astrocytes (H). Control GFP–hIPSAs did not express GLT1, while overexpression resulted in the majority of transplant-derived astrocytes expressing GLT1 (I). Less than 10% of transplant-derived cells continued to proliferate at D2, W2 and W4 (J). Results were expressed as means ± SEM. $^{***}p < 0.001$. n = 3 per group per time point in cell fate analysis.

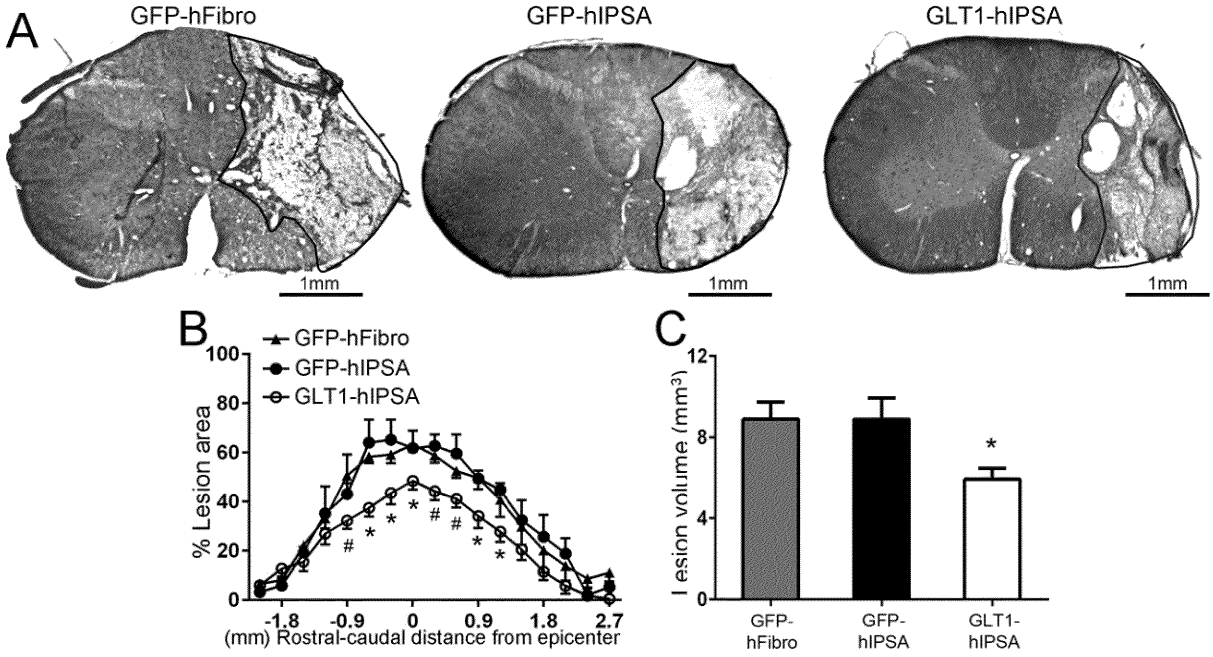

Fig. 5. GLT1 overexpressing hIPSA transplants reduced lesion size following cervical contusion SCI. At 4 weeks post-injury, we quantified Cresyl-violet stained transverse sections of the cervical spinal cord for the degree of ipsilesional tissue sparing by calculating the percentage of total ipsilateral hemi-cord area comprised of damaged tissue (A). Lesion area (B) and total lesion volume (C) analysis (combined for both white and gray matter) revealed that GLT1–hIPSA transplants significantly reduced lesion size at multiple locations surrounding the epicenter compared to both human fibroblast and control GFP–hIPSA transplant groups. Results were expressed as means ± SEM. $\#p < 0.05$, GLT1–hIPSA group *versus* GFP–hIPSA group only; $^*p < 0.05$, GLT1–hIPSA group *versus* both control groups. n = 6 per group for lesion area and volume analysis.

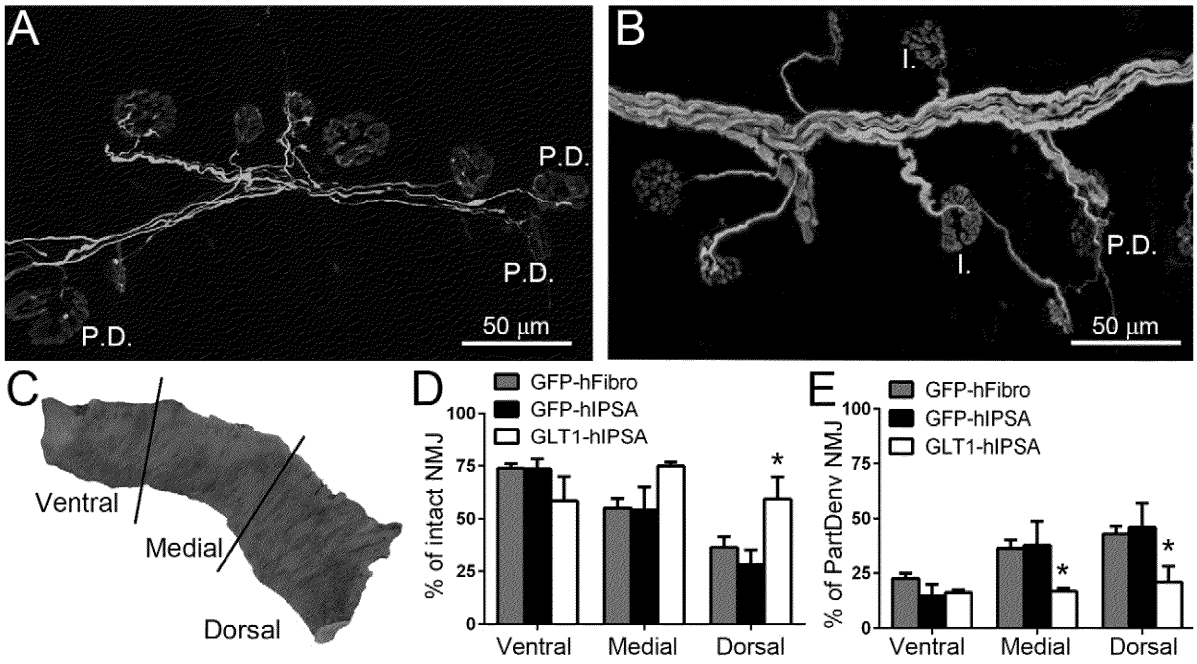

Fig. 6. GLT1 overexpressing hIPSA astrocyte transplants preserved diaphragm innervation by phrenic motor neurons following cervical contusion SCI. To examine pathological alterations at the diaphragm NMJ, hemi-diaphragm muscle ipsilateral to the contusion from the GFP–hFibro (A), GFP–hIPSA and GLT1–hIPSA (B) groups was examined at 4 weeks post-injury/transplantation. Individual NMJs were characterized as: intact (I.) and partially denervated (P.D.). For analysis, the hemi-diaphragm was divided into three anatomical regions (ventral, medial and dorsal) (C). At the dorsal region of the hemi-diaphragm, the percentage of intact NMJs in the GLT1–hIPSA group was significant greater than both control groups (D). GLT1–hIPSA transplants significantly reduced the percentage of partially denervated NMJs in the medial and dorsal hemi-diaphragm regions compared to both control groups (E). Results were expressed as means ± SEM. $^{*}p < 0.05$, GLT1–hIPSA group *versus* both control groups. n = 4–6 per group for NMJ analysis.

(Fig. 6D). GLT1–hIPSA transplants also significantly reduced the percentage of partially denervated NMJs in the medial and dorsal hemi-diaphragm regions compared to both control groups (Fig. 6E).

3.7. GLT1 overexpressing hIPSA transplants preserved diaphragm function following cervical contusion SCI

To determine the efficacy of preserving PMN-diaphragm innervation with respect to respiratory impairment, we characterized the *in vivo* functional effects of transplants on diaphragmatic function in cervical contusion rats. We recorded spontaneous EMG activity, which is indicative of PMN activation of diaphragm muscle due to central drive, at 4 weeks post-injury/transplantation (Fig. 7A). All groups showed reduced amplitude in rhythmic inspiratory EMG bursts associated with muscle contraction compared to uninjured animals (Nicaise et al., 2012). Integrated EMG analysis of this recording shows that the GLT1–hIPSA transplants significantly increased EMG amplitude in the dorsal region of the hemi-diaphragm compared to both control groups (Fig. 7B), again matching the anatomically-specific spinal cord and NMJ histological results. However, we observed no protective effects of GLT1–hIPSA transplants at either the medial or ventral regions, and the control GFP–hIPSA transplants showed no significant effects compared to control hFibroblast injection at all hemi-diaphragm locations (Fig. 7B). There were no significant differences in EMG burst frequency (Fig. 7C) or burst duration (Fig. 7D) amongst the three groups.

Following supramaximal phrenic nerve stimulus, we obtained compound muscle action potentials (CMAP) recordings from the ipsilateral hemi-diaphragm using a surface electrode (Fig. 7E). In all treatment groups, peak CMAP amplitude was significantly reduced compared to uninjured laminectomy only rats, whose CMAP amplitudes are approximately 7 mV (Nicaise et al., 2013). However, CMAP amplitudes in the GLT1–hIPSAtransplant group were significantly increased compared to the two control transplantation groups at weeks 2–4 post-injury (Fig. 7F). With the use of the surface electrode, we are recording from the entire hemi-diaphragm (or at least a significant portion of the muscle), yet we still observed this significant protective effect on overall muscle function, despite the fact that transplants only reduced central degeneration very near to the injury site and correspondingly preserved morphological innervation only in the dorsal hemi-diaphragm.

4. Discussion

The use of iPS cells as a source of mature cell types for therapeutic transplantation in CNS diseases represents an exciting direction in regenerative medicine. However, to date only a small number of studies have assessed the long-term fate and therapeutic efficacy of iPS cell-derived transplants in animal models of SCI.

A number of these studies reported significant therapeutic benefit when NSCs/NPCs derived from either mouse (Tsuji et al., 2010) or human (Fujimoto et al., 2012; Nori et al., 2011; Romanyuk et al., 2014) iPS cells were transplanted into contusion or cavity-type models of rodent SCI, as well as in non-human primate models (Kobayashi et al., 2012). Unlike our current work, these studies did not focus on, or achieve, targeted replacement of astrocytes in the injured spinal cord. In many cases, the cells were delivered in a multipotent NSC-like state and resulted in mixed differentiation into glial phenotypes, including astrocytes, and various neuronal subtypes. While these studies were able to achieve some functional benefit, future work may require more phenotypically targeted strategies, each of which depends on the nature of the SCI pathology (*e.g.* type of injury and anatomical locations affected) and the specific cell lineages being targeted for replacement. Nevertheless, these studies were able to nicely show promising properties of engrafted cells in the injured spinal cord environment, including synaptic integration into endogenous neuronal circuitry (Fujimoto et al., 2012; Nori et al., 2011). iPS cell-derived NSCs have also shown therapeutic promise in models of other spinal cord diseases such as spinal muscular atrophy (Simone et al., 2014).

A number of these studies with iPS cell transplantation reported a lack of beneficial outcomes in SCI models. Pomeshchik et al. (2014) did not observe functional improvement after transplantation of hIPS

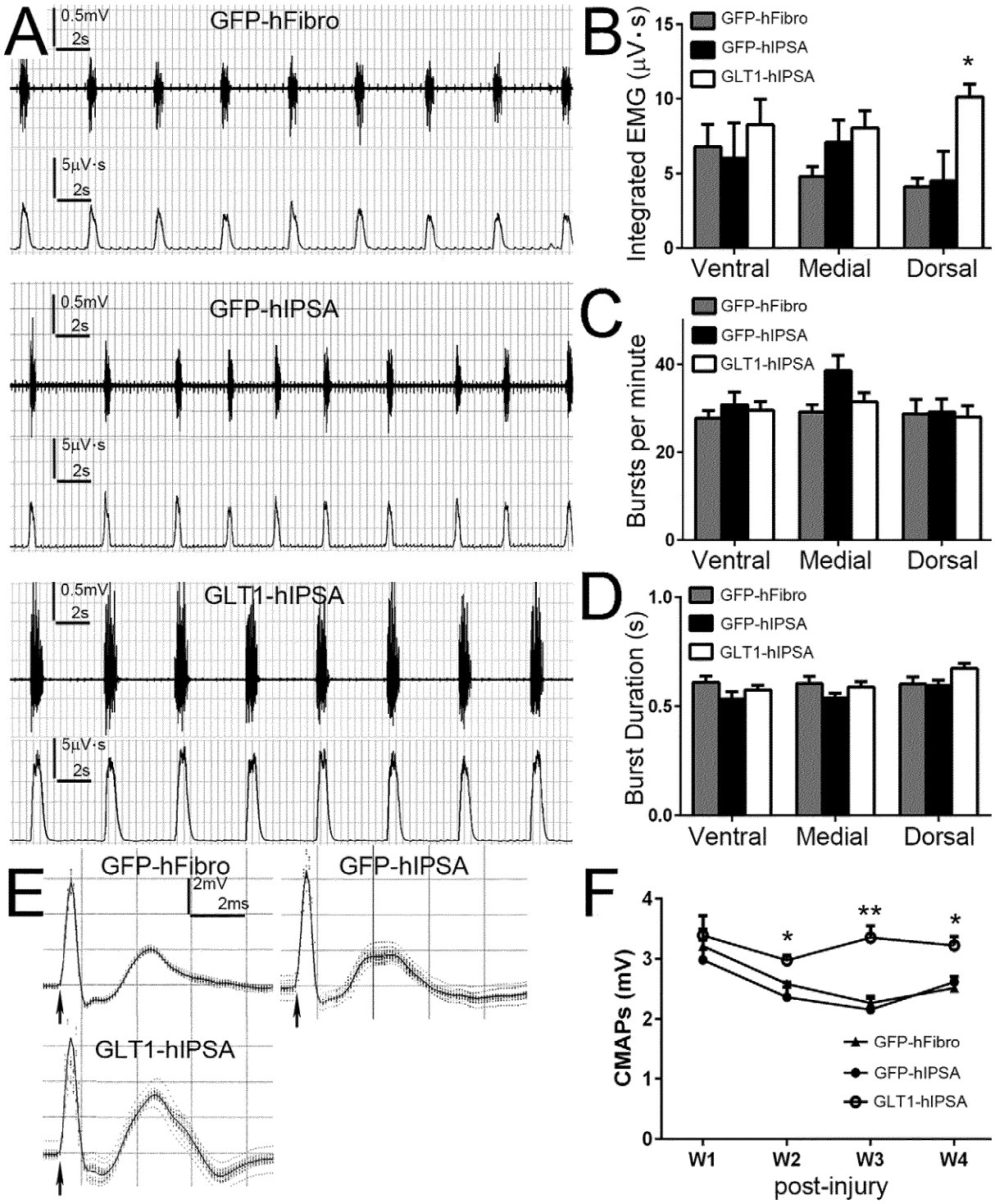

Fig. 7. GLT1 overexpressing hIPSA transplants preserved diaphragm function following cervical contusion SCI. Spontaneous EMG recordings from ipsilateral hemi-diaphram were obtained at 4 weeks post-injury/transplantation (A, *upper*: raw EMG; *lower*: integrated EMG). Integrated EMG amplitude (B), burst frequency (C), and burst duration (D) were analyzed. Following supramaximal phrenic nerve stimulation, we obtained compound muscle action potential (CMAP) recordings from the ipsilateral hemi-diaphragm using a surface electrode (E). CMAP amplitudes at different time points post-injury were analyzed (F). Results were expressed as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, GLT1–hIPSA group *versus* both control groups. n = 6 per group for EMG and CMAP analysis.

cell-derived NPCs in a contusion SCI model. However, they also did not find long term survival of grafted cells in these mice receiving a tacrolimus immune suppression regimen, unlike the robust and persistent integration that we observed in the present study using an immune suppression protocol consisting of both tacrolimus and rapamycin in mice or cyclosporine in rats. In addition to our work, other groups have reported impressive survival and differentiation of hIPS cells into mature CNS cell types after injection into adult spinal cord of similarly immunosuppressed rodents (Haidet-Phillips et al., 2014; Sareen et al., 2014).

An interesting study from the Horner group (Nutt et al., 2013) reported a lack of therapeutic improvement with transplantation of hIPS cell-derived NPCs in a SCI model, despite impressive graft integration. However, cells were delivered at a chronic time point, which may

represent an environment less amenable to transplant-induced plasticity, while we targeted early neuroprotection in this report.

A recent study from the Steward lab reported that transplantation of a mixed population of glial and neuronal progenitors into a transection model of SCI resulted in ectopic engraftment of large numbers of graft-derived cells in locations such as the central canal, ventricles and pial surface of the spinal cord (Steward et al., 2014), providing a note of caution when using transplantation of any class of NSC/NPC in SCI. This issue is particularly relevant to strategies employing cells derived from pluripotent sources such as ES and iPS cells given the possibility of incomplete and/or inefficient differentiation (Tsuji et al., 2010). In the current study and in our previous work (Lepore et al., 2004, 2005, 2006, 2008b, 2011b; Lepore and Fischer, 2005; Li et al., 2014), we never observed overt tumor formation or extensive migration away from injection sites beyond only a few spinal segments. In the current work, we did note the presence of a small residual population of proliferating transplant-derived cells even out to four weeks post-injection, though we never found any tumor formation. It will be important to assess very long-term time points post-transplantation in future experiments to establish the safety of these and similar types of cells before proceeding to the clinic. Unlike the Steward paper, we did not systematically assess distribution of transplant-derived cells throughout the neuraxis.

Mechanical allodynia (a form of neuropathic pain) was observed when mouse iPSAs were transplanted into a contusion SCI model (Hayashi et al., 2011). In addition to this work, other published studies have similarly reported sensory hypersensitivity in SCI models accompanying transplantation of progenitor-derived astrocytes (Davies et al., 2008; Hofstetter et al., 2005), possibly due to increased neuronal plasticity that is induced by transplantation of immature astrocyte populations (Smith et al., 1986). However, in a large body of work, we and others (Haas et al., 2012; Mitsui et al., 2005; Nutt et al., 2013) have not found such increased sensitivity, including following hIPSA transplantation (Nutt et al., 2013). The discrepancy amongst these studies may be due to heterogeneity in the subtypes of astrocytes being injected (Davies et al., 2008, 2011).

A number of practical issues that are beyond the scope of this discussion will need to be addressed before moving transplantation of iPS cells to the clinic in SCI and other diseases of the nervous system. Specifically with respect to targeting relative early events such as PhMN loss after cervical SCI, autologous derivation of cells will likely not be relevant given that PhMNs are lost within several days post-injury (Nicaise et al., 2013). Instead, cells to be used for transplantation will likely be obtained from banks of immune/HLA-matched cells (Zimmermann et al., 2012). Given the need to extensively test iPS cell lines prior to transplantation into a patient, as well as the costs and time that will be required for generating cells for each individual patient, this approach may actually be practically preferable to autologous derivation (Taylor et al., 2011). As human stem cell lines have shown donor variability in SCI models (Neuhuber et al., 2005), future studies will need to investigate *in vivo* properties and therapeutic efficacy of human iPS cells derived from multiple donors in an attempt to move this approach toward clinical translation.

Similar to our previous work using transplantation of astrocytes derived from rodent glial progenitors (Li et al., 2014), we find that GLT1-overexpresing hIPSAs promote significant preservation of diaphragm function and diaphragm innervation by PhMNs. In both studies, control unmodified transplant-derived astrocytes expressed relatively lower levels of GLT1 in the injured spinal cord, suggesting that the cells respond to the injured environment in a similar manner as host astrocytes that show extensive transporter downregulation. Interestingly, the unmodified hIPSA transplants, despite excellent survival and efficient differentiation, did not promote therapeutic benefit with respect to protection of diaphragmatic respiratory circuitry. These findings suggest that astrocyte replacement alone may insufficient when targeting certain pathological mechanisms (*e.g.* excitotoxocity) but that functional maturation of these astrocytes is necessary, which is not surprising given the diverse, complex and integral roles that astrocytes play in intact CNS function (Pekny and Nilsson, 2005).

We have made interesting observations over the course of a number of studies with respect to therapeutically targeting GLT1 following SCI. We have consistently observed significant GLT1 downregulation in endogenous reactive astrocyte populations in both contusion and crush, as well as both cervical and thoracic, models of SCI (Lepore et al., 2011a, 2011c; Li et al., 2015; Putatunda et al., 2014; Watson et al., 2014). When we selectively increased GLT1 expression in these endogenous astrocytes in the unilateral cervical contusion model using an AAV8 vector, we paradoxically found that secondary degeneration of PhMNs and diaphragm denervation were worsened (Li et al., 2015). This effect was due to compromise in the protective glial scar-forming properties of endogenous astrocytes, which resulted in unexpected expansion of the lesion. In the current study with hIPSAs and in our previous work with rodent-derived glial progenitors (Li et al., 2014), we found that delivery of an exogenous source of astrocytes that expresses high levels of functional GLT1 *via* transplantation (in the exact same cervical contusion model) results in significant preservation of PhMNs and diaphragm function. These findings, as well as other studies that tested the effects of pharmacologically elevating (Olsen et al., 2010) or genetically reducing (Lepore et al., 2011c) GLT1 in SCI, demonstrate that targeting GLT1 is a promising and powerful therapeutic strategy in SCI for targeting neuroprotection and possibly other outcomes of SCI such as neuronal hyperexcitability.

Despite the impressive therapeutic effect achieved in the present study, the degree of PhMN protection and diaphragm function preservation was only partial. In future work, we will need to optimize neuroprotective strategies such as hIPSA transplantation to enhance therapeutic effects, as well as combine these neuroprotective approaches with interventions aimed at promoting plasticity, axonal regrowth and targeted reconnection of the rVRG-PhMN-diaphragm circuit (Alilain et al., 2011). Preserving neural control of diaphragm function involves targeting a complex circuitry that extends beyond just protecting PhMNs (Lane et al., 2009). We focused on preservation of PhMNs centrally in the cervical spinal cord and NMJ innervation peripherally in the diaphragm. Nevertheless, our hIPSA intervention may have also exerted beneficial effects *via* protection of respiratory interneuron populations of the cervical spinal cord and/or descending bulbospinal input to PhMNs from the rVRG. hIPSA transplants may have also resulted in beneficial effects by promoting regrowth/regeneration and/or sprouting of rVRG axons and interneurons, which is possible given the growth-promoting properties of astrocyte transplants after SCI (Davies et al., 2006, 2008, 2011; Haas et al., 2012). However, we only observed therapeutic effects on diaphragm innervation and function with GLT1 overexpressing hIPSAs (but not with control unmodified hIPSAs), suggesting that neuroprotection mediated by increased GLT1 levels and consequent reduction in excitotoxicity was the likely mechanism, even if transplants also promoted some regrowth of respiratory axon populations. We also did not observe differences amongst groups in plasticity at the diaphragm NMJ such as sprouting or reinnervation, further supporting central neuroprotection as the responsible mechanism of therapeutic action.

In conclusion, we report exciting and novel results showing that targeted replacement of astrocyte GLT1 following cervical SCI using hIPSA transplantation significantly preserves diaphragmatic respiratory function. These findings are important for a number of reasons. We demonstrate the therapeutic efficacy and safety of hiPS transplantation in SCI, as well as the benefit of specifically addressing astrocyte dysfunction using this clinically-relevant source of cells. We also show mechanistically that targeting GLT1 using an astrocyte transplant-based approach has profound effects on functional and histopatholoigcal outcomes after SCI. Furthermore, we conducted these studies in a clinically-relevant SCI paradigm that models a large proportion of human disease cases. Excitingly, we find that this intervention results in therapeutic benefit on respiratory function, which has important implications for

SCI patients. Collectively, these studies lay the foundation for translating iPS cell transplantation to the treatment of SCI.

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References

Aguilar, R.M., Steward, O., 2010. A bilateral cervical contusion injury model in mice: assessment of gripping strength as a measure of forelimb motor function. Exp. Neurol. 221, 38–53.

Alilain, W.J., Horn, K.P., Hu, H., Dick, T.E., Silver, J., 2011. Functional regeneration of respiratory pathways after spinal cord injury. Nature 475, 196–200.

Awad, B.I., Warren, P.M., Steinmetz, M.P., Alilain, W.J., 2013. The role of the crossed phrenic pathway after cervical contusion injury and a new model to evaluate therapeutic interventions. Exp. Neurol. 248, 398–405.

Das, A.K., Pal, R., 2010. Induced pluripotent stem cells (iPSCs): the emergence of a new champion in stem cell technology-driven biomedical applications. J. Tissue Eng. Regen. Med. 4, 413–421.

Davies, J.E., Huang, C., Proschel, C., Noble, M., Mayer-Proschel, M., Davies, S.J., 2006. Astrocytes derived from glial-restricted precursors promote spinal cord repair. J. Biol. 5, 7.

Davies, J.E., Proschel, C., Zhang, N., Noble, M., Mayer-Proschel, M., Davies, S.J., 2008. Transplanted astrocytes derived from BMP- or CNTF-treated glial-restricted precursors have opposite effects on recovery and allodynia after spinal cord injury. J. Biol. 7, 24.

Davies, S.J., Shih, C.H., Noble, M., Mayer-Proschel, M., Davies, J.E., Proschel, C., 2011. Transplantation of specific human astrocytes promotes functional recovery after spinal cord injury. PLoS One 6, e17328.

Dowd, L.A., Robinson, M.B., 1996. Rapid stimulation of EAAC1-mediated Na+-dependent L-glutamate transport activity in C6 glioma cells by phorbol ester. J. Neurochem. 67, 508–516.

Fujimoto, Y., Abematsu, M., Falk, A., Tsujimura, K., Sanosaka, T., Juliandi, B., Semi, K., Namihira, M., Komiya, S., Smith, A., Nakashima, K., 2012. Treatment of a mouse model of spinal cord injury by transplantation of human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem cells. Stem Cells 30, 1163–1173.

Gage, F.H., 2000. Mammalian neural stem cells. Science 287, 1433–1438.

Gensel, J.C., Tovar, C.A., Hamers, F.P., Deibert, R.J., Beattie, M.S., Bresnahan, J.C., 2006. Behavioral and histological characterization of unilateral cervical spinal cord contusion injury in rats. J. Neurotrauma 23, 36–54.

Gwak, Y.S., Kang, J., Unabia, G.C., Hulsebosch, C.E., 2012. Spatial and temporal activation of spinal glial cells: role of gliopathy in central neuropathic pain following spinal cord injury in rats. Exp. Neurol. 234, 362–372.

Haas, C., Neuhuber, B., Yamagami, T., Rao, M., Fischer, I., 2012. Phenotypic analysis of astrocytes derived from glial restricted precursors and their impact on axon regeneration. Exp. Neurol. 233, 717–732.

Haidet-Phillips, A.M., Roybon, L., Gross, S.K., Tuteja, A., Donnelly, C.J., Richard, J.P., Ko, M., Sherman, A., Eggan, K., Henderson, C.E., Maragakis, N.J., 2014. Gene profiling of human induced pluripotent stem cell-derived astrocyte progenitors following spinal cord engraftment. Stem Cells Transl. Med. 3, 575–585.

Hayashi, K., Hashimoto, M., Koda, M., Naito, A.T., Murata, A., Okawa, A., Takahashi, K., Yamazaki, M., 2011. Increase of sensitivity to mechanical stimulus after transplantation of murine induced pluripotent stem cell-derived astrocytes in a rat spinal cord injury model. J. Neurosurg. Spine 15, 582–593.

Hofstetter, C.P., Holmstrom, N.A., Lilja, J.A., Schweinhardt, P., Hao, J., Spenger, C., Wiesenfeld-Hallin, Z., Kurpad, S.N., Frisen, J., Olson, L., 2005. Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. Nat. Neurosci. 8, 346–353.

Hulsebosch, C.E., 2008. Gliopathy ensures persistent inflammation and chronic pain after spinal cord injury. Exp. Neurol. 214, 6–9.

Kiskinis, E., Eggan, K., 2010. Progress toward the clinical application of patient-specific pluripotent stem cells. J. Clin. Invest. 120, 51–59.

Kobayashi, Y., Okada, Y., Itakura, G., Iwai, H., Nishimura, S., Yasuda, A., Nori, S., Hikishima, K., Konomi, T., Fujiyoshi, K., Tsuji, O., Toyama, Y., Yamanaka, S., Nakamura, M., Okano, H., 2012. Pre-evaluated safe human iPSC-derived neural stem cells promote functional recovery after spinal cord injury in common marmoset without tumorigenicity. PLoS One 7, e52787.

Lane, M.A., Fuller, D.D., White, T.E., Reier, P.J., 2008. Respiratory neuroplasticity and cervical spinal cord injury: translational perspectives. Trends Neurosci. 31, 538–547.

Lane, M.A., Lee, K.Z., Fuller, D.D., Reier, P.J., 2009. Spinal circuitry and respiratory recovery following spinal cord injury. Respir. Physiol. Neurobiol. 169, 123–132.

Lane, M.A., Lee, K.Z., Salazar, K., O'Steen, B.E., Bloom, D.C., Fuller, D.D., Reier, P.J., 2012. Respiratory function following bilateral mid-cervical contusion injury in the adult rat. Exp. Neurol. 235, 197–210.

Laskowski, M.B., Sanes, J.R., 1987. Topographic mapping of motor pools onto skeletal muscles. J. Neurosci. 7, 252–260.

Lee, J.H., Tigchelaar, S., Liu, J., Stammers, A.M., Streijger, F., Tetzlaff, W., Kwon, B.K., 2010. Lack of neuroprotective effects of simvastatin and minocycline in a model of cervical spinal cord injury. Exp. Neurol. 225, 219–230.

Lepore, A.C., Fischer, I., 2005. Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. Exp. Neurol. 194, 230–242.

Lepore, A.C., Maragakis, N.J., 2011. Stem cell transplantation for spinal cord neurodegeneration. Methods Mol. Biol. 793, 479–493.

Lepore, A.C., Han, S.S., Tyler-Polsz, C., Cai, J., Rao, M.S., Fischer, I., 2004. Differential fate of multipotent and lineage-restricted neural precursors following transplantation into the adult CNS. Neuron Glia Biol. 1, 113–126.

Lepore, A.C., Bakshi, A., Swanger, S.A., Rao, M.S., Fischer, I., 2005. Neural precursor cells can be delivered into the injured cervical spinal cord by intrathecal injection at the lumbar cord. Brain Res. 1045, 206–216.

Lepore, A.C., Neuhuber, B., Connors, T.M., Han, S.S., Liu, Y., Daniels, M.P., Rao, M.S., Fischer, I., 2006. Long-term fate of neural precursor cells following transplantation into developing and adult CNS. Neuroscience 139, 513–530.

Lepore, A.C., Dejea, C., Carmen, J., Rauck, B., Kerr, D.A., Sofroniew, M.V., Maragakis, N.J., 2008a. Selective ablation of proliferating astrocytes does not affect disease outcome in either acute or chronic models of motor neuron degeneration. Exp. Neurol. 211, 423–432.

Lepore, A.C., Rauck, B., Dejea, C., Pardo, A.C., Rao, M.S., Rothstein, J.D., Maragakis, N.J., 2008b. Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. Nat. Neurosci. 11, 1294–1301.

Lepore, A.C., O'Donnell, J., Bonner, J.F., Paul, C., Miller, M.E., Rauck, B., Kushner, R.A., Rothstein, J.D., Fischer, I., Maragakis, N.J., 2011a. Spatial and temporal changes in promoter activity of the astrocyte glutamate transporter GLT1 following traumatic spinal cord injury. J. Neurosci. Res. 89, 1001–1017.

Lepore, A.C., O'Donnell, J., Kim, A.S., Williams, T., Tuteja, A., Rao, M.S., Kelley, L.L., Campanelli, J.T., Maragakis, N.J., 2011b. Human glial-restricted progenitor transplantation into cervical spinal cord of the SOD1^{G93A} mouse model of ALS. PLoS One 6.

Lepore, A.C., O'Donnell, J., Kim, A.S., Yang, E.J., Tuteja, A., Haidet-Phillips, A., O'Banion, C.P., Maragakis, N.J., 2011c. Reduction in expression of the astrocyte glutamate transporter, GLT1, worsens functional and histological outcomes following traumatic spinal cord injury. Glia 59, 1996–2005.

Li, K., Nicaise, C., Sannie, D., Hala, T.J., Javed, E., Parker, J.L., Putatunda, R., Regan, K.A., Suain, V., Brion, J.P., Rhoderick, F., Wright, M.C., Poulsen, D.J., Lepore, A.C., 2014. Overexpression of the astrocyte glutamate transporter GLT1 exacerbates phrenic motor neuron degeneration, diaphragm compromise, and forelimb motor dysfunction following cervical contusion spinal cord injury. J. Neurosci. 34, 7622–7638.

Li, K., Javed, E., Hala, T.J., Sannie, D., Regan, K.A., Maragakis, N.J., Wright, M.C., Poulsen, D.J., Lepore, A.C., 2015. Transplantation of glial progenitors that overexpress glutamate transporter GLT1 preserves diaphragm function following cervical SCI. Mol. Ther. 23, 533–548.

Liu, D., Thangnipon, W., McAdoo, D.J., 1991. Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord. Brain Res. 547, 344–348.

Maragakis, N.J., Rothstein, J.D., 2004. Glutamate transporters: animal models to neurologic disease. Neurobiol. Dis. 15, 461–473.

Maragakis, N.J., Rothstein, J.D., 2006. Mechanisms of disease: astrocytes in neurodegenerative disease. Nat. Clin. Pract. Neurol. 2, 679–689.

McDonald, J.W., Becker, D., 2003. Spinal cord injury: promising interventions and realistic goals. Am. J. Phys. Med. Rehabil. 82, S38–S49.

Mitsui, T., Shumsky, J.S., Lepore, A.C., Murray, M., Fischer, I., 2005. Transplantation of neuronal and glial restricted precursors into contused spinal cord improves bladder and motor functions, decreases thermal hypersensitivity, and modifies intraspinal circuitry. J. Neurosci. 25, 9624–9636.

Neuhuber, B., Timothy Himes, B., Shumsky, J.S., Gallo, G., Fischer, I., 2005. Axon growth and recovery of function supported by human bone marrow stromal cells in the injured spinal cord exhibit donor variations. Brain Res. 1035, 73–85.

Nicaise, C., Hala, T.J., Frank, D.M., Parker, J.L., Authelet, M., Leroy, K., Brion, J.P., Wright, M.C., Lepore, A.C., 2012. Phrenic motor neuron degeneration compromises phrenic axonal circuitry and diaphragm activity in a unilateral cervical contusion model of spinal cord injury. Exp. Neurol. 235, 539–552.

Nicaise, C., Frank, D.M., Hala, T.J., Authelet, M., Pochet, R., Adriaens, D., Brion, J.P., Wright, M.C., Lepore, A.C., 2013. Early phrenic motor neuron loss and transient respiratory abnormalities after unilateral cervical spinal cord contusion. J. Neurotrauma 30, 1092–1099.

Nori, S., Okada, Y., Yasuda, A., Tsuji, O., Takahashi, Y., Kobayashi, Y., Fujiyoshi, K., Koike, M., Uchiyama, Y., Ikeda, E., Toyama, Y., Yamanaka, S., Nakamura, M., Okano, H., 2011. Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice. Proc. Natl. Acad. Sci. U. S. A. 108, 16825–16830.

Nutt, S.E., Chang, E.A., Suhr, S.T., Schlosser, L.O., Mondello, S.E., Moritz, C.T., Cibelli, J.B., Horner, P.J., 2013. Caudalized human iPSC-derived neural progenitor cells produce neurons and glia but fail to restore function in an early chronic spinal cord injury model. Exp. Neurol. 248, 491–503.

Olsen, M.L., Campbell, S.C., McFerrin, M.B., Floyd, C.L., Sontheimer, H., 2010. Spinal cord injury causes a wide-spread, persistent loss of Kir4.1 and glutamate transporter 1: benefit of 17 beta-oestradiol treatment. Brain 133, 1013–1025.

Panter, S.S., Yum, S.W., Faden, A.I., 1990. Alteration in extracellular amino acids after traumatic spinal cord injury. Ann. Neurol. 27, 96–99.

Park, E., Velumian, A.A., Fehlings, M.G., 2004. The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. J. Neurotrauma 21, 754–774.
Pekny, M., Nilsson, M., 2005. Astrocyte activation and reactive gliosis. Glia 50, 427–434.
Perego, C., Vanoni, C., Bossi, M., Massari, S., Basudev, H., Longhi, R., Pietrini, G., 2000. The GLT-1 and GLAST glutamate transporters are expressed on morphologically distinct astrocytes and regulated by neuronal activity in primary hippocampal cocultures. J. Neurochem. 75, 1076–1084.
Pomeshchik, Y., Puttonen, K.A., Kidin, I., Ruponen, M., Lehtonen, S., Malm, T., Akesson, E., Hovatta, O., Koistinaho, J., 2014. Transplanted human iPSC-derived neural progenitor cells do not promote functional recovery of pharmacologically immunosuppressed mice with contusion spinal cord injury. Cell Transplant.
Putatunda, R., Hala, T.J., Chin, J., Lepore, A.C., 2014. Chronic at-level thermal hyperalgesia following rat cervical contusion spinal cord injury is accompanied by neuronal and astrocyte activation and loss of the astrocyte glutamate transporter, GLT1, in superficial dorsal horn. Brain Res. 18, 64–79.
Romanyuk, N., Amemori, T., Turnovcova, K., Prochazka, P., Onteniente, B., Sykova, E., Jendelova, P., 2014. Beneficial effect of human induced pluripotent stem cell-derived neural precursors in spinal cord injury repair. Cell Transplant.
Salewski, R.P., Eftekharpour, E., Fehlings, M.G., 2010. Are induced pluripotent stem cells the future of cell-based regenerative therapies for spinal cord injury? J. Cell. Physiol. 222, 515–521.
Sandrow, H.R., Shumsky, J.S., Amin, A., Houle, J.D., 2008. Aspiration of a cervical spinal contusion injury in preparation for delayed peripheral nerve grafting does not impair forelimb behavior or axon regeneration. Exp. Neurol. 210, 489–500.
Sandrow-Feinberg, H.R., Izzi, J., Shumsky, J.S., Zhukareva, V., Houle, J.D., 2009. Forced exercise as a rehabilitation strategy after unilateral cervical spinal cord contusion injury. J. Neurotrauma 26, 721–731.
Sandrow-Feinberg, H.R., Zhukareva, V., Santi, L., Miller, K., Shumsky, J.S., Baker, D.P., Houle, J.D., 2010. PEGylated interferon-beta modulates the acute inflammatory response and recovery when combined with forced exercise following cervical spinal contusion injury. Exp. Neurol. 223, 439–451.
Sareen, D., Gowing, G., Sahabian, A., Staggenborg, K., Paradis, R., Avalos, P., Latter, J., Ornelas, L., Garcia, L., Svendsen, C.N., 2014. Human induced pluripotent stem cells are a novel source of neural progenitor cells (iNPCs) that migrate and integrate in the rodent spinal cord. J. Comp. Neurol. 522, 2707–2728.
Shanmuganathan, K., Gullapalli, R.P., Zhuo, J., Mirvis, S.E., 2008. Diffusion tensor MR imaging in cervical spine trauma. AJNR Am. J. Neuroradiol. 29, 655–659.
Simone, C., Nizzardo, M., Rizzo, F., Ruggieri, M., Riboldi, G., Salani, S., Bucchia, M., Bresolin, N., Comi, G.P., Corti, S., 2014. iPSC-derived neural stem cells act via kinase inhibition to exert neuroprotective effects in spinal muscular atrophy with respiratory distress type 1. Stem Cell Rep. 3, 297–311.
Smith, G.M., Miller, R.H., Silver, J., 1986. Changing role of forebrain astrocytes during development, regenerative failure, and induced regeneration upon transplantation. J. Comp. Neurol. 251, 23–43.
Stamegna, J.C., Felix, M.S., Roux-Peyronnet, J., Rossi, V., Feron, F., Gauthier, P., Matarazzo, V., 2011. Nasal OEC transplantation promotes respiratory recovery in a subchronic rat model of cervical spinal cord contusion. Exp. Neurol. 229, 120–131.
Steward, O., Sharp, K.G., Yee, K.M., Hatch, M.N., Bonner, J.F., 2014. Characterization of ectopic colonies that form in widespread areas of the nervous system with neural stem cell transplants into the site of a severe spinal cord injury. J. Neurosci. 34, 14013–14021.
Strakowski, J.A., Pease, W.S., Johnson, E.W., 2007. Phrenic nerve stimulation in the evaluation of ventilator-dependent individuals with C4- and C5-level spinal cord injury. Am. J. Phys. Med. Rehabil. 86, 153–157.
Stys, P.K., 2004. White matter injury mechanisms. Curr. Mol. Med. 4, 113–130.
Takahashi, K., Okita, K., Nakagawa, M., Yamanaka, S., 2007a. Induction of pluripotent stem cells from fibroblast cultures. Nat. Protoc. 2, 3081–3089.
Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007b. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872.
Taylor, C.J., Bolton, E.M., Bradley, J.A., 2011. Immunological considerations for embryonic and induced pluripotent stem cell banking. Philos. Trans. R. Soc. Lond. B Biol. Sci. 366, 2312–2322.
Tsuji, O., Miura, K., Okada, Y., Fujiyoshi, K., Mukaino, M., Nagoshi, N., Kitamura, K., Kumagai, G., Nishino, M., Tomisato, S., Higashi, H., Nagai, T., Katoh, H., Kohda, K., Matsuzaki, Y., Yuzaki, M., Ikeda, E., Toyama, Y., Nakamura, M., Yamanaka, S., Okano, H., 2010. Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury. Proc. Natl. Acad. Sci. U. S. A. 107, 12704–12709.
Watson, J.L., Hala, T.J., Putatunda, R., Sannie, D., Lepore, A.C., 2014. Persistent at-level thermal hyperalgesia and tactile allodynia accompany chronic neuronal and astrocyte activation in superficial dorsal horn following mouse cervical contusion spinal cord injury. PLoS One 9, e109099.
Wright, M.C., Son, Y.J., 2007. Ciliary neurotrophic factor is not required for terminal sprouting and compensatory reinnervation of neuromuscular synapses: re-evaluation of CNTF null mice. Exp. Neurol. 205, 437–448.
Wright, M.C., Cho, W.J., Son, Y.J., 2007. Distinct patterns of motor nerve terminal sprouting induced by ciliary neurotrophic factor vs. botulinum toxin. J. Comp. Neurol. 504, 1–16.
Wright, M.C., Potluri, S., Wang, X., Dentcheva, E., Gautam, D., Tessler, A., Wess, J., Rich, M.M., Son, Y.J., 2009. Distinct muscarinic acetylcholine receptor subtypes contribute to stability and growth, but not compensatory plasticity, of neuromuscular synapses. J. Neurosci. 29, 14942–14955.
Xu, G.Y., Hughes, M.G., Ye, Z., Hulsebosch, C.E., McAdoo, D.J., 2004. Concentrations of glutamate released following spinal cord injury kill oligodendrocytes in the spinal cord. Exp. Neurol. 187, 329–336.
Xu, G.Y., Hughes, M.G., Zhang, L., Cain, L., McAdoo, D.J., 2005. Administration of glutamate into the spinal cord at extracellular concentrations reached post-injury causes functional impairments. Neurosci. Lett. 384, 271–276.
Zimmer, M.B., Nantwi, K., Goshgarian, H.G., 2007. Effect of spinal cord injury on the respiratory system: basic research and current clinical treatment options. J. Spinal Cord Med. 30, 319–330.
Zimmermann, A., Preynat-Seauve, O., Tiercy, J.M., Krause, K.H., Villard, J., 2012. Haplotype-based banking of human pluripotent stem cells for transplantation: potential and limitations. Stem Cells Dev. 21, 2364–2373.

Drug Dosages for Mice and Rats

Amoxicillin
Mice- Dose: 150mg/8oz. Using a gram scale, measure 2.14g of Trimox powder (250mg/5mL). Add to 8oz water bottle and shake well. If using 14oz bottle, add 3.7g, if using 13.5oz Hydropac, add 3.6g. Solution should be changed weekly or bi-weekly.
*** Contains sugar.
Rats-Dose: 100mg/kg. Concentration 1mg/mL. Using a gram scale measure 6.75g Trimox powder (250mg/5ml) and add to a 16oz water bottle, 5.9g to a 14oz water bottle, or 5.7g to a 13.5oz Hydropac. Shake well. Solution should be changed weekly or bi-weekly. **Contains sugar.

Baytril (antibiotic injection / used in water)
Loading dose: 25mg/kg. For 25gm mouse give 0.02mL of full strength Baytril (22.7mg/mL) SQ. Dilute
Maintenance dose: 4mg/kg. Use diluted Baytril Solution: 0.1mL of full strength (22.7mg/mL) Baytril with 9.9mL sterile water. Solution yields 0.22mg/mL solution.
For 25gm mouse give 0.45ml SQ.
Baytril Water
Mice-Dose: 25mg/kg/day. Use 1.7mL full strength (22.7mg/mL) Baytril in 8oz of water, 3mL in a 14oz bottle, or 2.9mL in a 13.5oz Hydropac. Change weekly.
Rats-Dose: 25 mg/kg/day. Add 5.2mL of 22.7 mg/lm Baytril into 16oz of water, 4.6mL to a 14oz bottle, or 4.4mL to a 13.5oz Hydropac. Change weekly.

Buprenex (pain injection)
Dose: 0.025mg/kg-0.05mg/kg. Dilute 0.1mL of 0.3mg/mL Buprenex to 0.9mL of sterile water to yield 0.03mg/mL solution.
For 25gm mouse at 0.05mg/kg give 0.04mL of diluted solution SQ.
Or calculate for higher dose: example; 2.5mg/kg dose for a 25mg mouse, give 0.06mg or 2mLs of the diluted solution.
Store at room temperature. Label with date of dilution. Expires on same date of the next month.
SR Buprenorphine-dose is 0.5mg/kg. For a 25gm mouse give 0.0125ml SQ.

Calcium Gluconate ***(*Use first for dystocia-see Oxytocin and dystocia guidelines)***
Dose: 100mg/kg given intraperitoneal 10 minutes prior to Oxytocin inj.
Stock Solution: 98mg/mL.
For average 25 gm mouse give 0.02mL full strength solution or 2.5mg.

Carprofen (pain injection)
Dose: 5mg/kg. Use diluted solution. Dilute 1mL of full strength Carprofen (50mg/mL) with 3mLs of Sterile Water yielding a 12.5mg/ml solution.
For 25gm mouse give 0.01mL SQ.
Keep refrigerated. Label with date of dilution. Expires on same date the next month.

Doxycycline Powder
Use gram scale to measure out 0.25g of powder per 8oz of water.

Ibuprofen (for pain; used in the water)
Mice-Stock solution is 20mg/mL = 100mg/5mL. Use diluted solution. Add 2.5ml of 20mg/mL Ibuprofen suspension to 8oz water bottle (0.2mg/mL solution.), 4.4mL to a 14oz bottle, or 4.2mL to a 13.5oz Hydropac. Change weekly or bi-weekly. **Contains sugar.
Rats-Add 7.6ml (20mg/mL) ibuprofen into 16oz water bottle, 6.7ml to a 14oz bottle, or 6.4mL to a 13.5oz Hydropac. Change weekly or bi-weekly. ** Contains sugar. Results in a dose of 32mg/kg.

Ivermectin (parasiticide in drinking water for pinworms)
Dose is 0.08% sheep drench diluted as follows:
Mice: 2.4mL/8oz of water.
Rats: 12mL/16oz water.

Updated 3/5/2018 (ELS)

Or 192mL/5 gallons of water for one mouse rack.

Ketoprofen (pain injection)
Dose: 5mg/kg. Use diluted solution. Dilute 1mL of 100mg/mL Ketoprofen injection to 9mLs of sterile water to yield 10mg/mL solution. For 25gm mouse give 0.0125mL subcutaneously.
Store at room temperature. Label with date of dilution. Expires on same date of the next month.

Mouse Mix (for anesthesia)
Dose: 0.02mL/gm IP.
Full volume: Mix 0.22mL Xylazine (20mg/mL), 0.65mL Ketamine (100mg/mL), 9.13 mL Sterile water.
Half volume: Mix 0.11mL Xylazine, 0.32mL Ketamine, 4.56mL Sterile water.
Solution expires after 10-14 days.

Meloxicam (analgesic)
Mice: 1mg/kg/day in the water
Add 0.19 mL of 5mg/ml of injectable Meloxicam to an 8oz bottle, 0.33mL to a 14oz bottle, or 0.32mL to a 13.5oz Hydropac. Change weekly.
Mice: 5 mg/kg for injection SC once daily

Rats: 1mg/kg/day in the water
Add 0.94mL of 5mg/ml of injectable Meloxicam in a 16 oz. water bottle, or 0.79mL to a 13.5oz Hydropac.
Rats: 1mg/kg SC once daily

Neomycin (Antibiotic in water for irradiation experiments in Mice and Rats)
Mice- Dose: 2mg/mL.
Prepare as follows: Use 25g bottle of powdered Neomycin (732mc/mg) to 223mL of R/O water from sink.
This yields an 82mg/mL solution. For a 2mg/mL solution, add 6mL of the prepared solution to an 8oz water bottles, 10.5mL to a 14oz bottle, or 10.1mL to a 13.5oz Hydropac. Solution should be changed weekly.
Rats-Dose 3.2mg/ml. Reconstitute powdered Neomycin as per mice instructions to a 82mg/ml solution. Add 18.5mL of this solution into a 16 oz water bottle, 16.2ml to a 14oz bottle, or 15.6mL to a 13.5oz Hydropac.

Ondansetron (anti-nausea medication)
Rats – Dose: 0.1mg/kg SQ
Dilute full strength (2mg/mL) 1mL in 9ml sterile water to make a 0.2mg/mL solution. Inject 0.25 mL for a 500g rat.

Oxytocin (Injection for dystocia- see dystocia guidelines)
Dose: 1.0IU/kg. Use diluted solution.
From 20 IU Oxytocin, use 0.1mL to 9.9 mL sterile water to yield a 0.21 IU/mL solution. Give 0.125mL SQ for an average 25 gm mouse every 30min- hour.
If mouse has not given birth after three doses, euthanasia is necessary.

Rat Mix (for anesthesia)
Dose: 0.9mL of prepared mix/kg IP.
Prepare as follows: 5mL Ketamine (100mg/mL), 1.6mL Xylazine (20mg/mL).
After calculating dose, dilute further with 2mL of sterile water before giving IP.
Full strength mix expires in approximately one week.

Sucralfate oral suspension (20mg/ml)
Rats – Dose: 0.5ml per os BID

SMZ-TMP
Dose is approximately 54mg/kg/day.
Mice: Use 1mL of full strength (Sulfa 80mg/mL & Trimeth 16mg/mL) per 8oz of drinking water, or 1.7mL per 13.5oz Hydropac.
Rats: Use 3mLs of full strength per 16oz of drinking water, or 2.5mL per 13.5oz Hydropac.
*** Diluted drug labels are filed in filing cabinet under "Diluted Drug Labels"

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Monday, August 10, 2020 2:02 PM
To: Molly K. Lucas
Subject: Fw: Additional Vet Questions for Protocol 4174-03
Attachments: response to comments on animal protocol- Hai Zhang.docx

Hi Molly,

How are you? I (finally) received the revision for Dr. Zhang's protocol last week, and was able to finish looking through it this afternoon. Overall, I think it's looking good. They incorporated most (if not all) of your suggestions. I just sent the protocol back to you in HoverBoard. Attached are the additional questions that you sent via email, along with their responses. Let me know if you have additional follow-up for the group.

Thanks,
Aubrey

FERPA
RCW 42.56.070(1)

From: [redacted]
Sent: Tuesday, August 4, 2020 10:25 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: Additional Vet Questions for Protocol 4174-03

Hi Dr. Schoenleben,
Here are the responses to the questions you sent me by email. Thanks for your efforts and time to help us with the protocol! Hope the revised protocol is good enough to perform this time:-)
Cheers,
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年7月31日 18:12
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: Additional Vet Questions for Protocol 4174-03

Hi [redacted]

Good question! I would recommend adding your response to each question in the document that I sent you, and then emailing that document back to me. Please also incorporate the response/any needed edits in to the protocol itself.

Hope this helps! Any other questions, let me know.

Cheers,
Aubrey

From: [redacted]
Sent: Friday, July 31, 2020 5:04 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: Additional Vet Questions for Protocol 4174-03

Hi Dr. Schoenleben,
Thanks for your prompt respond! Your answers are very helpful.
You sent me the additional comments raised by the vets. But I am not sure where to attach these questions/suggestions and our replies in the IACUC system. Would you please send me an instruction on that? Thanks a lot!
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年7月31日 16:40
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: Additional Vet Questions for Protocol 4174-03

Hi [redacted]

Its good to hear from you! I hope you've been doing well. In response to your questions:

- That sounds reasonable. I would recommend including that information in response to the vet question, and asking for alternate anesthesia options if ketamine/xylazine is not recommended for that procedure.
- Absolutely - the surgery training only needs to be completed prior to performing surgeries (not prior to approval of the protocol).

Take care,
Aubrey

From: [redacted]
Sent: Friday, July 31, 2020 11:45 AM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: Additional Vet Questions for Protocol 4174-03

Hi Dr. Schoenleben,
Hope you are doing well!
Thank you for all the support you provided. The vets helped me on the analgesic dosing, so I can answer most of the questions now, except for 2 issues:

1. We haven't found a micro CT that equiped with inhalation anesthesia apparatus. So we are not sure that we can use isoflurane at imaging procedures. As the vets didn't totally deny the ketamine/xylazine, can we still adopt this kind of anesthesia?
2. Because of the COVID pandemic and other ongoing study, it seems that I won't have enough time to accomplish the socket preservation surgery. I only have 2 months to be here, and we

want to start on the blood collection experiment as soon as possible. The reviewer kindly reminded me to contact the training group, to reserve a vet's observation over my surgery procedure. In this case, can I only receive a training on blood collection, rather than the tooth extraction surgery?

Thank you very much!

[redacted]

FERPA
RCW 42.56.070(1)

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年6月27日 15:37
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: Additional Vet Questions for Protocol 4174-03

Hi [redacted]

Absolutely- the best email address for the vets is vsreview@uw.edu.

Hope you're having a good weekend,
Aubrey

Sent from my iPhone

On Jun 27, 2020, at 2:33 PM, [redacted] wrote:

Hi Dr. Schoenleben,
I am doing well, I hope you are doing good too.
Thank you for informing me of the update. I appreciate the suggestions from the vets. I have some questions about the dose of the recommended medicine. May I know how to contact the vets who gave the comments?
Thank you!
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年6月26日 17:51
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Additional Vet Questions for Protocol 4174-03

Hi [redacted]

How are you? The vets have a few additional questions/suggestions for your protocol. Since the protocol is currently in your court, we're not able to add these questions as Reviewer Notes in HoverBoard, so I am attaching them here. Please be sure to address these questions along with the 8 questions that the vets and I sent to you on 6/3/20. If you have any questions or if I can help with anything, let me know.

Take care,
Aubrey

AUBREY SCHOENLEBEN, PhD, CPIA
Scientific Liaison & Review Scientist
Office of Animal Welfare

Health Sciences Building, Box 357160
1705 NE Pacific Street, Seattle, WA 98195-7160
vm: 206.685.6923 / fax: 206.616.5664
aubreys@uw.edu / oaw.washington.edu

<Outlook-1471462127.png>

Thank you for all the valuable comments and the timely help you provided. Following are the questions/suggestions and corresponding replies:

Q1. Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team): Since tooth extractions are known to be moderately painful, multimodal analgesia following this surgery is recommended unless scientifically contraindicated. The 72 hr meloxicam procedure that is already here is a good choice for one type of analgesic. Other types of analgesics that can be added on to the NSAID already in place are local anesthesia and an opioid:

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Please comment/edit as needed.

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-Q #3: Re: the statement, "…using a sterilized round bur and copious amount of sterilized saline for cooling." How will aspiration of the saline be prevented?

A6. Thank you for your suggestion about anesthetic re-dosing. We added re-dosing with half of the starting dose of ketamine to the procedure “Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team)”.

Thank you for pointing out this critical issue in the surgery. To avoid aspiration, the head of the rat will be set lower than body while preparing the bony defect. We added this description to the surgical procedure.

Activity	Author	Activity Date
Letter Sent	Schoenleben, Aubrey	9/23/2020 8:09 AM
Correspondence_for_PROTO202000003.doc		
Letter Prepared	Schoenleben, Aubrey	9/23/2020 8:08 AM
Correspondence_for_PROTO202000003.doc		
Approval Period Edited	Schoenleben, Aubrey	9/23/2020 8:07 AM
Designated Member Review Submitted	Robinson, Farrel R.	9/22/2020 4:19 PM
Designated Reviewers Assigned	Huang, Stephanie W	9/22/2020 11:02 AM
Assigned to Designated Review	Huang, Stephanie W	9/22/2020 11:02 AM
Agenda Item Removed	Huang, Stephanie W	9/22/2020 11:00 AM
Ancillary Review Submitted	Cashman, Judy L	9/21/2020 3:11 PM
Ancillary Reviews Managed	Cashman, Judy L	9/21/2020 3:10 PM
Tags Managed	Cashman, Judy L	9/21/2020 3:10 PM
OHRs attached	Cashman, Judy L	9/21/2020 3:10 PM
Private Comment Added	Robinson, Farrel R.	9/21/2020 11:20 AM
I have read this new protocol application and I have no questions.		
Meeting Assigned	Schoenleben, Aubrey	9/14/2020 12:32 PM
Pre-Review Submitted	Schoenleben, Aubrey	9/14/2020 12:32 PM
Vet Consult Submitted	Lucas, Molly K	9/14/2020 12:00 PM
Do you accept the submission? yes no further questions/comments		
Vet Consult Sent	Schoenleben, Aubrey	9/14/2020 10:21 AM
Vet review of revision		
Response Submitted	[redacted]	9/13/2020 10:31 PM
Thank you for your valuable advices. We appreciate your efforts to help us make the protocol better!		
Comment Added	Schoenleben, Aubrey	9/4/2020 7:18 AM
Hi [redacted] How are you? Just checking in to see how this last round of revisions is coming along. If there is anything that I can do to help, please let me know. Thanks!		
Clarification by Pre-Reviewer Requested	Schoenleben, Aubrey	8/19/2020 9:23 AM
Hi [redacted] How are you? A few last suggests from the vets. Please see the new Reviewer Notes (5 total) and edit/comment as needed. Any questions, please let me know. Thanks!		
Vet Consult Submitted	Lucas, Molly K	8/14/2020 1:47 PM
Do you accept the submission? no Would like to review response regarding antibiotics		
Vet Consult Sent	Schoenleben, Aubrey	8/10/2020 1:59 PM
Vet review of revision		
Response Submitted	[redacted]	8/4/2020 10:12 PM
Thanks for your valuable comments!		

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RCW 42.56.070(1)

Thanks for your valuable comments!

Comment Added	Schoenleben, Aubrey	7/21/2020 11:26 AM

Hi [redacted] I hope you're doing well and enjoying the summer! Just checking in to see if you needed any help with the revision. If there is anything that I can do, please let me know. Thanks!

Comment Added	Schoenleben, Aubrey	6/17/2020 6:19 AM

Hi [redacted] How are you? Just checking in again to see how the revision is coming along. If there is anything that I can do to help, please let me know. Thanks!

Clarification by Pre-Reviewer Requested	Schoenleben, Aubrey	6/3/2020 3:45 PM

Hi [redacted] How are you? The vets have completed the pre-review of your protocol. Please see the new Reviewer Notes (8 total) and edit the protocol/procedures as needed. Any questions, let me know. Thanks!

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RCW 42.56.070(1)

	Vet Consult Submitted	Lucas, Molly K	6/3/2020 3:38 PM
Do you accept the submission? no			
	Vet Consult Sent	Lucas, Molly K	6/2/2020 8:33 AM
	Vet Consult Sent	Schoenleben, Aubrey	5/26/2020 7:49 AM
	Response Submitted	[redacted]	5/24/2020 5:46 PM
Thanks very much for your comments!			
	Tags Managed	Williams, Ashley E	5/19/2020 10:11 AM
	Clarification by Pre-Reviewer Requested	Schoenleben, Aubrey	5/18/2020 12:49 PM
Hi [redacted] Really nice work on the revision - the protocol is looking much better! I have a few last questions to address before we send the protocol for vet review. Please see the new Reviewer Notes (4 total) and edit the protocol/procedures as needed. Any questions, let me know. Thanks!			
	Response Submitted	[redacted]	5/13/2020 2:39 PM
Thanks for your comments! We made modification in our protocol accordingly. Hope we will receive your positive response later!			
	Comment Added	Schoenleben, Aubrey	4/16/2020 4:48 PM
Hi [redacted] How are you? Just checking in again to see how the revision is coming along. If there is anything that I can do to help, please let me know. Thanks!			
	Comment Added	Schoenleben, Aubrey	3/23/2020 4:22 PM
Hi [redacted] How are you? Just checking in to see how the revision is coming along. If there's anything I can do to help, please let me know. Thanks!			
	Tags Managed	Huang, Stephanie W	3/17/2020 11:49 AM
	Tags Managed	Kunsman, Robyn	3/9/2020 1:48 PM
	Clarification by Pre-Reviewer Requested	Schoenleben, Aubrey	3/5/2020 10:40 AM
Hi [redacted] How are you? I've completed the initial pre-review of your protocol. There are a couple of questions we should address before sending to the vets for review. Please see the Reviewer Notes (7 total) and edit the protocol/procedures as needed. Any questions, let me know. Thanks!			
	Comment Added	Jimenez, Selesteen	3/2/2020 2:12 PM
Hi Aubrey, please see attached email, PI decided to remove himself from surgery procedures. Thanks RE_ Action Required for Training #4174-03.pdf			
	Coordinator Assigned	Schoenleben, Aubrey	3/2/2020 8:43 AM
Assigned to Aubrey Schoenleben			
	Coordinator Assigned	Jimenez, Selesteen	3/2/2020 8:29 AM
Assigned to OAW Purple Team			
	Assigned Portfolio ID	Jimenez, Selesteen	3/2/2020 8:27 AM
	Tags Managed	Jimenez, Selesteen	3/2/2020 8:27 AM
Hold for PI rat lab Hold for Mei rat lab Action Required for Training #4174-03.pdf			
	Submitted	[redacted]	2/28/2020 1:43 PM
	PI Proxy Assigned	Zhang, Hai	1/22/2020 12:29 PM
PI Proxies Added: [redacted]			
	Protocol Created	Zhang, Hai	1/22/2020 11:55 AM

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RCW 42.56.070(1)

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Tuesday, June 2, 2020 3:07 PM
To: Emily W. Clark
Subject: hoverboard question

Hi Emily,

I'm currently doing the vet review for a new protocol (Hai Zhang) that I think would be great for an upcoming protocol review class with the residents. But that isn't starting for a few weeks. What do you think is the best way to save it so I can use it in a month or so? E.g., I want a way for the residents to look at it, without any of my vet review comments OR the groups' edits/responses to them, i.e., so it would look to them like it does to me today.

I know I ask this periodically but this is the first time I have a concrete example of one I'd like to do now. I just looked at the Printer Version and what the options are with "save as" (I was wondering if it could be pdf'ed- ?) but the 3 "save as" options were all as web pages.

Right now I don't have any questions entered because I'm drafting them in Word, but I should probably get them in and the vet consult turned in within the next few days.

What do you think is the best way to "freeze"/save this document in today's form so I can use it in a few weeks? Any advice much appreciated!

Thanks,
Molly

1. Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team): Since tooth extractions are known to be moderately painful, multimodal analgesia following this surgery is recommended unless scientifically contraindicated. The 72 hr meloxicam procedure that is already here is a good choice for one type of analgesic. Other types of analgesics that can be added on to the NSAID already in place are local anesthesia and an opioid:

-Local anesthesia (e.g., lidocaine/bupivacaine): Could be injected in a specific location as a maxillary nerve block (similar to what is done in human dentistry), or could be applied as a "splash block" (dripped onto the extraction site). The former is probably more technically challenging, and I'm not sure if there are any concerns about the latter interfering with the implant? If you are interested in pursuing the nerve block, vet services and AUTS could likely work with you to practice/develop the technique, and you can contact vsreview@uw.edu for more information/resources.

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Journal of the American Association for Laboratory Animal Science
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Vol 53, No 3
May 2014
Pages 301–306

Antibiotic Administration in the Drinking Water of Mice

James O Marx,[1,2,*] Daljit Vudathala,[3] Lisa Murphy,[3] Shelley Rankin,[4] and F Claire Hankenson[1,2]

Although antibiotics frequently are added to the drinking water of mice, this practice has not been tested to confirm that antibiotics reach therapeutic concentrations in the plasma of treated mice. In the current investigation, we 1) tested the stability of enrofloxacin and doxycycline in the drinking water of adult, female C57BL/6 mice; 2) measured the mice's consumption of water treated with enrofloxacin, doxycycline, amoxicillin, or trimethoprim–sulfamethoxazole; and 3) used HPLC to measure plasma antibiotic concentrations in mice that had ingested treated water for 1 wk. Plasma concentrations of antibiotic were measured 1 h after the start of both the light and dark cycle. The main findings of the study were that both enrofloxacin and nonpharmaceutical, chemical-grade doxycycline remained relatively stable in water for 1 wk. In addition, mice consumed similar volumes of antibiotic-treated and untreated water. The highest plasma antibiotic concentrations measured were: enrofloxacin, 140.1 ± 10.4 ng/mL; doxycycline, 56.6 ± 12.5 ng/mL; amoxicillin, 299.2 ± 64.1 ng/mL; and trimethoprim–sulfamethoxazole, 5.9 ± 1.2 ng/mL. Despite the stability of the antibiotics in the water and predictable water consumption by mice, the plasma antibiotic concentrations were well below the concentrations required for efficacy against bacterial pathogens, except for those pathogens that are exquisitely sensitive to the antibiotic. The findings of this investigation prompt questions regarding the rationale of the contemporary practice of adding antibiotics to the drinking water of mice for systemic antibacterial treatments.

Abbreviations: C_{max}, peak plasma concentration; MIC, minimum inhibitory concentration; TMS, trimethoprim–sulfamethoxazole.

The use of antibiotics to treat bacterial infections is a standard of care in veterinary medicine. In many species, the administration of antibiotics is a routine procedure with proven efficacy. Unfortunately, this is not the case for laboratory mice used in biomedical research, where the delivery of antibiotics may be associated with stress to animals and where confirming that drugs reach therapeutic concentrations in the blood has proven challenging.

The administration of antibiotics to mice either parenterally or bolused enterally involves handling of the mice and induces stress in the animal.[3] To ameliorate this handling-associated stress, medications—including antibiotics—frequently are added to the drinking water. This method is time-efficient for laboratory animal personnel and is thought to be of added therapeutic benefit, because it provides continuous accessibility to the medication. As with any route, there are potential limitations to this route of delivery to mice: first, the antibiotic must remain stable in the drinking water and be available for consumption by the mouse; second, mice must drink predictable volumes of treated water; and, last, sufficient concentrations of antibiotic must be maintained in the bloodstream to achieve systemic antibacterial efficacy.

To date, few studies have tested the stability of antibiotics in the drinking water of laboratory mice. For example, one study[16] that tested the stability of amoxicillin–clavulanic acid and trimethoprim–sulfamethoxazole (TMS) in acidified and reverse-osmosis (RO)–treated water found that amoxicillin was stable in RO water but had an immediate drop in concentration to approximately 50% in acidified water, whereas clavulanic acid dropped to 40% in RO over 7 d and immediately was degraded in acidified water. TMS showed variability over the course of 7 d, making reliable dosing with this drug difficult.[16] The cited study did not measure the consumption or systemic absorption of the antibiotics in the mice; therefore although these antibiotics exhibit variable stability in the drinking water, it is unknown whether these drugs reach concentrations sufficient to eliminate pathogenic bacteria.

When antibiotics are administered in drinking water, it is challenging to estimate accurately the total amount of water consumed by the mice. Many factors complicate this measurement, including: spillage of water from the bottle into the bedding; altered taste of the antibiotic-treated water, which may alter the daily water consumption by the mice; ill or unhealthy mice, which may consume less water than would clinically healthy animals, resulting in dehydration and inadequate antibiotic ingestion; and the diurnal pattern of water intake in laboratory mice, which tend to consume most of their daily water intake at the beginning of the dark cycle, creating potential circadian changes in the ingestion of the antibiotics, with the highest dosing occurring at night.[7,19] Therefore, despite the common practice of adding drugs to drinking water, using this route for dosage of antibiotics to mice is unpredictable.

Even when consumption is sufficient, it is complicated to determine whether the antibiotics reach plasma concentrations adequate to eliminate the pathogenic bacteria responsible for the infection. When inadequate antibiotic concentrations occur, there is an increased risk of selecting for drug-resistant pathogens and eliminating normal flora. The risks of failure to

Received: 09 Sep 2013. Revision requested: 08 Oct 2013. Accepted: 13 Nov 2013.
[1]University Laboratory Animal Resources, [2]Department of Pathobiology, [3]PADLS New Bolton Center, Toxicology Laboratory, Department of Pathobiology, and [4]Clinial Microbiology Laboratory, Veterinary Hospital, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania.
[*]Corresponding author. Email: marx@upenn.edu

achieve adequate drug concentrations are augmented further in laboratory mice, given that many mice are immunosuppressed due to genetic manipulation, radiation, or pharmacologic immunosuppression. These laboratory mice will be almost totally dependent on the bactericidal activity of the antibiotic, with little contribution of the immune system, in the resolution of an infection. The minimum inhibitory concentration (MIC) is the lowest concentration of antibiotic that will effectively inhibit bacterial growth.[10,24] Another important parameter when discussing antimicrobial susceptibility of a given bacterial species is the MIC_{90}. The MIC_{90} represents the MIC value at which 90% of the bacterial strains within a test population containing multiple independent isolates of the same species are inhibited.

An important factor that potentially limits the efficacy of antibiotics in mice is allometric scaling (also referred to as scaling). Scaling relates to the change in physiologic parameters in species in relationship to body size. Obvious examples of scaling can be recognized as changes in heart rate, gestation, and life expectancy with body size. Factors influencing drug pharmacokinetics, such as metabolic rate, glomerular filtration rate, and hepatic blood flow also scale relative to overall body size, resulting in increased metabolic clearance and decreased drug half lives in small species, such as mice.[4,17,22] Pharmacokinetic features of both enrofloxacin and doxycycline have been demonstrated to have significant scaling effects,[6,21] and amoxicillin is reported to have potential scaling effects.[22] The drugs that comprise TMS do not demonstrate evidence of a significant scaling effect, although this drug combination has yet to be tested against scaling parameters other than body size, which may yield a more accurate representation of the effects of scaling on the metabolism of these drugs.[14,15] This increase in the metabolic clearance of antibiotics may limit their ability to achieve the necessary plasma concentrations in mice required for antibiotic efficacy.

The purpose of the current study was to analyze the limitations of the administration of 4 commonly used antibiotics in the drinking water of mice. The antibiotics studied were doxycycline, which typically is administered for the control of gene expression in genetically manipulated mice,[28] and TMS, amoxicillin, and enrofloxacin, which are broad-spectrum antibiotics that have been added to the drinking water of mice.[5,16,25] The first experiment tested the stability of 2 of the antibiotics, enrofloxacin and doxycycline, in tap and acidified water, and enrofloxacin in hyperchlorinated water, all of which are commonly used in laboratory mouse vivaria. The second experiment measured the consumption of the 4 antibiotics from the drinking water of mice and the plasma concentrations of the antibiotics that were achieved. In light of anecdotal evidence of a positive therapeutic effect of antibiotics administered in the drinking water of mice, we hypothesized that 1) the antibiotics would be stable in the drinking water, 2) the treated water would be consumed normally by the mice, and 3) therapeutic plasma concentrations would be achieved.

Materials and Methods

Experiment 1: stability of enrofloxacin and doxycycline in drinking water. The dosages and concentrations of the antibiotics in the drinking water were based on published antibacterial doses and the projected daily consumption of 5 mL by an adult mouse (Table 1). The products used were injectable enrofloxacin (Baytril 100 mg/mL, Bayer HealthCare Animal Health Division, Shawnee Mission, KS); oral pharmaceutical-grade doxycycline calcium (Vibramycin calcium 5 mg/mL, Pfizer Labs, New York, NY); and chemical, nonpharmaceutical-grade doxycycline HCl (Research Products International, Mt Prospect, IL).

Table 1. Antibiotic doses and concentration of antibiotic in the drinking water

Drug	Daily oral dose (mg/kg; [reference])	Antibiotic concentration (mg/mL) in water
Enrofloxacin	50 (5)	0.25
Doxycycline	10 (18)	0.05
Amoxicillin	50 (23)	0.25
TMS	160 (16)	0.8

Sample collection. The stability of enrofloxacin in tap, acidified, and hyperchlorinated water was tested over 7 d, and that of doxycycline in tap and acidified water was tested over 7 d (Table 2), in light of the water systems available at the University of Pennsylvania. The antibiotic-treated water was maintained in a standard, clear mouse water bottle (265 mL; Polysulfone Water Bottles, Ancare, Bellmore, NY) and was placed in a complete mouse cage setup that was empty of animals in a mouse holding room. Facility temperatures were maintained at 22.2 ± 1.1 °C (72 ± 2 °F); humidity was between 30% and 70% with 10 to 15 air changes hourly, as recommended by the *Guide*.[12] The cages were 7.5 in. × 11.5 in. × 5 in. polycarbonate, static isolation cages (Ancare, Bellmore, NY) with 1/4-in. corn cob bedding (Animal Specialties and Provisions, Quakertown, PA). All treated water bottles were shaken daily by the research staff. Samples of treated water were collected on days 0 and 7. At the time of sample collection, 10 mL of treated water was collected by syringe from the end of the sipper tube from each water bottle. Care was taken to disturb the water bottle as little as possible before the sample was collected, to obtain a representative sample of the water that would be available to the mice from the sipper tubes. The samples were stored in centrifuge tubes and frozen at −80 °C until analysis. The effect of the antibiotics on the pH of the water was tested by measuring its pH (pH 510 Benchtop Meter, Oakton Instruments, Vernon Hills, IL) before and after the addition of the antibiotic.

Analysis of antibiotic concentrations in water. Water samples were analyzed by using Shimadzu (Columbia, MD) liquid chromatography with a diode array detector. Water samples containing enrofloxacin were diluted 1:10 with 0.5% formic acid containing 10% acetonitrile. Doxycycline-containing water samples were diluted 1:1 into the same diluent. Control samples of both antibiotics were prepared by dissolving in methanol to obtain a concentration of 1 mg/mL of free drug. Standards were prepared that reflected the expected concentrations for each drug: enrofloxacin standards were 0, 5, 10, 25, and 50 µg/mL; and doxycycline standards were 0, 10, 50, 100, and 200 µg/mL. Enrofloxacin was analyzed by using water and acetonitrile (20:80, both containing 0.1% formic acid) in an isocratic run with a flow rate of 0.6 mL/min. Water samples containing doxycycline were analyzed by gradient chromatography using 0.1% formic acid with 0.005 M EDTA and acetonitrile at a flow rate of 0.8 mL/min; the gradient was as follows: 20% acetonitrile for first 2 min, ramp to 70% acetonitrile over 1 min and then held constant for 3 min, and back to original conditions over 1 min. The system was equilibrated for 5 min prior to the next injection. The diode array detector was monitored from 190 to 320 nm, with quantification done at 280 nm for enrofloxacin and at 265 nm for doxycycline. Acetonitrile, methanol, formic acid, and EDTA were purchased from Thermo-Fisher Scientific (Fair Lawn, NJ). Enrofloxacin and doxycycline hyclate standards were obtained from Sigma-Aldrich (St Louis, MO).

Table 2. Antibiotic concentrations (mg/mL± SE; n = 4 water bottles) during experiment 1

	Tap water		Acidified water		Hyperchlorinated water	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
Enrofloxacin	0.239 ± 0.006	0.237 ± 0.006	0.250 ± 0.004	0.248 ± 0.005	0.246 ± 0.002	0.155 ± 0.019
Doxycycline						
Pharmaceutical grade	0.017 ± 0.001	0.017 ± 0.003	0.048 ± 0.001	0.043 ± 0.001	not tested	not tested
Chemical grade	0.052 ± 0.004	0.032 ± 0.001	0.037 ± 0.002	0.042 ± 0.002	not tested	not tested

Experiment 2: consumption of treated water and serum antibiotic concentrations. Young adult (6 to 10 wk) female C57BL/6J mice (*Mus musculus*, Jackson Laboratory, Bar Harbor, ME) were used in this investigation. The mice were housed in polycarbonate cages with bedding, as described earlier, with free access to autoclaved food (Lab Diet 5010, Animal Specialties and Provisions, Quakertown, PA) and were maintained on a 12:12-h light:dark cycle. Prior to the start of the study, the mice were allowed at least 1 wk to acclimate to the housing facility and conditions. Sentinel mice were tested routinely and were free of pinworms by cecal exam and of fur mites by fur pluck and were antibody-negative for tested pathogens including mouse hepatitis virus, mouse parvoviruses, rotavirus, ectromelia virus, Sendai virus, pneumonia virus of mice, Theiler murine encephalomyelitis virus, reovirus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, and polyomavirus. All aspects of the current investigation were approved by the University of Pennsylvania IACUC.

Mice were pair-housed and randomly assigned to receive 1 of the 4 antibiotics (n = 8 mice for each antibiotic) during the study. The antibiotics tested were enrofloxacin and chemical-grade doxycycline as in experiment 1, amoxicillin (50 mg/mL, Sardoz, Princeton, NJ), and TMS (48 mg/mL, Hi-Tech Pharmacal, Amityville, NY; Table 1). The mice were weighed at the start of the study, and daily water consumption was measured by weighing the water bottles for each pair of mice for 7 d. On day 7, the mice were weighed again, and the antibiotic was added to fresh tap water. The water bottles were shaken daily, and water consumption was measured over an additional 7 d. At the day 14 endpoint, the mice were weighed, and a random half of the mice (n = 4 per group) underwent blood collection into heparinized tubes at 0700; the remaining mice had blood collected at 1900. All blood collection in the study was by retroorbital bleeding under isoflurane anesthesia. Mice were induced at 3% isoflurane until they lost the righting reflex, after which they were maintained at 2.25% for 3 min. This protocol allowed sufficient anesthesia time after removing the mice from the anesthetic to safely collect the blood. Approximately 200 µL blood was collected into heparinized centrifuge tubes at each time point. The mice then were allowed to recover and were returned to their home cages. Two days later, the mice underwent a terminal blood collection at either 0700 or 1900, so that blood was collected from each mouse during both the morning and evening. The blood sample was centrifuged and the plasma separated and frozen at −80 °C until analysis.

To detect the highest possible plasma enrofloxacin concentration, 2 additional groups of 4 mice each were studied. In these mice, the blood was collected at 0100, in an effort to measure the concentration when mice are likely to recently have consumed the greatest water volume (and thus largest therapeutic dose of antibiotic). In addition, the enrofloxacin dose was increased in one group of mice to increase the plasma antibiotic concentration. Specifically, one group of 4 mice received the 50-mg/kg daily dose used in the previous mice, and remaining mice received 100 mg/kg daily.

Analysis of antibiotic concentrations in plasma. Due to the higher sensitivity required for plasma samples compared with water samples, plasma samples were analyzed by using an API 4000 (AB Sciex, Foster City, CA) liquid chromatography–tandem mass spectrometry system. The system was equipped with a Luna C18 (150 × 4.6 mm, 5-µm particle size) analytical column (Phenomenex, Torrance, CA). For each sample, 50 µL plasma was mixed with 0.1 mL acetonitrile containing 1% formic acid. The mixture was vortexed, centrifuged, and filtered through a 0.22-µm nylon filter prior to analysis. Plasma samples containing doxycycline, enrofloxacin, or TMS were analyzed by using a gradient run with 0.1% formic acid and 85% methanol containing 0.1% formic acid at a flow rate of 0.5 mL/min. The gradient conditions were as follows: methanol for the first 2 min, ramp to 95% methanol over the next 3 min, hold at 95% methanol for 4.5 min, return to the original conditions over 0.5 min, and then hold for 4 min. Samples containing amoxicillin were analyzed by using the same gradient conditions but with 0.1% formic acid and acetonitrile instead of methanol. The following ion transitions were selected to quantitate each antibiotic: doxycycline, 445.4 /154; enrofloxacin, 360/316.2; TMS, 291/261.1; and amoxicillin, 366/143.9. The test samples were quantified against curves obtained by analyzing control bovine serum spiked with antibiotic in concentrations ranging from 0.001 to 0.5 µg/mL. Methanol was purchased from Thermo-Fisher Scientific, and control bovine serum was obtained from Sigma-Aldrich.

Statistical analysis. Antibiotic concentrations in water were compared by ANOVA (SigmaPlot 12.3, Systat Software, San Jose, CA). Morning and evening plasma antibiotic concentrations were compared by repeated-measures ANOVA. Statistical significance was defined as a P value of less than 0.05.

Results

Enrofloxacin. Enrofloxacin remained stable in both tap and acidified water throughout the 7-d test period (Table 2). The addition of the injectable enrofloxacin immediately and dramatically increased the pH of both the tap and acidified water (Table 3). In the hyperchlorinated water, a precipitate rapidly formed over the first 24 h. This precipitate was absent from the untreated tap and acidified water, and once formed, the precipitate remained throughout the entire 7-d period. The time 0 sample had the expected antibiotic concentration; however at day 7, only 62% of the antibiotic remained in solution and was available to mice.

Doxycycline. The pharmaceutical-grade oral doxycycline immediately dissolved in the acidified water and remained at stable concentrations for the entire 7-d period (Table 2). However when the drug was added to tap water, a precipitate immediately formed and quickly settled to the bottom of the water bottle. HPLC analysis of the water samples revealed that the concentrations of doxycycline at days 0 and 7 were ap-

Table 3. pH of antibiotic-treated water after drug addition

	Tap water (pH = 6.99 before addition)	Acidified water (pH = 3.29 before addition)
Enrofloxacin	9.54	8.78
Doxycycline	7.02	3.26
Amoxicillin	6.89	3.50
TMS	6.92	3.39

Note the profound effect of the addition of injectable enrofloxacin on the pH of both the tap and acidified water.

proximately 30% of the expected value (Table 2). The sample then was acidified to a pH of 3.0 with hydrochloric acid and remeasured. This action resulted in a doxycycline concentration that was 90.8% of that expected, indicating that the majority of the active ingredient was present but unavailable for consumption by the mice because it was in the precipitate at the bottom of the water bottle.

The nonpharmaceutical, chemical-grade doxycycline powder was tested in both tap and acidified water. In acidified water, the chemical-grade doxycycline immediately dropped to approximately 75% of the expected concentration and then remained stable over 7-d period. There was no significant difference between the day 0 and day 7 doxycycline measurements. In tap water, the initial concentration was approximately 100% of the expected value, dropping significantly ($P < 0.05$) to 64% of the expected concentration at day 7. The addition of chemical-grade doxycycline had little effect on the pH of either the tap or the acidified water (Table 3).

Consumption of antibiotic-treated water and measurement of body weight. The pairs of mice in experiment 2 consumed 9.6 ± 0.2 mL water daily when no antibiotic was added (Table 4). Neither baseline body weight nor water consumption differed between any of the groups. Only the enrofloxacin group had a significant ($P < 0.05$) change in water consumption during the week of antibiotic administration; consumption increased from 9.7 ± 0.4 mL/d to 11.4 ± 0.2 mL/d per pair of mice. Initial body weight did not differ between any of the groups at the start of the experiments, and mice in all 4 groups gained weight over the next 2 wk.

Plasma antibiotic concentrations. Plasma concentrations (Table 4) showed no significant differences between the morning and evening sampling time points for any of the antibiotics. To maximize the measured plasma enrofloxacin concentration, 2 additional groups of mice were tested at 0100, during the dark cycle. The 0100 plasma enrofloxacin concentrations for the group receiving the 50-mg/kg dose was similar to those of the earlier time points; and the plasma antibiotic concentration of the group that received 100 mg/kg enrofloxacin was 174.8 ± 55.5 ng/mL.

Discussion

Achieving therapeutic concentrations of antibiotic in patients is critical to the efficacy of any antibiotic, independent of species. The current investigation demonstrates that, although the antibiotics tested remained stable in the drinking water and the mice consumed predictable volumes of antibiotic-treated water, plasma concentrations above the reported MIC values for most common pathogenic bacteria (Table 5) were not attained. These findings question the rationale for the common practice of antibiotic administration in the drinking water of mice.

The efficacy of antibiotics depends on the pharmacodynamics of the antibiotic–bacteria interaction. Antibiotics are commonly divided into 2 groups according to their pharmacodynamic characteristics: time-dependent, such as β-lactam drugs, in which the efficacy of the drug is determined by the total time the plasma antibiotic concentration is above the MIC of the organism being targeted, and concentration-dependent, such as fluroquinolones, in which efficacy is associated with the peak plasma concentration of the antibiotic.[1,20] Administering antibiotics in the drinking water of mice will optimize the performance of the time-dependent antibiotics, maintaining elevated concentrations of antibiotics in the blood stream whenever mice drink water. In the current study, the amoxicillin plasma concentrations were similar at the start of both the light and dark cycles. However, dosing by water resulted in plasma concentrations that were well below the MIC of most common bacterial pathogens, so that only exquisitely sensitive organisms would be effectively killed by this route of dosing (Table 5).[2,20] The results of the current study are similar to those reported previously[16] regarding plasma levels achieved after antibiotic administration in animals' food.

Providing enrofloxacin in the drinking water failed to achieve effective plasma concentrations. Enrofloxacin is a concentration-dependent drug, which means that the peak serum concentration (C_{max}) achieved has been shown to be a critical factor in the efficacy of bactericidal activity. An C_{max}:MIC value greater than 10 has been shown to predict efficacy.[10,24] Oral bolus dosing of enrofloxacin in dogs has been shown to achieve C_{max} values of 2.1 to 5.2 µg/mL, whereas the plasma concentration measured in the current murine experiment were only 112.2 ± 11.7 ng/mL at 0700 and 140.1 ± 10.4 ng/mL at 1900. We hypothesized that the peak plasma concentration would occur in the middle of the dark cycle, when mice tend to drink the most water,[7,13] so we measured plasma enrofloxacin concentrations in mice in the middle of the dark cycle and found that, surprisingly, this value (117.5 ± 16.9) was lower than the 1900 value. In an effort to maximize C_{max}, a second group of mice for which the enrofloxacin dose was doubled were tested in the middle of the dark cycle, but this adjustment resulted in an average plasma concentration of only 174.8 ± 55.5 ng/mL. Considering that the goal is to achieve a C_{max}:MIC ratio of 10 or greater, these findings indicate that providing enrofloxacin in the drinking water of mice likely will be ineffective against most pathogenic bacteria.[20]

The plasma concentrations of both TMS and doxycycline were well below the MIC_{90} values (Table 5) for common pathogenic bacteria, indicating that the administration of these antibiotics by this route for the treatment of systemic infections in mice should be discouraged. The doses reported in the literature for mice are similar to those used in other species. This dosing regimen fails to take into account the effects of allometric scaling on drug metabolism, which as discussed earlier, will result in an increase in drug metabolism and a subsequent decrease in plasma concentration. Both doxycycline and TMS are used frequently with success in laboratory mice for purposes other than systemic bacterial infections. Doxycycline is used most often in genetically manipulated mice in the control of 'Tet-on' gene expression, by using a tetracycline-sensitive promoter gene to control either the expression or inhibition of gene expression.[28] TMS frequently is added to the drinking water of mice after ionizing irradiation to prevent bacterial sepsis by reducing the number of potential pathologic bacteria within the gastrointestinal tract.[8]

Increasing the amount of antibiotic consumed by the mice can be accomplished by either increasing the concentration of antibiotic in the drinking water or by increasing the amount of water consumed by the mice.[11] However, according to our

Table 4. Water consumption (mL; mean ± SE; n = 4 cages) and plasma antibiotic concentration (ng/mL; mean ± SE)

	Consumption[a]		Plasma antibiotic concentration[b] at		
Antibiotic	Control water	Antibiotic water	0700	1900	0100
Enrofloxacin	9.7 ± 0.8	11.4 ± 0.3[c]	112.2 ± 11.7	140.1 ± 10.4	117.5 ± 16.9
Doxycycline	9.3 ± 0.3	10.1 ± 0.9	56.6 ± 12.5	42.9 ± 7.8	not tested
Amoxicillin	9.3 ± 0.4	8.8 ± 0.8	299.2 ± 64.1	275.2 ± 50.2	not tested
TMS	10.1 ± 1.3	11.2 ± 1.5	5.7 ± 2.3	5.9 ± 1.2	not tested

[a]Consumption data represent 2 mice per cage.
[b]n = 8 mice per antibiotic, except for the 0100 enrofloxacin sample (n = 4).
[c]Value significantly ($P < 0.05$) different from that for consumption of control water.

Table 5. MIC of various antibiotics for common bacteria

	Bacteria	MIC_{90} (ng/mL; [reference])
Enrofloxacin	*E. coli*	30-125 (20)
	S. aureus	120-250 (20)
	Enterococcus spp.	1000-2000 (20)
	Pseudomonas aeruginosa	1000-8000 (20)
	P. multocida	500 (27)
Doxycycline	*M. pneumonia*	500 (26)
	Pasteurella spp.	125 (27)
Amoxicillin	***S. aureus***	**50 (20)**
	E. coli	5000 (20)
	S. pseudintermedius	2000 (20)
	C. perfringens	**50 (20)**
	P. multocida	**250 (27)**
TMS	*S. xylosus*	>2000 (25)
	K. pneumonia	<500 (20)
	E. coli	<500 (20)
	β-hemolytic streptococci	2000 (20)
	Pasteurella spp.	250 (27)

The bacteria–antibiotic combinations represent common pathogenic bacteria in veterinary medicine. MIC_{90} values for ampicillin were used interchangeably with those for amoxicillin. Systemic infections with bacteria in bold can be treated reasonably effectively with the corresponding antibiotic. Note that most of the bacteria isolated during common murine infections lack published MIC_{90} values for various antibiotics.

findings, plasma concentrations would need to be increased by 10-fold to achieve effective plasma concentrations through the drinking water or those that are achieved with oral bolus dosing in other species. Further compounding these difficulties are that the plasma concentrations may not increase linearly with increasing doses, meaning it may take more than a 10-fold increase in the amount of antibiotic consumed to achieve the desired increase in plasma concentration.

A potential use of administration of antibiotics in the drinking water of mice involves the treatment of localized infections in mice. Both amoxicillin and enrofloxacin are concentrated in the urine due to renal excretion.[18] This concentration may enable these antibiotics to achieve sufficient urinary concentrations to be effective for the treatment of cystitis and renal infections in mice. The results of the first experiment indicate care must be taken to ensure the stability of the antibiotic–water combination. Enrofloxacin is stable in both tap and acidified water but radically alters hyperchlorinated water, making the addition of this drug to hyperchlorinated water a poor option. When in either tap or acidified water, this antibiotic appeared to be minimally affected by light over the brief time period studied, given that the water concentrations remained stable over the 7-d period. Ultimately, however, bolus dosing of some antibiotics, particularly enrofloxacin (which works in a concentration-dependent fashion), is more likely to achieve effective plasma concentrations.

Several different water treatments are used to prevent the exposure of immunosuppressed mice to bacterial pathogens, particularly *Pseudomonas aeruginosa*. These include acidification, hyperchlorination, and reverse-osmosis.[9] The stability and solubility of the antibiotics we tested was dependent on the type of water and the formulation of the antibiotic. Preliminary experiments used a pharmaceutical-grade, oral doxycycline suspension and showed that it dissolved into and was stable in acidified water but remained as a suspension in tap water. We then tested a chemical nonpharmaceutical-grade doxycycline powder that is used by many research laboratories for control of gene expression using the Tet promoter, and the drug demonstrated mild degradation over the 7-d observation period. This distinction is an important one to make for institutional committees that review the use of doxycycline for research purposes, because investigators typically are expected to justify the use of nonpharmaceutical chemical-grade products for research animals. This difference in solubility would be a scientific justification for investigators to choose the chemical-grade product over the pharmaceutical grade product. Similar findings occurred with enrofloxacin, which was soluble in both acidified and tap water but precipitated in hyperchlorinated water. Finally, injectable enrofloxacin had a profound effect on the pH of the water, both acidified and tap, whereas the other antibiotics had little effect on this parameter. The effects of drugs on the drinking water's pH is an important consideration, particularly when adding drugs to acidified water, given that a loss of acidification may favor the growth of *Pseudomonas* spp. in the water of vulnerable immunosuppressed mice. The finding that the mice drank more of the enrofloxacin-treated water when compared with the untreated control was surprising, considering that enrofloxacin is reported to have a bitter taste. It is possible that the novel taste of the water appealed to the mice and promoted increased drinking during the week of treatment. Future work examining the taste preferences of mice will be valuable in an effort to increase their consumption of medicated water.

The findings of the current study demonstrate that the administration of antibiotics in the drinking water of mice does not result in plasma antibiotic concentrations that are effective against most pathogenic bacteria. Although this oral administration route may be adequate for treatment of some bacterial infections, such as when the antibiotic is concentrated at the site

of infection, it is inappropriate for general systemic bacterial infections in which the sensitivity of the pathogenic bacteria has not been identified.

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References

1. **Aarestrup FM.** 2006. Antimicrobial resistance in bacteria of animal origin. Washington (DC): American Society for Microbiology Press.
2. **Adams HR.** 2001. Veterinary pharmacology and therapeutics. Ames (IA): Iowa State University Press.
3. **Balcombe JP, Barnard ND, Sandusky C.** 2004. Laboratory routines cause animal stress. Contemp Top Lab Anim Sci **43:**42–51.
4. **Boxenbaum H.** 1984. Interspecies pharmacokinetic scaling and the evolutionary–comparative paradigm. Drug Metab Rev **15:**1071–1121.
5. **Brook I, Elliott TB.** 1991. Quinolone therapy in the prevention of mortality after irradiation. Radiat Res **128:**100–103.
6. **Cox SK, Cottrell MB, Smith L, Papich MG, Frazier DL, Bartges J.** 2004. Allometric analysis of ciprofloxacin and enrofloxacin pharmacokinetics across species. J Vet Pharmacol Ther **27:**139–146.
7. **Dole VP, Ho A, Gentry RT.** 1983. An improved technique for monitoring the drinking behavior of mice. Physiol Behav **30:**971–974.
8. **Duran-Struuck R, Dysko RC.** 2009. Principles of bone marrow transplantation (BMT): providing optimal veterinary and husbandry care to irradiated mice in BMT studies. J Am Assoc Lab Anim Sci **48:**11–22.
9. **Fox JG.** 2002. Laboratory animal medicine. New York (NY): Academic Press.
10. **Gillespie EL, Kuti JL, Nicolau DP.** 2005. Pharmacodynamics of antimicrobials: treatment optimisation. Expert Opin Drug Metab Toxicol **1:**351–361.
11. **Hojman P, Eriksen J, Gehl J.** 2007. Tet-On induction with doxycycline after gene transfer in mice: sweetening of drinking water is not a good idea. Anim Biotechnol **18:**183–188.
12. **Institute for Laboratory Animal Research.** 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Pres.
13. **Kutscher CL.** 1974. Strain differences in drinking in inbred mice during ad libitum feeding and food deprivation. Physiol Behav **13:**63–70.
14. **Mahmood I.** 1998. Interspecies scaling of renally secreted drugs. Life Sci **63:**2365–2371.
15. **Mahmood I, Sahajwalla C.** 2002. Interspecies scaling of biliary excreted drugs. J Pharm Sci **91:**1908–1914.
16. **McIntyre AR, Lipman NS.** 2007. Amoxicillin–clavulanic acid and trimethoprim– sulfamethoxazole in rodent feed and water: effects of compounding on antibiotic stability. J Am Assoc Lab Anim Sci **46:**26–32.
17. **Mordenti J.** 1986. Man versus beast: pharmacokinetic scaling in mammals. J Pharm Sci **75:**1028–1040.
18. **Plumb DC.** 2011. Plumb's veterinary drug handbook. Ames (IA): Wiley.
19. **Possidente B, Hegmann JP, Elder B, Carlson L.** 1980. Dissociation of circadian rhythms for food and water consumption in mice. Physiol Behav **25:**279–281.
20. **Prescott JF, Baggot JD, Walker RD.** 2000. Antimicrobial therapy in veterinary medicine. Ames (IA): Iowa State University Press.
21. **Riond JL, Riviere JE.** 1990. Allometric analysis of doxycycline pharmacokinetic parameters. J Vet Pharmacol Ther **13:**404–407.
22. **Riviere JE, Martin-Jimenez T, Sundlof SF, Craigmill AL.** 1997. Interspecies allometric analysis of the comparative pharmacokinetics of 44 drugs across veterinary and laboratory animal species. J Vet Pharmacol Ther **20:**453–463.
23. **Russell RJ, Haines DC, Anver MR, Battles JK, Gorelick PL, Blumenauer LL, Gonda MA, Ward JM.** 1995. Use of antibiotics to prevent hepatitis and typhlitis in male SCID mice spontaneously infected with *Helicobacter hepaticus*. Lab Anim Sci **45:**373–378.
24. **Rybak MJ.** 2006. Pharmacodynamics: relation to antimicrobial resistance. Am J Med 119:S37–44; discussion S62–70.
25. **Sader HS, Jones RN, Dowzicky MJ, Fritsche TR.** 2005. Antimicrobial activity of tigecycline tested against nosocomial bacterial pathogens from patients hospitalized in the intensive care unit. Diagn Microbiol Infect Dis **52:**203–208.
26. **Waites KB, Crabb DM, Duffy LB.** 2003. Inhibitory and bactericidal activities of gemifloxacin and other antimicrobials against *Mycoplasma pneumoniae*. Int J Antimicrob Agents **21:**574–577.
27. **Yu VL, Merigan TC, Barriere SL, editors.** 1999. Antimicrobial therapy and vaccines. Baltimore (MD): Williams and Wilkins.
28. **Zhu Z, Zheng T, Lee CG, Homer RJ, Elias JA.** 2002. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. Semin Cell Dev Biol **13:**121–128.

Jourdan Brune
Protocol Review
Assignment #1 Zhang 4173-03

FERPA
RCW 42.56.070(1)

General

1. [redacted] and Hai Zhang are the only team members listed on this protocol but neither are certified to perform independent surgery in rodents. Until certified, they must perform surgery under the supervision of a veterinarian from Animal Use and Training or under the supervision of a team member certified in independent rodent surgery who will need to be added to the protocol. What are your plans for implementing supervision of surgery or adding a certified team member to the protocol?
2. Under animal use location, the protocol describes that the researcher may leave to get equipment essential for surgery. Please plan to move animals from their housing room to the surgery/procedure room after all supplies have been acquired and brought to the procedure room. In general, animals should not be left unattended in procedure rooms in the vivarium. This section is meant to describe animal procedure spaces within the PIs laboratory outside of the vivarium. If you intend to do all work in the vivarium, this section may not be relevant for your protocol.

Procedures

1. Cyclosporine is a potent immunosuppressant that poses a risk for toxicity at high doses. The regime in this protocol, cites a xenograft transplantation study which may necessitate robust immunosuppression. As this protocol proposes an allograft transplantation a tapered or reduced cyclosporine dosing protocol may be more appropriate for the studies proposed. Additionally, cyclosporine can be administered in the water and after an initial period of administration by injection, transitioning to oral administration by placing cyclosporine in the cage water bottle may improve animal welfare and reduce stress that could be associated with daily injections. Please reconsider the proposed administration route and dose/duration of cyclosporine administration based on this feedback.
2. In the 7-day post-operative monitoring period, will the rats be weighed daily or will only body condition scores be taken? Weighing the rats daily will provide a more objective measure of weight loss potentially related to discomfort from tooth extraction. Additionally, depending on if significant weight loss is seen, soft food may need to be offered for longer than 2 days.
3. I recommend that you plan to use a smaller gauge needle (20-25G) over the 15G needle described in your protocol. Additionally, if you enter the thorax with only the needle, without opening the chest, you are likely to get larger blood volumes by maintaining the negative pressure of the chest cavity.
4. Daily IP injections of antibiotics may cause additional stress to your rats in the post-operative period and negatively impact their recovery. Amoxicillin administration in the water would be a refinement and provide slightly enhanced antibacterial activity. Penicillin is mainly effective against only gram-positive aerobic organisms. Enrofloxacin is another antibiotic with even broader antibiotic activity than amoxicillin and it also penetrates bone well. I recommend either amoxicillin or enrofloxacin administered through the cage water bottle over penicillin given by IP injection.

5. Would the use of an opioid analgesic be contraindicated for your study? Dental extractions and surgery are considered to be category 3 (invasive, moderate to severe pain, longer duration expected) by the UW IACUC and multimodal analgesia is recommended. I recommend adding at least one dose of slow-release buprenorphine to your post-operative analgesics for the tooth extraction procedure. Additional doses may be necessary post-operative observations of rat if signs of discomfort and weight loss from reduced food intake are apparent.
6. Intraperitoneal injections should be performed with a 25G needle or smaller. (Larger number indicates smaller gauge)
7. Please double check the volume and units of bone mineralization matrix you plan to administer. 16 mL is likely a typo.
8. Please clarify for consistency where dental defects will be made. The CT imaging procedure describes a mandibular defect while maxillary extraction and defect are described elsewhere.
9. Bioluminescence imaging typically requires cells to be transfected with luciferase enzyme and for D-luciferin substrate to be administer to animals immediately prior to imaging. Please add D-luciferin as a substance administration to your protocol if you intend to use this. Please describe if all cells will be transfected to express luciferase enzyme or if cells used in Experiment 3 "Socket Preservation-Full Study" will not be transfected.
10. Bioluminescence imaging is also typically of short duration and isoflurane anesthesia is sufficient and available in IVIS machines. Please clarify if you have access to an IVIS machine with isoflurane anesthesia available as this is preferred over ketamine/xylazine anesthesia for this procedure.
11. Similarly, please clarify if you have access to a CT machine with isoflurane anesthesia available as this is preferred over ketamine/xylazine anesthesia for this short procedure.

Experiments

1. Please see above comment regarding cardiac blood draw technique. With the recommended technique, you can expect the blood volume of the draw to be approximately 3% of body weight and are likely to draw more than 4ml based on weight of the rat you intend to use. Avoid specifying the volume you intend to draw and simply state "blood will be collected from the heart; the rat will be euthanized by exsanguination".
2. In the total animal number justification for experiment 3, please edit your justification to reflect that the pilot study has not been conducted and that the effect size for the power justification is an estimation at this time.

Basic Information

1. *** Select research team:**
   Zhang, H
2. *** Title of protocol:**
   Effect of magnesium, I50 and iPSC on rat extraction socket preservation
3. *** Short title:**
   4174-03: Magnesium Stem Cell
4. *** Summary of research:**
   This research uses rat molar extraction model to determine the effects of magnesium ion, induced pluripotent stem cells (iPSCs) and Tie2 super agonist I50 on socket preservation. The general approach is as followed: magnesium ion, osteogenic pre-induced rat derived iPSCs (abbreviated as riPOBs, which will be generated from rat peripheral blood mononuclear cells) and I50 will be mixed with deproteinized bovine bone mineralized matrix (BMM) and applied in the maxillary first molar extraction sockets of 12-week-old SD rats individually and in combination. Bone formation in the sockets and the dimension of alveolar ridge in height and width will be evaluated by high-resolution micro-CT at 2 weeks (live animals) and 6 weeks (sacrificed animals). At 6 weeks, the alveolar bone samples will be harvested and submitted for histology. New blood vessel formation will be evaluated by H&E staining and immunohistochemical staining.
5. *** Principal investigator:**
   Hai Zhang
6. *** What is the intention of the animal protocol?**
   Experimental Research

Experimental Research Protocol Addition

1. * **Will the protocol include breeding?**
   ○ Yes ● **No**

Protocol Team Members

1. Identify each additional person involved in the design, conduct, or reporting of the research:

Name	Role	Involved in Animal Handling	Authorized To Order Animals	E-mail	Phone
[redacted]	Graduate Student	yes	yes	[redacted]	
TBD PI Must Assign	Other	yes	no		

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2. If veterinary care will be provided by individuals outside of DCM or WaNPRC, provide the name, credentials and contact information below:

N/A

Funding Sources

1. Identify each organization supplying funding for the protocol:

	Funding Organization	eGC1 Number(s)
View	Restorative Dentistry	N/A

Scientific Aims

1. *** Scientific aims of the research:**

   One aim is to establish a rat derived induced pluripotent stem cell (riPSC) cell line from rat peripheral blood mononuclear cell (rPBMC) and induce its initial differentiation towards riPSCs derived pre-osteoblasts (riPOBs). Another aim is to determine the effects of magnesium ion, riPOBs and I50 in a socket preservation model *in vivo*.

   We hypothesize that the riPSC cell line will be successfully established, and magnesium ion can promote the proliferation and osteogenic differentiation of riPOBs in the rat socket preservation model. In addition, I50 can accelerate new blood vessel ingrowth in socket, thus prompting bone formation to achieve better socket preservation outcome.

2. *** Using language understandable to non-scientists, describe the goals and significance of the protocol to humans, animals and science:**

   This research addresses a critical clinical problem (bone loss after tooth extraction resulting in deficient foundation tissue for implant or prosthetic treatment) that has a significant impact in the field of restorative dentistry. Many patients still need an operation to acquire enough bone for implant placement. The entire treatment is lengthy, costly and accompanied with morbidity. This has significantly affected patient's acceptance of implant therapy and quality of life during the treatment.
   In recent years, magnesium, induced pluripotent stem cells (iPSCs, which is a new kind of stem cell that can differentiate to different body cells) and agent promoting vessel growth showed promising potential in bone regeneration. This research will generate iPSCs, and evaluate the effect of magnesium, iPSCs and an agent promoting vessel growth (which is called I50 in this study) on bone growth in a rat extraction socket. The results of this research will provide insights for new approaches either by applying them individually or combined. The outcome of the bone formation in the socket will be much improved and the need of additional surgical procedure will be significantly reduced. In the meantime, patient's satisfaction will be significantly improved due to the reduced surgical procedures and treatment time.

3. *** Provide a statement to address the potential harm to the animals on this study (e.g., pain, distress, morbidity, mortality) relative to the benefits to be gained by performing the proposed work:**

   The animals in this study will lose one maxillary first molar, and experience post-operation local pain which can be controlled by analgesics, just as the human patients who undergo tooth extraction do. The chewing efficiency will decrease at the beginning, but will be gradually compensated by other teeth. The results of this research will provide insights for new approaches of socket preservation by evaluating the effect of magnesium, riPSCs and I50 on bone formation in rat extraction socket as well as the ridge dimension.

Experiments

Note: If you will be administering cells, cell lines, sera or other biologicals to rodents, contact the Rodent Health Monitoring Program (RHMP, rhmp@uw.edu). Testing may be required prior to administration to rodents.

1. * Define the experiments to be used in this protocol:

Name	Species	USDA	Count	Count by Pain Category	Procedures	Husbandry Exception Types
01. Blood Collection for riPSC Cell Line Generation	Rats	no	2	B: 0 C: 0 D: 2 E: 0	▪ Other: Body Condition Score (Standard) ▪ Substance Administration: Anesthesia, Terminal, Ketamine and Xylazine (Standard) ▪ Tissue/Blood Collection: Zhang: Intracardiac Blood Collection Under Anesthesia (Team)	Rats - No husbandry or enrichment exceptions.

Name	Species	USDA	Count	Count by Pain Category	Procedures	Husbandry Exception Types
02. Socket Preservation - Pilot Study	Rats	no	6	B: 0 C: 0 D: 6 E: 0	▪ Euthanasia: CO2 followed by Secondary Method (>10 days of age) (Standard) ▪ Imaging: Zhang: Bioluminescence Imaging (Team) ▪ Other: Body Condition Score (Standard) ▪ Substance Administration: Zhang: Administration of Cyclosporine (Team) ▪ Substance Administration: Zhang: Analgesia, Local Infiltration, Lidocaine (Team) ▪ Substance Administration: Zhang: Baytril/Metronidazole Application (Team) ▪ Substance Administration: Analgesia, Buprenorphine or Buprenorphine SR (72 hours) (Standard) ▪ Substance Administration: Zhang: Filling Reagents in Tooth Socket (Team) ▪ Substance Administration: Zhang: D-Luciferin Administration (Team) ▪ Substance Administration: Zhang: Anesthesia, Ketamine and Xylazine with Option to Re-Dose (Team) ▪ Substance Administration: Anesthesia, Isoflurane, Short Duration (<1 hour) (Standard) ▪ Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team)	Rats - Special/medicated water Rats - No husbandry or enrichment exceptions.

Name	Species	USDA	Count	Count by Pain Category	Procedures	Husbandry Exception Types
03. Socket Preservation - Full Study	Rats	no	72	B: 0 C: 0 D: 72 E: 0	▪ Euthanasia: CO2 followed by Secondary Method (>10 days of age) (Standard) ▪ Imaging: Zhang: Micro CT Imaging (Team) ▪ Other: Body Condition Score (Standard) ▪ Substance Administration: Zhang: Anesthesia, Ketamine and Xylazine with Option to Re-Dose (Team) ▪ Substance Administration: Zhang: Analgesia, Local Infiltration, Lidocaine (Team) ▪ Substance Administration: Anesthesia, Ketamine and Xylazine (Standard) ▪ Substance Administration: Zhang: Baytril/Metronidazole Application (Team) ▪ Substance Administration: Analgesia, Buprenorphine or Buprenorphine SR (72 hours) (Standard) ▪ Substance Administration: Anesthesia, Isoflurane, Short Duration (<1 hour) (Standard) ▪ Substance Administration: Zhang: Administration of Cyclosporine (Team) ▪ Substance Administration: Zhang: Filling Reagents in Tooth Socket (Team) ▪ Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team)	Rats - No husbandry or enrichment exceptions. Rats - Special/medicated water

2. Will any single animal undergo more than one survival surgery? (include any animal that underwent surgery prior to use on this protocol) ○ Yes ● **No**

Procedure Personnel Assignment

1. * Select the team members who will be performing each procedure:

Procedure	Species	Is USDA Species	Team Members
Euthanasia: CO2 followed by Secondary Method (>10 days of age), ver. 2 (Standard)	Rats	no	[redacted]
Imaging: Zhang: Bioluminescence Imaging, ver. 1 (Team)	Rats	no	
Imaging: Zhang: Micro CT Imaging, ver. 1 (Team)	Rats	no	
Other: Body Condition Score , ver. 1 (Standard)	Rats	no	
Substance Administration: Analgesia, Buprenorphine or Buprenorphine SR (72 hours), ver. 2 (Standard)	Rats	no	TBD PI Must Assign
Substance Administration: Anesthesia, Isoflurane, Short Duration (<1 hour), ver. 2 (Standard)	Rats	no	[redacted]
Substance Administration: Anesthesia, Ketamine and Xylazine, ver. 1 (Standard)	Rats	no	
Substance Administration: Anesthesia, Terminal, Ketamine and Xylazine, ver. 2 (Standard)	Rats	no	
Substance Administration: Zhang: Administration of Cyclosporine, ver. 1 (Team)	Rats	no	
Substance Administration: Zhang: Analgesia, Local Infiltration, Lidocaine, ver. 1 (Team)	Rats	no	TBD PI Must Assign
Substance Administration: Zhang: Anesthesia, Ketamine and Xylazine with Option to Re-Dose, ver. 1 (Team)	Rats	no	TBD PI Must Assign
Substance Administration: Zhang: Baytril/Metronidazole Application, ver. 1 (Team)	Rats	no	TBD PI Must Assign
Substance Administration: Zhang: D-Luciferin Administration, ver. 1 (Team)	Rats	no	[redacted]
Substance Administration: Zhang: Filling Reagents in Tooth Socket, ver. 1 (Team)	Rats	no	

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Procedure	Species	Is USDA Species	Team Members
Survival Surgery: Zhang: Rat Tooth Extraction and Implantation, ver. 1 (Team)	Rats	no	TBD PI Must Assign
Tissue/Blood Collection: Zhang: Intracardiac Blood Collection Under Anesthesia, ver. 1 (Team)	Rats	no	

2. Team member training:

First Name	Last Name	Training

[redacted]

Course	Category	Source	Stage	Stage Number	Completion Date	Expiration Date
Annual DCM Facility Access Training (Rodent)	General	Online	Basic Course	Stage 1	2/13/2020	2/28/2021
Animal Use Medical Screening	General	Online	Basic Course	Stage 1	2/19/2020	2/28/2023
Rat Hands-On Laboratory	Animal Handling	In Person	Basic Course	Stage 1	3/5/2020	
Surgery Laboratory Part 2	Surgery	In Person	Basic Course	Stage 1	3/6/2020	
Surgery Laboratory Part 1A	Surgery	In Person	Basic Course	Stage 1	3/2/2020	
Animal Use Laws & Regulations	General	Online	Basic Course	Stage 1	10/8/2019	10/8/2024

No experience data to display

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TBD **PI Must Assign**

No training data to display

No experience data to display

Hai **Zhang**

Course	Category	Source	Stage	Stage Number	Completion Date	Expiration Date
Animal Use Laws & Regulations	General	Online	Basic Course	Stage 1	3/8/2017	3/8/2022
Foege Facility Orientation	Orientation	In Person	Basic Course	Stage 1	7/18/2014	
Annual DCM Facility Access Training (Rodent)	General	Online	Basic Course	Stage 1	3/1/2020	3/31/2021

No experience data to display

Course	Category	Source	Stage	Stage Number	Completion Date	Expiration Date
Rat Online Course: Working with Rats at UW	Animal Handling	Online	Basic Course	Stage 1	3/5/2020	
Cervical Dislocation, Mouse Anesthetized	Procedure	In Person	Basic Course	Stage 1	1/19/2011	
Cervical Dislocation, Mouse Unanesthetized	Procedure	In Person	Basic Course	Stage 1	1/19/2011	
Mouse Hands-On Laboratory	Animal Handling	In Person	Basic Course	Stage 1	1/19/2011	
Animal Use Medical Screening	General	Online	Basic Course	Stage 1	1/17/2019	1/31/2022

Animal Details

1. *** How are animals acquired?**
   Purchased
2. **Describe the acquisition for:**
   a. **Not purchasing through DCM or WaNPRC:**
      N/A
3. **Identification of individual animals (other than cage cards):**
   a. **Method(s) (e.g., ear punch/tag, tattoo, tagging/banding, radio collar, etc.) (Note: If method is implantation (e.g. PIT tag), create or select an Implant procedure to describe the details. If method is surgical (e.g., satellite tag), create or select Survival Surgery procedure to describe the details):**
      Ear tag
   b. **Will external identification be replaced if it falls off/out? If yes, describe the plan for replacement:**
      No
   c. **Will external identification be removed as part of the protocol (e.g., radio collars on field animals)? If yes, describe the plan for removal:**
      No
4. **Identify strain/stock for rodents and genetically modified animals:**

	Species	Is USDA Species	Strain	Genetically Modified Strain	Phenotype Description
View	Rats	no	Crl:CD(SD); Hsd:SD; NTac:SD (Sprague-Dawley)	no	No anticipated deleterious phenotypes.

Animal Number Adjustments

"Animals Identified in Experiments" is the total number of animals per pain category listed in all experiments on this protocol. If more or fewer animals will be used on the protocol (see Help Text for examples), click Update to enter this new number in the corresponding "Adjusted Animal Count" column. **Only input numeric values in this field; 0 is acceptable.**
If no adjustment is required, the values in the "Animals Identified in Experiments" and "Adjusted Animal Count" columns must match. Click Update in each Pain Category row to input the matching value.
For questions about adjusting animal numbers, contact OAW.

1. * Click Update to adjust the number of animals to be used or produced for this protocol:

	Species	USDA Covered Species	Pain Category	Animals Identified in Experiments	Adjusted Animal Count
View	Rats	no	Pain Category B	0	0
View	Rats	no	Pain Category C	0	0
View	Rats	no	Pain Category D	80	80
View	Rats	no	Pain Category E	0	0

2. If you adjusted the number of animals for this protocol, explain why:

N/A

3. If you will be using animals to train personnel or to practice procedures included in this protocol, describe below:

N/A

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

Alternatives and Duplication Searches

Display Procedures that cause pain or distress:

- Survival Surgery: Zhang: Rat Tooth Extraction and Implantation, ver. 1 (Team)

1. Record all searches for any previous research that this protocol might duplicate:

	Search Date	Searched Databases	Other
View	5/11/2020	EMBASE (searches multiple databases) Web of Science (searches multiple databases)	N/A
View	5/11/2020	EMBASE (searches multiple databases) Web of Science (searches multiple databases)	N/A

2. Briefly describe the results of your searches and why you can or cannot incorporate the findings. Or, if a literature search was not performed, describe the methods used to determine that alternatives are not available or feasible:

I50 used in this protocol is a newly synthesized compound, which hasn't been reported by any former articles. The effect of magnesium on riPSC hasn't been investigated, and riPSCs haven't been applied in animal ridge preservation model in combination with magnesium. So there's no duplicate of this protocol. The results of searches for alternatives don't yield any practical methods for this protocol. The results include culturing cells in various kinds of scaffolds. However, the in vitro models cannot totally mimic in vivo microenvironment of tooth socket, and cannot simulate bone maturation and angiogenesis at the same time. Thus, the animal experiments in this protocol cannot be replaced by in vitro studies.

3. Confirm that you have made every effort to ensure that this protocol is not unnecessary duplication of previous research: ☑

Housing and Use

Housing and use outside of the vivarium is not allowed without strong scientific justification.

1. Identify each location where animals will be housed:

	Facility	Species	Justification for Housing Outside Vivarium
View	ARCF ABSL1	Rats	N/A

2. Identify each location where animals will be used:

	Facility	Use	Species	Justification for Use Outside Vivarium
View	ARCF ABSL1	All procedures will be performed here. A cleared place will be used for operation and a power source will be needed for portal dental drill motor.	Rats	N/A

Disposition

1. **Disposition plans for the animals when this research is complete:**
   (check all that apply)
   Euthanasia
2. **If other, provide an animal disposition description:**
   N/A
3. **If protocol involves fixing tissues, list agents (e.g., paraformaldehyde, formalin):**
   4% paraformaldehyde for fixing the bone tissue that is collected after euthanasia.

Refinement, Replacement and Reduction

1. Describe below how the three R's (refinement, replacement and reduction) have been employed on this project. Include alternatives that were considered for the procedures above that cause pain or distress:

* Refinement (use of methods to decrease animals' sensitivity to pain)

Surgical refinements to reduce animal discomfort and stress will always be considered. Analgesia will begin before the surgery, and anesthesia will be given properly during tooth extraction to reduce pain to the largest extent.

* Replacement (include in vitro tests, use of less sentient animals)

Established iPSC cell line will be used prior to the animal experiments, and in vitro cell culture constitutes the bulk of the research done for our project. We will use in vitro experiment to determine the optimal concentrations of the MgCl2 solution for iPSCs osteogenic differentiation, instead of testing the concentrations in animal model. When strong and positive results are observed in vitro, these results must then will be confirmed in animal models- the living system. In vitro bone cultures have not been successful in our lab and others, so animal model is still irreplaceable to date.

* Reduction (use of fewer animals to attain statistical significance)

Larger sample size will provide higher statistical power for the study, however, use of power analysis (based on pilot work) helped us identify the minimal sample size needed to draw a valid conclusion. Whenever a reduction is possible without compromising the findings, it is undertaken.

2. Describe the rationale for using animals and the appropriateness of the species proposed:

The in vitro cell experiments can't totally mimic the environment of live animal. In this study, bone formation is influenced not only by the reagents filled in the bony defect, but also by the cytokines circulating with the blood and microenvironment in 3D live tissue. So the animal model is non-substitutable. Rat and mouse are most commonly used animals in dentistry experiment. In our research, rats are more suitable because the larger size of molar makes it easier to perform the operation.

Supporting Documents

1. **Attach supporting files:**

	Document Name	Date Modified
	flow chart (1).pptx	5/11/2020 1:04 PM

Procedures Appendix:

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Bioluminescence Imaging

1. *** Name of the procedure or surgery:**
   Zhang: Bioluminescence Imaging
2. *** Select procedure type:**
   Imaging
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

Imaging

1. **Imaging types:**
   Optical Imaging (e.g., IVIS, 2-Photon)
2. **If Other, specify:**
3. **Select the anesthesia and analgesia procedures to be used:**

Anesthesia, Isoflurane, Short Duration (<1 hour)	Substance Administration	2 Standard

4. **Frequency, including minimum time between imaging sessions and the maximum number of sessions (enter specific, detailed procedure timing in the Experiment):**
   See experiment for timing and frequency.
5. **Duration of imaging session:**
   Approximately 15-30 minutes
6. **Purpose:**
   To detect transplanted cell survival with bioluminescence imaging
7. **Will supportive care of animals be necessary during the imaging session?**
   Yes No
8. **If yes, describe:**
   Upon removal from the scanner, rats are placed in a recovery cage until they resume normal ambulation. The recovery cage is warmed to 37°C with a small animal heating pad.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Procedure Identification

Procedure Identification: Body Condition Score

1. *** Name of the procedure or surgery:**
   Body Condition Score
2. *** Select procedure type:**
   Other
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

Other

1. Description of Procedure:

Rats are handled gently during palpation of bony prominences over the shoulders, spinal column, and pelvis. This is usually performed with rats standing comfortably on the wiretop of the cage with minimal tail-base restraint.

A numerical Body Condition Score (BCS) (see attached diagram from Hickman and Swan, 2010) is assigned for each individual animal. Frequency of BCS assessment is described in the experiment.

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
Hickman and Swan, 2010.pdf	10/6/2016 5:25 PM

Procedure Identification: Zhang: Anesthesia, Ketamine and Xylazine with Option to Re-Dose

1. *** Name of the procedure or surgery:**
   Zhang: Anesthesia, Ketamine and Xylazine with Option to Re-Dose
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Ketamine (Ketaset, Ketaflo, Vetalar)	Standard	Intraperitoneal	22.5 – 68.2 mg/kg	N/A	Up to 10 microliters per gram of body weight	N/A
View	Xylazine	Standard	Intraperitoneal	1.1 – 4.4 mg/kg	N/A	Up to 10 microliters per gram of body weight	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Ketamine/Xylazine (45 - 68.2 mg/kg ketamine and 1.1 - 1.4 mg/kg xylazine) is mixed together and diluted in sterile pharmaceutical grade saline. The cocktail is administered IP to induce 25-30 minutes of general anesthesia. Appropriate depth of anesthesia is monitored by respiratory rate, corneal reflex, and response to front toe pinch. Heat support and eye lubrication will be provided.

If needed, rats may be re-dosed once with half of the starting dose of ketamine only (22.5 - 34.1 mg/kg) to maintain the surgical level anesthesia until the surgery ends.

3. Describe the intended effects of administering the substance(s):

General anesthesia

4. Describe any potential adverse reactions to administering the substance(s):

Respiratory and cardiac depression, including bradycardia and hypotension

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Needles must not be recapped unless a recapping device is used.

Gloves must be worn when handling these agents.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

Procedure Documents

1. Supporting documents:

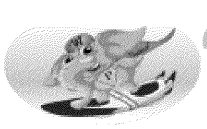
Document Name	Date Modified
There are no items to display	

1. *** Substance:**
   Ketamine (Ketaset, Ketaflo, Vetalar)
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   22.5 – 68.2 mg/kg
4. **Frequency and duration of dosages:**
   Once at full dose (45-68.2 mg/kg); option to re-dose once at half dose (22.5 - 34.1 mg/kg)
5. **Volume (for rodents or intracranial injections):**
   Up to 10 microliters per gram of body weight
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Ketamine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

1. *** Substance:**
   Xylazine
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   1.1 – 4.4 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Up to 10 microliters per gram of body weight
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Xylazine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A



Procedure Identification: Zhang: Administration of Cyclosporine

1. *** Name of the procedure or surgery:**
   Zhang: Administration of Cyclosporine
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats

4. * **Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)	Standard	Subcutaneous	5-10mg/kg	N/A	250-500 uL	1
View	Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)	Standard	Oral - Via Drinking Water	N/A	50-100ug/ml	N/A	2

2. * Describe step-by-step the procedure for administering the substance(s):

The rats will receive cyclosporine 5-10 mg/kg/day subcutaneously for 7 days starting 2 days prior to grafting, followed by 50-100 ug/ml in the drinking water for 10 days minimum up through time of euthanasia.

3. Describe the intended effects of administering the substance(s):

To suppress transplant rejection.

4. Describe any potential adverse reactions to administering the substance(s):

High doses cause renal and hepatic toxicity.

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

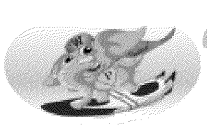
1. *** Substance:**
   Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)
2. **Route:**
   Subcutaneous

   If you indicated Other, specify the route:
3. **Dose:**
   5-10mg/kg
4. **Frequency and duration of dosages:**
   See Q2/experiment for duration
5. **Volume (for rodents or intracranial injections):**
   250-500 uL
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Cyclosporine A will be pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   1

View: Custom: Create Substance

1. *** Substance:**
   Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)
2. **Route:**
   Oral - Via Drinking Water

   If you indicated Other, specify the route:
3. **Dose:**
   N/A
4. **Frequency and duration of dosages:**
   See Q2/experiment for duration
5. **Volume (for rodents or intracranial injections):**
   N/A
6. **Concentration:**
   50-100ug/ml
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   This agent will be pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   2



View: Custom SF: Procedure Identification

Procedure Identification: Anesthesia, Ketamine and Xylazine

1. *** Name of the procedure or surgery:**
   Anesthesia, Ketamine and Xylazine
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Ketamine (Ketaset, Ketaflo, Vetalar)	Standard	Intraperitoneal	45 – 68.2 mg/kg	N/A	Up to 10 microliters per gram of body weight	N/A
View	Xylazine	Standard	Intraperitoneal	1.1 – 4.4 mg/kg	N/A	Up to 10 microliters per gram of body weight	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Ketamine/Xylazine is mixed together and diluted in sterile pharmaceutical grade saline. The cocktail is administered IP to induce 25-30 minutes of general anesthesia. Appropriate depth of anesthesia is monitored by respiratory rate, corneal reflex, and response to front toe pinch. Heat support and eye lubrication will be provided.

3. Describe the intended effects of administering the substance(s):

General anesthesia

4. Describe any potential adverse reactions to administering the substance(s):

Respiratory and cardiac depression, including bradycardia and hypotension

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Needles must not be recapped unless a recapping device is used.

Gloves must be worn when handling these agents.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

1. *** Substance:**
   Ketamine (Ketaset, Ketaflo, Vetalar)
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   45 – 68.2 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Up to 10 microliters per gram of body weight
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Ketamine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   Xylazine
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   1.1 – 4.4 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Up to 10 microliters per gram of body weight
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Xylazine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Rat Tooth Extraction and Implantation

1. *** Name of the procedure or surgery:**
   Zhang: Rat Tooth Extraction and Implantation
2. *** Select procedure type:**
   Survival Surgery
3. *** Species:**
   Rats

4. * Will administering this procedure cause any more than momentary pain or distress? **Yes** No

If yes,

i. **Identify expected symptoms from administering this procedure:**
This procedure is expected to cause discomfort that should be relieved by anesthesia and/or analgesia. Please see procedure description and/or experimental description for monitoring plan, including specific behavioral and clinical signs to be monitored.

ii. **Identify criteria under which animals will be removed from research:**
Please see experimental description for end point criteria.

Survival Surgery

1. *** Surgery Type:**
   Major
2. *** Describe how the animal, surgeon, and instruments will be prepared for surgery:**
   Surgical instruments including dental explorer and dental bur will be autoclaved prior to the initial surgery. Several sets of sterile instruments that are sufficient for one day use will be prepared.

   Sterile surgical gloves and face mask will be used. Surgical gloves will be changed between animals. No food/water restriction will be needed. Animal will be weighed prior to surgery.

   Extraction site will be scrubbed by sterile gauze soaked with 1% chlorhexidine and dried using sterile gauze prior to tooth extraction. Rats will receive eye lubricant in each eye to prevent corneal drying. Rats will be anesthetized through injection of ketamine and xylazine cocktail mixed with sterile 0.9% saline and mounted on a jaw retraction board.
3. *** Describe the surgical procedure, including any deficits expected as a result of the surgery:**

   The rats will be kept warm on a warm-water pad during the surgical procedure. After anesthetized by intraperitoneal injection with ketamine and xylazine, sterile eye lubricant will be placed in each eye. Buprenorphine will be injected subcutaneously, and 2% lidocaine will be slowly injected into the gingiva around the left maxillary first molars 5 mins prior to surgery.

   The left maxillary first molars will be extracted, and a standardized bone defect (approximately 3 mm in length, 2.6 mm in width and 2 mm in depth) will be created in the extraction area with approximately 0.5 mm to the mesial of maxillary second molar using a sterilized round bur and copious amount of sterilized saline for cooling. While preparing the bony defect, the head of the rat will be set lower than body to avoid aspiration. Depending on the experiment/group, the defects will be (1) left unfilled, or (2) filled by bone mineralized matrix (Bio-Oss®) solely, or (3) filled by bone mineralized matrix (Bio-Oss®) mixed with different reagents ($MgCl_2$ solution, Tie2 super agonist, osteogenic pre-induced riPSCs (riPOBs) solely or in combination). See related experiments for filling reagents in each group. Collagen membrane (Bio-Gide®, approximately 3×3 mm^2) will be sutured to gingival margin by 5-0 absorbable suture to seal the wound.

   Duration of procedure: up to 40 minutes.

   Redosing of anesthetic: If there are signs showing the rat is recovering from the surgery level of anesthesia, re-dose with half of the starting dose of ketamine.

   Expected Deficits: difficulty with eating during 24-48 hours post-surgery. Soft food (moistened chow and a soft dietary supplement such as Nutri-Cal) will be provided during this period.
4. *** Select associated substance administration procedures, including anesthesia and analgesia procedures to be used:**

Analgesia, Buprenorphine or Buprenorphine SR (72 hours)	Substance Administration	2 Standard
Zhang: Analgesia, Local Infiltration, Lidocaine	Substance Administration	1 Team
Zhang: Anesthesia, Ketamine and Xylazine with Option to Re-Dose	Substance Administration	1 Team
Zhang: Baytril/Metronidazole Application	Substance Administration	1 Team

5. Describe how animals will be monitored during the procedure:

Front toe pinch will be used to monitor depth of Ketamine/Xylazine anesthesia. Heart rate, respiration, temperature, movement, relaxed jaw, corneal reflex will be monitored until the rats recover from anesthesia. If there are signs showing the rat is recovering from the surgery level of anesthesia, re-dose with half of the starting dose of ketamine.

6. Describe the routine for postoperative care: (including removal of sutures, if applicable)

The rats will be placed in the recovery cage and monitored every 5 minutes for breathing rate and body temperature. After righting reflex has been regained, rats will be placed into a clean cage with gel on the cage floor and immediately placed back into the rack in the housing room.

We anticipate the administration of buprenorphine will be efficient to relieve pain for 72 hours post-surgery. If rats are exhibiting signs of pain or distress, we will consult with Vet Services.

All animals will also be under Baytril/metronidazole treatment for at least 8 days, starting from one day before the operation to prevent infection. If prominent reduction of iPOB longevity is proved in the pilot study, immunosuppressant (cyclosporine) will be administered in iPOB-transplanted groups in formal experiment, starting 2 days prior to grafting until sacrifice. See procedures "Substance Administration: Zhang: Baytril/metronidazole application" and "Substance Administration: Zhang: Administration of Cyclosporine (Team)"for details.

For the first week post-surgery, animals will be monitored daily for signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. Body weight will be monitored every other day. Because the wound will be closed by absorbable sutures, the removal of sutures will not be needed.

7. Describe how postoperative pain and distress will be assessed:

(including need for further care)

The analgesic (subcutaneous injection(s) of buprenorphine) will be administered as described in the related procedure. Animals will be monitored daily for the first week post-surgery for signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. If rats are exhibiting signs of pain or distress, we will consult with Vet Services.

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
diagram for rat socket preservation model.docx	2/18/2020 3:40 PM

Procedure Identification: Analgesia, Buprenorphine or Buprenorphine SR (72 hours)

1. *** Name of the procedure or surgery:**
   Analgesia, Buprenorphine or Buprenorphine SR (72 hours)
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Buprenorphine HCl (Buprenex, Simbadol)	Standard	Subcutaneous	0.05 mg/kg	N/A	Total volume will not exceed 10 microliters per gram of body weight.	N/A
View	Buprenorphine SR (Zoopharm)	Standard	Subcutaneous	1.0-1.2 mg/kg	N/A	Total volume will not exceed 10 microliters per gram of body weight.	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Buprenorphine will be diluted with sterile saline or water to the appropriate concentration and then injected subcutaneously (SC) at the time of the procedure, and then administered every 8-12 hours for 72 hours.

Alternatively, buprenorphine SR will be injected subcutaneously (SC) at the time of the procedure.

Either buprenorphine or buprenorphine SR will be given, not both, unless under veterinary direction.

If signs of pain are noted despite buprenorphine or buprenorphine SR administration, or following this period, Veterinary Services will be consulted.

Note: Many Category 2 or 3 procedures require multimodal analgesia and more than one type of analgesic is ideally administered. Please consult with Veterinary Services if questions. Additional information can also be found in the IACUC policy on "Analgesia in Research Animals."

3. Describe the intended effects of administering the substance(s):

Provide analgesia for 72 hours.

4. Describe any potential adverse reactions to administering the substance(s):

Overdose can result in dysphoria, pica, respiratory depression, and gastrointestinal pain.

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and

Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Needles must not be recapped unless a recapping device is used.

Gloves must be worn when handling these agents.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

1. *** Substance:**
   Buprenorphine HCl (Buprenex, Simbadol)
2. **Route:**
   Subcutaneous

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   0.05 mg/kg
4. **Frequency and duration of dosages:**
   Once at the time of procedure, then every 8-12 hours for 72 hours.
5. **Volume (for rodents or intracranial injections):**
   Total volume will not exceed 10 microliters per gram of body weight.
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   All substances will be pharmaceutical grade.
8. **Complication remediation:**
   When using ketamine/xylazine anesthesia, the first dose will be administered as the animal is recovering from anesthesia.

   When using isoflurane anesthesia, the first dose will be given at least 30 minutes prior to recovery from surgery.
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   Buprenorphine SR (Zoopharm)
2. **Route:**
   Subcutaneous

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   1.0-1.2 mg/kg
4. **Frequency and duration of dosages:**
   Once (provides approximately 72 hours of analgesia)
5. **Volume (for rodents or intracranial injections):**
   Total volume will not exceed 10 microliters per gram of body weight.
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Buprenorphine SR is pharmaceutical grade.
8. **Complication remediation:**
   When using ketamine/xylazine anesthesia, the first dose will be administered as the animal is recovering from anesthesia.

   When using isoflurane anesthesia, the first dose will be given at least 1-2 hours prior to surgery for most procedures. Consult with Veterinary Services on timing.
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Anesthesia, Terminal, Ketamine and Xylazine

1. *** Name of the procedure or surgery:**
   Anesthesia, Terminal, Ketamine and Xylazine
2. *** Select procedure type:**
   Substance Administration

3. *** Species:**
   Rats

4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Ketamine (Ketaset, Ketaflo, Vetalar)	Standard	Intraperitoneal	≥68.2 mg/kg	N/A	Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.	N/A
View	Xylazine	Standard	Intraperitoneal	≥4.4 mg/kg	N/A	Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Ketamine/Xylazine is mixed together and diluted in sterile pharmaceutical grade saline or water. The cocktail is administered IP to induce anesthesia appropriate for a short (<20 minutes) terminal procedure such as perfusion.

Deep anesthesia is confirmed by lack of response to toe pinch, change in respiratory character and decreased respiratory rate.

3. Describe the intended effects of administering the substance(s):

Anesthesia for short (<20 minutes) terminal procedure

4. Describe any potential adverse reactions to administering the substance(s):

N/A

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Needles must not be recapped unless a recapping device is used.

Gloves must be worn when handling this agent.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA)**

paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or ehsbio@uw.edu**.**

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

1. *** Substance:**
   Ketamine (Ketaset, Ketaflo, Vetalar)
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   ≥68.2 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Ketamine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   Xylazine
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   ≥4.4 mg/kg
4. **Frequency and duration of dosages:**
   Once
5. **Volume (for rodents or intracranial injections):**
   Total volume of ketamine/xylazine mixture will not exceed 10 microliters per gram of body weight.
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Xylazine is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Baytril/Metronidazole Application

1. *** Name of the procedure or surgery:**
   Zhang: Baytril/Metronidazole Application
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats

4. * **Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Enrofloxacin (Baytril)	Standard	Oral - Via Drinking Water	25 mg/kg/day in drinking water	N/A	Ad libitum	N/A
View	Metronidazole (Flagyl)	Standard	Oral - Other	10-40 mg/kg/day	N/A	0.25-1ml	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

From one day before survival surgery, Baytril will be added to the drinking water at 25 mg/kg/day for a minimum of 8 days and a max of ~30 days. Water bottles will be changed 1x weekly. At the same duration, metronidazole will be administrated orally via syringe.

3. Describe the intended effects of administering the substance(s):

Prevention of infection following surgery

4. Describe any potential adverse reactions to administering the substance(s):

Alterations in gastrointestinal microbiota, gastrointestinal upset. Transient neutopenia, leukopenia.

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

1. *** Substance:**
   Enrofloxacin (Baytril)
2. **Route:**
   Oral - Via Drinking Water

   If you indicated Other, specify the route:
3. **Dose:**
   25 mg/kg/day in drinking water
4. **Frequency and duration of dosages:**
   Ad libitum in drinking water for 8-30 days
5. **Volume (for rodents or intracranial injections):**
   Ad libitum
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   It is pharmaceutical grade
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   Metronidazole (Flagyl)
2. **Route:**
   Oral - Other

   If you indicated Other, specify the route:
3. **Dose:**
   10-40 mg/kg/day
4. **Frequency and duration of dosages:**
   Daily for 8-30 days
5. **Volume (for rodents or intracranial injections):**
   0.25-1ml
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   It is pharmaceutical grade
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Analgesia, Local Infiltration, Lidocaine

1. *** Name of the procedure or surgery:**
   Zhang: Analgesia, Local Infiltration, Lidocaine
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

Administration of Substances

1. *** Substances:**

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Lidocaine	Standard	Oral - Other	2-4 mg/kg	2% (20mg/ml)	~60 ul	N/A

2. *** Describe step-by-step the procedure for administering the substance(s):**

   Lidocaine (2%) will be slowly injected into the gingiva around the left maxillary first molars 5 mins prior to surgery.

3. **Describe the intended effects of administering the substance(s):**

   Local analgesia

4. **Describe any potential adverse reactions to administering the substance(s):**

   None anticipated.

5. **If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.**

   Needles must not be recapped unless a recapping device is used.

   Gloves must be worn when handling these agents.

6. *** Does this procedure include the use of a paralytic agent?**

   Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Lidocaine
2. **Route:**
   Oral - Other

   If you indicated Other, specify the route:
   infiltration in to gingiva
3. **Dose:**
   2-4 mg/kg
4. **Frequency and duration of dosages:**
   Once, 5 mins prior to surgery
5. **Volume (for rodents or intracranial injections):**
   ~60 ul
6. **Concentration:**
   2% (20mg/ml)
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   All substances, including diluent (saline), will be pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Anesthesia, Isoflurane, Short Duration (<1 hour)

1. *** Name of the procedure or surgery:**
   Anesthesia, Isoflurane, Short Duration (<1 hour)
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats

4. * **Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Isoflurane	Standard	Inhalation	1-5%	N/A	N/A	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

The rat is placed in an induction chamber and 1-5% isoflurane is administered until the rat is recumbent. If more than momentary anesthesia is required, the rat is removed from the chamber and positioned in a nose cone or intubated, with 1-5% isoflurane administered to maintain anesthesia. Adequate depth of anesthesia is monitored by respiratory rate, corneal reflex, and response to toe pinch. Heat support and eye lubrication will be provided.

3. Describe the intended effects of administering the substance(s):

General anesthesia

4. Describe any potential adverse reactions to administering the substance(s):

Respiratory depression, hypotension, cardiac arrhythmia

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

Isoflurane is administered using an anesthesia machine that has been adequately tested and certified.

Waste gas is scavenged using either an activated charcoal canister (e.g., F/Air), active scavenging system, or by conducting the work within a certified fume hood.

Isoflurane is an irritant and may cause reproductive problems in women. Refer to Occupational Health Recommendations.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   Isoflurane
2. **Route:**
   Inhalation

   If you indicated Other, specify the route:
   N/A
3. **Dose:**
   1-5%
4. **Frequency and duration of dosages:**
   Continuous for <1 hour (estimated)
5. **Volume (for rodents or intracranial injections):**
   N/A
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Isoflurane is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Filling Reagents in Tooth Socket

1. *** Name of the procedure or surgery:**
   Zhang: Filling Reagents in Tooth Socket
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats

4. * **Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	Bone mineralized matrix (Bio-Oss®)	Team	Other	N/A	N/A	Approximately 16 cubic milliliter	N/A
View	I53-50 (abbreviated as I50)	Team	Other	N/A	1000ng/ml of F-domains	20µl	N/A
View	magnesium chloride	Team	Other	N/A	0.8, 1.8, 5, 10 or 20 mM (decided by in vitro experiments)	20µl	N/A
View	riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)	Team	Other	20,000 cells per site in media	N/A	20µl	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

Bone mineralized matrix (Bio-Oss®) alone or together with one or more of the below reagents will be implanted into the tooth socket of rats (prepared into a box-like defect) during a survival surgery (see related survival surgery for details):

- riPOBs (riPOBs used in pilot study will be labelled with luciferase prior to implantation; cells will be derived from Sprague Dawley rats)
- magnesium chloride solution
- I50

See the treatment of each group in experiment protocol for combination of the reagents in each group.

Note: The optimal concentration of magnesium chloride will be verified by cell proliferation and osteogenic differentiation experiments on riPOBs (riPSCs derived pre-osteoblasts) in vitro, and will be applied in grafting procedure. The concentration will be chosen from 0.8, 1.8, 5, 10 and 20 mM.

3. Describe the intended effects of administering the substance(s):

riPOBs labelled with luciferase: To test the longevity of transplanted riPOBs in rat tooth socket with or without immunosuppressive therapy.

riPOBs used in full study: To promote bone growth in rat tooth socket.

I50 is a Tie2 super agonist that can activate Ang-1/Tie2 pathway, thus to accelerate angiogenesis and promote bone growth in rat tooth socket.

Magnesium chloride: To reduce bone resorption after tooth extraction, and promote bone growth in tooth socket.

4. **Describe any potential adverse reactions to administering the substance(s):**
   No
5. **If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.**
6. *** Does this procedure include the use of a paralytic agent?**
   Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

1. *** Substance:**
   Bone mineralized matrix (Bio-Oss®)
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket
3. **Dose:**
   N/A
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   Approximately 16 cubic milliliter
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Bone mineralized matrix (Bio-Oss®) is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

1. *** Substance:**
   I53-50 (abbreviated as I50)
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket together with bone matrix
3. **Dose:**
   N/A
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   20µl
6. **Concentration:**
   1000ng/ml of F-domains
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   I50 is an investigational new compound, not available in pharmaceutical grade. It will be dissovled in Dulbecco's Modified Eagle Medium (or similar media) and sterile filtered prior to use.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

1. *** Substance:**
   magnesium chloride
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket together with bone matrix
3. **Dose:**
   N/A
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   20µl
6. **Concentration:**
   0.8, 1.8, 5, 10 or 20 mM (decided by in vitro experiments)
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Magnesium chloride is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom: Create Substance

1. *** Substance:**
   riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)
2. **Route:**
   Other

   If you indicated Other, specify the route:
   Grafting into the tooth socket together with bone matrix
3. **Dose:**
   20,000 cells per site in media
4. **Frequency and duration of dosages:**
   Once at surgery
5. **Volume (for rodents or intracranial injections):**
   20µl
6. **Concentration:**
   N/A
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   Not available pharmaceutical grade; cell mixture will be prepared in sterile culture hood prior to administration.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: CO2 followed by Secondary Method (>10 days of age)

1. *** Name of the procedure or surgery:**
   CO2 followed by Secondary Method (>10 days of age)
2. *** Select procedure type:**
   Euthanasia
3. *** Species:**
   Rats

4. * **Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

Euthanasia

1. *** Method of euthanasia:**
   CO2 Overdose
2. **Describe procedure:**
   CO2 will be administered from a compressed commercial cylinder utilizing a flow meter to deliver 30-70% of the chamber volume per minute. Total gas exposure will be at least 10 minutes, with gas flow being maintained for at least 1 minute after apparent clinical death. A timer will be used to ensure adequate length of exposure.

   Secondary method will be one of the following: placed in a bag filled with CO2, decapitation, exsanguination, thoracotomy/tissue collection.
3. *** Will anesthesia be used?** Yes **No**
4. **Describe how death will be confirmed:**
   Death will be confirmed by lack of respirations and heartbeat.
5. **Is this method approved by the AVMA Guidelines on Euthanasia (2013)?**
   Yes No

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

Procedure Identification: Zhang: D-Luciferin Administration

1. *** Name of the procedure or surgery:**
   Zhang: D-Luciferin Administration
2. *** Select procedure type:**
   Substance Administration
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

Administration of Substances

1. * Substances:

	Substance	Substance Scope	Route	Dose	Concentration	Volume	Substance Order for the Procedure
View	D-luciferin	Team	Intraperitoneal	150mg/kg	15 mg/ml	up to 2.5-3ml (for a 250-300g rat)	N/A

2. * Describe step-by-step the procedure for administering the substance(s):

D-luciferin will be diluted with DPBS (no calcium or magnesium) to a final concentration of 15 mg/ml, and then injected intraperitoneal 10-20 min before bioluminescent imaging.

3. Describe the intended effects of administering the substance(s):

The injection of D-luciferin allows for the real-time monitoring of luciferase labeled stem cells implanted in the rat model through Bioluminescence Imaging.

4. Describe any potential adverse reactions to administering the substance(s):

Not reported

5. If working with hazardous agents, protocol personnel will read and follow the Occupational Health Recommendations (OHRs) and Biological Use Authorization letter (BUA), if applicable. The OHRs and the BUA can be found on the protocol workspace.

6. * Does this procedure include the use of a paralytic agent?

Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

Procedure Documents

1. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom: Create Substance

1. *** Substance:**
   D-luciferin
2. **Route:**
   Intraperitoneal

   If you indicated Other, specify the route:
3. **Dose:**
   150mg/kg
4. **Frequency and duration of dosages:**
   Single injection 10-20 min before bioluminescent imaging
5. **Volume (for rodents or intracranial injections):**
   up to 2.5-3ml (for a 250-300g rat)
6. **Concentration:**
   15 mg/ml
7. **Confirm the agents used will be pharmaceutical grade. If you must use non-pharmaceutical grade agents, provide scientific justification for their use and describe how the agent will be prepped and sterilized prior to use:**
   The agent is pharmaceutical grade.
8. **Complication remediation:**
   N/A
9. **Substance order for the procedure:**
   N/A

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Intracardiac Blood Collection Under Anesthesia

1. *** Name of the procedure or surgery:**
   Zhang: Intracardiac Blood Collection Under Anesthesia
2. *** Select procedure type:**
   Tissue/Blood Collection
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
N/A

ii. **Identify criteria under which animals will be removed from research:**
N/A

Live Tissue/Blood Collection

1. *** Identify tissues to be collected:**
   Blood from heart
2. **Describe timing and frequency of collection and amount to be collected:**
   Volume: 2-4 ml. Once during terminal procedure under anesthesia.
3. **Select the anesthesia and analgesia procedures to be used:**

Anesthesia, Terminal, Ketamine and Xylazine	Substance Administration	2 Standard

4. **If withholding anesthesia/analgesia when normally required, provide scientific justification:**
   N/A
5. **Describe any potential complications from collection:**
   None anticipated.
6. *** Describe the collection procedure:**
   The rat is anesthetized. Front toe pinch is used to monitor depth of ketamine/xylazine anesthesia. A syringe is primed with EDTA to prevent clotting. Palpate strongest beat on left thoracic wall near flexed elbow. Remove hair on (and around) the point described and aseptically prepare with betadine scrub and alcohol. Introduce the needle between ribs at point described and a terminal blood sample is collected.

View: SF: Procedure Documents

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Procedure Identification

Procedure Identification: Zhang: Micro CT Imaging

1. *** Name of the procedure or surgery:**
   Zhang: Micro CT Imaging
2. *** Select procedure type:**
   Imaging
3. *** Species:**
   Rats
4. *** Will administering this procedure cause any more than momentary pain or distress?** Yes **No**

If yes,

i. **Identify expected symptoms from administering this procedure:**
   N/A

ii. **Identify criteria under which animals will be removed from research:**
   N/A

Imaging

1. **Imaging types:**
   Computed Tomography (CT)
2. **If Other, specify:**
3. **Select the anesthesia and analgesia procedures to be used:**

Anesthesia, Isoflurane, Short Duration (<1 hour)	Substance Administration	2 Standard
Anesthesia, Ketamine and Xylazine	Substance Administration	1 Standard

4. **Frequency, including minimum time between imaging sessions and the maximum number of sessions (enter specific, detailed procedure timing in the Experiment):**
   See experiment for timing and frequency.
5. **Duration of imaging session:**
   20 minutes
6. **Purpose:**
   Within experiment assessment of bone formation in maxillary defect.
7. **Will supportive care of animals be necessary during the imaging session?**
   Yes No
8. **If yes, describe:**
   Upon removal from microCT scanner, rats are placed in a recovery cage until they resume normal ambulation. The recovery cage is warmed to 37°C with a small animal heating pad.

Procedure Documents

1. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

Substances Appendix:

Substance Information: riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)

1. *** Name:**
   riPSC (rat derived induced pluripotent stem cells) derived pre-osteoblast (riPOB)
2. *** Substance types:** (select all that apply)
   Cell, Cell Line, or Tissue - Other
3. *** Is this a hazardous agent:** Yes **No**

   NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

Substance Information: I53-50 (abbreviated as I50)

1. *** Name:**
   I53-50 (abbreviated as I50)
2. *** Substance types:** (select all that apply)

Chemical Agent

3. *** Is this a hazardous agent:** Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: magnesium chloride

1. *** Name:**
   magnesium chloride
2. *** Substance types:** (select all that apply)
   Chemical Agent
3. *** Is this a hazardous agent:** Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Buprenorphine SR (Zoopharm)

1. *** Name:**
   Buprenorphine SR (Zoopharm)
2. *** Substance types:** (select all that apply)
   Analgesic
   Reproductive Hazard/Teratogen

3. *** Is this a hazardous agent:** **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Bone mineralized matrix (Bio-Oss®)

1. *** Name:**
   Bone mineralized matrix (Bio-Oss®)
2. *** Substance types:** (select all that apply)
   Other
3. *** Is this a hazardous agent:** Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Ketamine (Ketaset, Ketaflo, Vetalar)

1. *** Name:**
   Ketamine (Ketaset, Ketaflo, Vetalar)
2. *** Substance types:** (select all that apply)
   Anesthetic
   Reproductive Hazard/Teratogen

3. *** Is this a hazardous agent:** **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Xylazine

1. *** Name:**
   Xylazine
2. *** Substance types:** (select all that apply)
   Anesthetic
3. *** Is this a hazardous agent:** **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Buprenorphine HCl (Buprenex, Simbadol)

1. *** Name:**
   Buprenorphine HCl (Buprenex, Simbadol)
2. *** Substance types:** (select all that apply)
   Analgesic
   Reproductive Hazard/Teratogen
3. *** Is this a hazardous agent:** **Yes** No

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: D-luciferin

1. * Name:

D-luciferin

2. * Substance types: (select all that apply)

Other

3. * Is this a hazardous agent: Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Enrofloxacin (Baytril)

1. * Name:

Enrofloxacin (Baytril)

2. * Substance types: (select all that apply)

Antibiotic

3. * Is this a hazardous agent: Yes **No**

NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Isoflurane

1. *** Name:**
   Isoflurane
2. *** Substance types:** (select all that apply)
   Anesthetic
   Reproductive Hazard/Teratogen
3. *** Is this a hazardous agent:** **Yes** No

   NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**
4. **Supporting documents:**

Document Name	Date Modified
There are no items to display	

View: Custom SF: Substance Information

Substance Information: Lidocaine

1. *** Name:**
   Lidocaine
2. *** Substance types:** (select all that apply)
   Analgesic
   Anesthetic
3. *** Is this a hazardous agent:** Yes **No**

   NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**
4. **Supporting documents:**

Document Name	Date Modified

There are no items to display

Document Name	Date Modified

View: Custom SF: Substance Information

Substance Information: Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)

1. *** Name:**
   Cyclosporine (Sandimmune, Atopica, Neoral, Optimmune, Restasis)
2. *** Substance types:** (select all that apply)
   Analgesic
   Carcinogen
   Immunosuppressant
   Reproductive Hazard/Teratogen
3. *** Is this a hazardous agent:** **Yes** No

   NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**
4. **Supporting documents:**

Document Name	Date Modified

There are no items to display

View: Custom SF: Substance Information

Substance Information: Metronidazole (Flagyl)

1. *** Name:**
   Metronidazole (Flagyl)
2. *** Substance types:** (select all that apply)
   Antibiotic
   Antiparasitic
   Carcinogen
3. *** Is this a hazardous agent:** Yes **No**

   NOTE: Working with biohazardous agents requires a separate approval from the Institutional Biosafety Committee (IBC). Submit the Biological Use Authorization **(BUA) paperwork to initiate this process. If you have questions, contact EH&S Research and Occupational Safety at 206-221-7770 or** ehsbio@uw.edu**.**

4. Supporting documents:

Document Name	Date Modified
There are no items to display	

1. *** Select the funding organization:**
   Restorative Dentistry

   If Other was selected in question 1, provide Funding Organization:

2. *** All animal use projects must be reviewed for scientific merit prior to initiating animal use. Choose the required reviews for this project:**
   Has already been conducted and approved by a funding agency

3. **Provide name of the committee or the department reviewer** (Required if "Has been conducted by my department or school and has been found to be scientifically meritorious" was selected):
   Dr. Marty Anderson, Margaret Spencer Fund Committee Chair

4. **eGC1 Number(s):**(assigned internally)
   N/A

Experiments Appendix:

01. Blood Collection for riPSC Cell Line Generation

1. *** Experiment name:**
   01. Blood Collection for riPSC Cell Line Generation
2. *** Species:**
   Rats
3. **If other was selected, provide a species:**
4. **What is the scientific goal of this experiment:**
   To establish a rat induced pluripotent stem cell (riPSC) cell line with rat peripheral blood mononuclear cells (rPBMC).
5. *** Describe the animal experience in the experiment, from enrollment in the study to the final endpoint, including all procedures in chronological order and the minimum time between procedures. We encourage using bullet points, timeline, table, or a flow chart as appropriate:**

   1. Rats will be anesthetized by ketamine/xylazine cocktail overdose before blood collection.

   2. **Two to four milliliter blood will be collected from heart ventricle. Rat will be euthanized by exsanguination.**

   Animal Sex:
   Female

   Animal Ages:
   3 months

   Animal Size:
   250-300g
6. **Select experimental procedures:**

Name	Type	Version	Scope
Body Condition Score	Other	1	Standard
Anesthesia, Terminal, Ketamine and Xylazine	Substance Administration	2	Standard
Zhang: Intracardiac Blood Collection Under Anesthesia	Tissue/Blood Collection	1	Team

7. **Monitoring protocol, including frequency and specific behavioral and clinical signs to be monitored. Include humane endpoints (criteria for euthanasia):**
   The rats will be monitored once every week for weight, body condition score and

other general condition before euthanasia.

Rats will be euthanized before blood collection according to the following criteria:

1. More than 20% weight loss;
2. Body condition score of 2 or less. See Body Condition Score Procedure for detailed evaluation method.
3. Inability or reluctance to move when stimulated, or moribund condition.
4. Impairment of ability to eat, drink, or ambulate normally.
5. Labored breathing.
6. Hypothermia.
7. Ulcerated tumors.

8. If there is expected mortality (spontaneous death) in this experiment:

a. Procedure/condition associated with mortality:
N/A

b. Estimated mortality rate, i.e. percentage of animals expected to die spontaneously (not via euthanasia) or need to be euthanized as a result of the procedure. (Be sure to account for this in your animal number calculations):
N/A

c. Explain why euthanasia is not possible or appropriate:
N/A

9. Will some animals live out their natural lifespan as part of this experiment? If so, indicate their use and describe the monitoring plan for aged animals (e.g., rodents >18 months of age), including frequency, behavioral and clinical signs to be monitored and criteria for euthanasia.
No

10. * Total number of animals used in this experiment:(including all the animals to be produced)
2

a. Justify total number of animals used in this experiment:

Two milliliter blood is needed for isolation of the monocytes at one time according to manufacturer's instruction (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/General_Information/1/ge-isolation-of-mononuclear-cells.pdf). It is difficult to collect this volume of blood from other sites, and blood sample is easy to get contaminated by other survival blood collection techniques. So exsanguination by this open method is more suitable for our cell line generation purpose. One rat is needed for single blood collection procedure. One more rat will be prepared for additional blood collection in case the iPSCs generation fails.

11. Number of animals by pain and distress category:(include each animal only once in the highest pain category)

B: 0
C: 0
D: 2
E: 0

a. Justify the need for any animals in pain category E:

N/A

12. * Identify husbandry exceptions:

	Exception Type	Description and Justification
View	Rats - No husbandry or enrichment exceptions.	N/A

13. Supporting documents:

Document Name	Date Modified
There are no items to display	

1. *** Exception type:**

   Rats - No husbandry or enrichment exceptions.

2. **Description and justification:**

   N/A

02. Socket Preservation - Pilot Study

1. *** Experiment name:**
   02. Socket Preservation - Pilot Study
2. *** Species:**
   Rats
3. **If other was selected, provide a species:**
4. **What is the scientific goal of this experiment:**
   To evaluate the longevity of transplanted riPSCs derived pre-osteoblasts (riPOBs) in rat tooth socket, and the efficacy of immunosuppressive therapy on survival of riPOBs.
5. *** Describe the animal experience in the experiment, from enrollment in the study to the final endpoint, including all procedures in chronological order and the minimum time between procedures. We encourage using bullet points, timeline, table, or a flow chart as appropriate:**

   1. **Three-month-old SD rats will be enrolled in this study. Female rats will be selected because the size is smaller and easier to handle compared to male ones.**
   2. **Animals will be divided into 2 groups (n=3 in each group). All rats will receive left maxillary first molar extraction and bony defect creation, with defect filled by riPOBs and** Bio-oss**. The immunosuppressive therapy group will receive immunosuppressant administration, while control group will not. See procedure** Zhang: Rat Tooth Extraction and Implantation **for detailed process of surgery, and** Zhang: Administration of Cyclosporine **for details of** immunosuppressant administration**.**
   3. **A**nalgesic **and antibiotic will be administered as described in the related procedures. Immunosuppressant will be administrated in** immunosuppressive therapy group**.** See procedures **Analgesia, Buprenorphine or Buprenorphine SR (72 hours)**, Zhang: Analgesia, Local Infiltration, lidocaine**, Zhang: Baytril/Metronidazole Application**, and Zhang: Administration of Cyclosporine for details of drug administration.
   4. **Transplanted cell survival will be monitored at 2 weeks and 6 weeks post-operative via bioluminescent imaging (BLI)**. See procedures Zhang: D-Luciferin Administration **and** Zhang: Bioluminescence Imaging for details of D-luciferin administration and BLI.

5. Euthanasia will be conducted a**t 6 weeks post-operation.**

Animal Sex:
Female

Animal Ages:
3 months

Animal Size:
250-300g

6. Select experimental procedures:

Name	Type	Version	Scope
CO2 followed by Secondary Method (>10 days of age)	Euthanasia	2	Standard
Zhang: Bioluminescence Imaging	Imaging	1	Team
Body Condition Score	Other	1	Standard
Analgesia, Buprenorphine or Buprenorphine SR (72 hours)	Substance Administration	2	Standard
Anesthesia, Isoflurane, Short Duration (<1 hour)	Substance Administration	2	Standard
Zhang: Administration of Cyclosporine	Substance Administration	1	Team
Zhang: Analgesia, Local Infiltration, Lidocaine	Substance Administration	1	Team
Zhang: Anesthesia, Ketamine and Xylazine with Option to Re-Dose	Substance Administration	1	Team
Zhang: Baytril/Metronidazole Application	Substance Administration	1	Team
Zhang: D-Luciferin Administration	Substance Administration	1	Team
Zhang: Filling Reagents in Tooth Socket	Substance Administration	1	Team
Zhang: Rat Tooth Extraction and Implantation	Survival Surgery	1	Team

7. Monitoring protocol, including frequency and specific behavioral and clinical signs to be monitored. Include humane endpoints (criteria for euthanasia):
For the first week post-surgery, animals will be monitored daily for signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. Body weight will be monitored every other day. Then, the rats will be monitored 3 times a week for the same signs throughout to the endpoint .

Criteria for euthanasia:

1. More than 20% weight loss;
2. Body condition score of 2 or less. See Body Condition Score Procedure for detailed evaluation method.
3. Inability or reluctance to move when stimulated, or moribund condition.
4. Impairment of ability to eat, drink, or ambulate normally.
5. Labored breathing.
6. Ulcerated tumors.
7. Severe infection in surgical site.

8. If there is expected mortality (spontaneous death) in this experiment:

a. Procedure/condition associated with mortality:
N/A

b. Estimated mortality rate, i.e. percentage of animals expected to die spontaneously (not via euthanasia) or need to be euthanized as a result of the procedure. (Be sure to account for this in your animal number calculations):
N/A

c. Explain why euthanasia is not possible or appropriate:
N/A

9. Will some animals live out their natural lifespan as part of this experiment? If so, indicate their use and describe the monitoring plan for aged animals (e.g., rodents >18 months of age), including frequency, behavioral and clinical signs to be monitored and criteria for euthanasia.
N/A

10. * Total number of animals used in this experiment:(including all the animals to be produced)
6

a. Justify total number of animals used in this experiment:

This is only a pilot study, and we only want to observe the trend of cell survival, and want to include the least number of animals possible while still drawing a valid conclusion. We will use 3 rats per group, and have 2 groups (control, immunosuppressive therapy).

We based our choice in group number on similar work performed by Li et al (Li K, Javed E, Scura D, Hala TJ, Seetharam S, Falnikar A, et al. Human iPS cell-derived astrocyte transplants preserve respiratory function after spinal cord injury. Experimental Neurology 2015;271:479-92), which included 3 animals in each group for each time point, and allowed them to see positive results. We will start with this sample size. If it doesn't work, we will amend the protocol to expand the sample size (based on the acquired pilot data) and/or amend the immunosuppressive therapy as needed.

11. Number of animals by pain and distress category:(include each animal only once in the highest pain category)

B: 0
C: 0
D: 6
E: 0

a. Justify the need for any animals in pain category E:

N/A

12. * Identify husbandry exceptions:

	Exception Type	Description and Justification
View	Rats - No husbandry or enrichment exceptions.	N/A
View	Rats - Special/medicated water	Rats may receive cyclosporine via drinking water for a maximum of ~6 weeks. Rats may also receive Baytril via drinking water for up to 30 days. See experiment description and related procedures for details.

13. Supporting documents:

Document Name	Date Modified
There are no items to display	

1. *** Exception type:**

   Rats - No husbandry or enrichment exceptions.

2. **Description and justification:**

   N/A

1. *** Exception type:**

   Rats - Special/medicated water

2. **Description and justification:**

   Rats may receive cyclosporine via drinking water for a maximum of ~6 weeks. Rats may also receive Baytril via drinking water for up to 30 days. See experiment description and related procedures for details.

03. Socket Preservation - Full Study

1. *** Experiment name:**
   03. Socket Preservation - Full Study
2. *** Species:**
   Rats
3. **If other was selected, provide a species:**
4. **What is the scientific goal of this experiment:**
   To determine the effects of magnesium ion, riPOBs and I50 in a socket preservation model in vivo.
5. *** Describe the animal experience in the experiment, from enrollment in the study to the final endpoint, including all procedures in chronological order and the minimum time between procedures. We encourage using bullet points, timeline, table, or a flow chart as appropriate:**

   a. **Three-month-old SD rats will be enrolled in this study. Female rats will be selected because the size is smaller and easier to handle compared to male ones.**

   b. **Animals will be divided into 9 groups (n=8 in each group). All rats will receive left maxillary first molar extraction and bony defect creation, with defect filled by different combination of Bio-oss, magnesium chloride solution, I50 and riPOBs or left untreated. Treatment design for each group is listed in the following table. See procedure** Zhang: Rat Tooth Extraction and Implantation **for detailed process of surgery.**

Table: Groups in Animal Study

Group Number	Bone Mineralized Matrix (BMM)	riPOBs	$MgCl_2$	I50
1	Applied			
2	Applied	Applied		
3	Applied		Applied	
4	Applied			Applied
5	Applied	Applied	Applied	
6	Applied	Applied		Applied

7	Applied		Applied	Applied
8	Applied	Applied	Applied	Applied
9				

3. Analgesic and antibiotic will be given as described in the related procedures. Immunosuppressant will be administrated if immune rejection is proven to be prominent and immunosuppressant is verified to be necessary in pilot study (see Expt 02). See procedures Analgesia, Buprenorphine or Buprenorphine SR (72 hours), Zhang: Analgesia, Local Infiltration, Lidocaine, Zhang: Baytril/Metronidazole Application, and Zhang: Administration of Cyclosporine for details of drug administration.

4. We will evaluate animals at 2 weeks post-operation by micro CT under anesthesia.

5. Euthanasia will be conducted at 6 weeks post-operation.

Animal Sex:
Female

Animal Ages:
3 months

Animal Size:
250-300g

6. Select experimental procedures:

Name	Type	Version	Scope
CO2 followed by Secondary Method (>10 days of age)	Euthanasia	2	Standard
Zhang: Micro CT Imaging	Imaging	1	Team
Body Condition Score	Other	1	Standard
Analgesia, Buprenorphine or Buprenorphine SR (72 hours)	Substance Administration	2	Standard
Anesthesia, Isoflurane, Short Duration (<1 hour)	Substance Administration	2	Standard
Anesthesia, Ketamine and Xylazine	Substance Administration	1	Standard
Zhang: Administration of Cyclosporine	Substance Administration	1	Team
Zhang: Analgesia, Local Infiltration, Lidocaine	Substance Administration	1	Team

Name	Type	Version	Scope
Zhang: Anesthesia, Ketamine and Xylazine with Option to Re-Dose	Substance Administration	1	Team
Zhang: Baytril/Metronidazole Application	Substance Administration	1	Team
Zhang: Filling Reagents in Tooth Socket	Substance Administration	1	Team
Zhang: Rat Tooth Extraction and Implantation	Survival Surgery	1	Team

7. **Monitoring protocol, including frequency and specific behavioral and clinical signs to be monitored. Include humane endpoints (criteria for euthanasia):**
For the first week post-surgery, animals will be monitored daily for signs of dehydration and pain/distress such as hunched posture, decreased activity, and rough coat. Body weight will be monitored every other day. Then, the rats will be monitored 3 times a week for the same indicators throughout to the endpoint.

   Criteria for euthanasia:
   1. More than 20% weight loss;
   2. Body condition score of 2 or less. See Body Condition Score Procedure for detailed evaluation method.
   3. Inability or reluctance to move when stimulated, or moribund condition.
   4. Impairment of ability to eat, drink, or ambulate normally.
   5. Labored breathing.
   6. Ulcerated tumors.
   7. Severe infection in surgical site.

8. **If there is expected mortality (spontaneous death) in this experiment:**

   a. Procedure/condition associated with mortality:
   N/A

   b. Estimated mortality rate, i.e. percentage of animals expected to die spontaneously (not via euthanasia) or need to be euthanized as a result of the procedure. (Be sure to account for this in your animal number calculations):
   N/A

   c. Explain why euthanasia is not possible or appropriate:
   N/A

9. **Will some animals live out their natural lifespan as part of this experiment? If so, indicate their use and describe the monitoring plan for aged animals (e.g., rodents >18 months of age), including frequency, behavioral and clinical signs to be monitored and criteria for euthanasia.**
N/A

10. *** Total number of animals used in this experiment:**(including all the animals to be produced)
72

a. Justify total number of animals used in this experiment:

We used an effect size of 0.75 which was the effect size found in the pilot study. Using ANOVA with a significance level of 0.05, there will be 90% power to detect an effect size of 0.75. This applies to the effects of each of the 3 factors (riPOBs, $MgCl_2$ and I50) being tested. Therefore we concluded that 8 animals in each group is an appropriate sample size for our study. We have 9 groups in total, so 72 is the total sample size for this study.

11. Number of animals by pain and distress category:(include each animal only once in the highest pain category)

B: 0
C: 0
D: 72
E: 0

a. Justify the need for any animals in pain category E:

N/A

12. * Identify husbandry exceptions:

	Exception Type	Description and Justification
View	Rats - No husbandry or enrichment exceptions.	N/A
View	Rats - Special/medicated water	Rats may receive cyclosporine via drinking water for a maximum of ~6 weeks. Rats may also receive Baytril via drinking water for up to 30 days. See experiment description and related procedures for details.

13. Supporting documents:

Document Name	Date Modified

There are no items to display

1. *** Exception type:**

   Rats - No husbandry or enrichment exceptions.

2. **Description and justification:**

   N/A

1. *** Exception type:**

   Rats - Special/medicated water

2. **Description and justification:**

   Rats may receive cyclosporine via drinking water for a maximum of ~6 weeks. Rats may also receive Baytril via drinking water for up to 30 days. See experiment description and related procedures for details.

1. * Identify the location where animals will be used:

ARCF ABSL1

a. For locations that are lab managed, provide justification for housing outside of the vivarium:

N/A

2. * What species will be housed in this location?

Common Name	Scientific Name
Rats	Rattus

1. **Campus:**
   Vivarium
2. **Vivarium:**
   ARCF (Animal Research & Care Facility)
3. *** BSL Level:**
   ARCF ABSL1

1. * Identify the location where animals will be used:

ARCF ABSL1

a. For locations that are outside of the vivarium, provide justification for the use of this space:

N/A

2. * What species will be used in this location?

Common Name	Scientific Name
Rats	Rattus

3. Describe how this location will be used:

All procedures will be performed here.

A cleared place will be used for operation and a power source will be needed for portal dental drill motor.

4. * If animals are left unattended in this location, provide an explanation and include maximum duration:

The researcher maybe leave to get equipment essential for surgery. The animals won't be under unattended longer than half an hour.

5. Describe how animals will be transported to and from this location, including container and route. (Note: use of private vehicles requires IACUC approval):

The animal will be purchased through AOps/DCM, and the animals will be transported by Vendor's vehicle in cage. When transported in buildings, the animals will be in draped cages.

1. Campus:

Vivarium

2. Vivarium:

ARCF (Animal Research & Care Facility)

3. * BSL Level:

ARCF ABSL1

Leandra Mosca
Proto202000003 Comments

Refinement, Replacement and Reduction

Are there other reasons that rats are more suitable besides their larger molar size?

Substances

Xylazine dose 1.1-4.4 mg/kg and ketamine dose 48 – 68.2 mg/kg initially written as such, then later for terminal study listed as ≥4.4 mg/kg and ≥68.2 mg/kg respectively. What exactly does that mean?

Should the tie-2 agonist (I50) be ABSL-2?

Experiments

Can a local block (ex: lido:bupivacaine 50:50) be placed before surgery? Do you think meloxicam 72 hours post-op is sufficient analgesia?

Duration of procedure: 40 min may exceed duration of ketamine/xylazine anesthesia. What is your plan if the animal exits the appropriate surgical plane of anesthesia mid-procedure? Will you re-dose? How will you monitor these animals differently post-op?

Is five days of penicillin/ampicillin treatment going to be sufficient for immunosuppressed animals?

If you see signs of infection what will you do? Will the animal remain on study in conjunction with clinical care? Will this interfere with your bone samples/imaging?

Can you be more specific about the endpoint criteria: "Impairment of ability to eat, drink, or ambulate normally."

How will you measure severity of infection, if one occurs? Will you treat milder cases? Will a repair surgery be necessary if the graft is ever compromised?

Zabrecky Vet Review Questions
Protocol 4174-03 Magnesium Stem Cell
Protocol Class 6/18/20

Survival Surgery

- Surgery type: [This is more for a discussion point and not a question for the group. It is more for my own understanding]
    - Since this surgery is generally and outpatient procedure. Would this be classified as minor?
- Describe prep: In the protocol, you describe scrubbing the extraction site with gauze in 1% chlorhexidine solution. Due to the nature of the procedure, this is not necessary.
- Describe procedure: You describe copious amounts of sterilized saline will cool the round bur. How will you ensure that this does not get aspirated by the rats during the procedure?
- Analgesic: Have you considered a local maxillary block to assist with analgesia for your rats? There have been some studies investigating its use in rat tooth pulp analgesia. For other species, local blocks are indicated in extractions.
    - [mostly a point of discussion for us that I was thinking about for this study]
- Post op care: You describe the use of penicillin and ampicillin in your protocol. These are similar, but not the same antibiotic. Do you need an antibiotic for your experiment? In your substance section, the compounds are either sterile filtered or cultured or pharmaceutical grade.
    - Due to the nature of the procedure. Providing wet or moistened food for the first few days after surgery is indicated for these rats.

Anesthesia agent: Most of these procedures are fairly short such as imaging. Can isoflurane be also used as an anesthetic?

Bioluminescent imaging: Do you inject a compound, such as luciferin, to allow for imaging? If so, this will need to be added to the protocol.

Animal numbers: Do you want to add animal numbers for blood collection in case you can't use one rat or do not get enough blood during your collection?

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Friday, August 14, 2020 2:03 PM
To: dcmresidents@uw.edu
Subject: rats, antibiotics, anaerobes
Attachments: Drug Dosages for Mice and Rats.doc; jaalas2014000301.pdf

Hi everyone,

Just wanted to let you know what I responded (pasted below). If it happens to come to one of you (e.g., via vsreview) before me for advice on penicillin-derivative dosing specifics, please let me know so we can collaborate on that response. We used to use amoxicillin water sometimes, but hardly ever do anymore. It was seen to "settle out" so there were concerns about solubility. But Thea pointed out this paper in which amoxicillin water actually came out looking relatively decent (attached). If they did go with the water, I'd want them to shake it daily, as they did in this study.

I also attached the VS mouse and rat formulary, which includes an amoxi water recipe. If you do the math, the dose here is higher than what is in the JAALAS paper (more like 125 mg/kg if you go with ~25 g mouse and 5 ml/day water cons.).

I think there are pros/cons to all the options, but we'll see what happens.

Molly

Zhang: Administration of Penicillin G sodium:

I appreciate that activity against anaerobes is desired for this project (in addition to broad activity against other bacteria). With that in mind, there are options available that are much preferable to daily IM injections of Pen G. IM injections are painful in rodents (small muscle mass) and very strong justification would be required for 5 days of daily IM injections of this drug when other options exist.

One alternative which would provide a broader spectrum of activity than Pen G in addition to not requiring injections is to administer both Baytril water and oral metronidazole (dosed orally via syringe, 10-40 mg/kg once a day, which could be done at the same time as cyclosporine A SC injections). Vet services (vsreview@uw.edu) can provide information about ordering metronidazole from a compounding pharmacy for oral administration (the pharmacy can formulate it to make it as palatable as possible). For Baytril water, the dose is 25 mg/kg/day. This can be made by adding 5.7 ml of 22.7 mg/ml Baytril to 16 oz water (or 4.6 ml in 14 oz bottle), and once a bottle is made it is good for a week. Vet services recommends starting any medicated water 1-2 days prior to surgery, to reduce the risk of development of taste aversion (association of a new taste with a painful event). If you want to add Baytril water, your OAW liaison can probably help with that procedure as other groups already have this in their protocols.

If you prefer a penicillin-type drug over the Baytril/metronidazole option, there are

formulations that could be given orally by syringe (amoxicillin or ampicillin), orally in the drinking water (amoxicillin), or injected subcutaneously (e.g., amoxicillin) if injection is preferred over oral delivery. I think the Baytril/metronidazole combination would have the broadest spectrum, but I can also provide more additional details on dose, etc. for these other options.

Please comment/edit as needed.

Drug Dosages for Mice and Rats

Amoxicillin
Mice- Dose: 150mg/8oz. Using a gram scale, measure 2.14g of Trimox powder (250mg/5mL). Add to 8oz water bottle and shake well. If using 14oz bottle, add 3.7g, if using 13.5oz Hydropac, add 3.6g. Solution should be changed weekly or bi-weekly.
*** Contains sugar.
Rats-Dose: 100mg/kg. Concentration 1mg/mL. Using a gram scale measure 6.75g Trimox powder (250mg/5ml) and add to a 16oz water bottle, 5.9g to a 14oz water bottle, or 5.7g to a 13.5oz Hydropac. Shake well. Solution should be changed weekly or bi-weekly. **Contains sugar.

Baytril (antibiotic injection / used in water)
Loading dose: 25mg/kg. For 25gm mouse give 0.02mL of full strength Baytril (22.7mg/mL) SQ. Dilute
Maintenance dose: 4mg/kg. Use diluted Baytril Solution: 0.1mL of full strength (22.7mg/mL) Baytril with 9.9mL sterile water. Solution yields 0.22mg/mL solution.
For 25gm mouse give 0.45ml SQ.
Baytril Water
Mice-Dose: 25mg/kg/day. Use 1.7mL full strength (22.7mg/mL) Baytril in 8oz of water, 3mL in a 14oz bottle, or 2.9mL in a 13.5oz Hydropac. Change weekly.
Rats-Dose: 25 mg/kg/day. Add 5.2mL of 22.7 mg/lm Baytril into 16oz of water, 4.6mL to a 14oz bottle, or 4.4mL to a 13.5oz Hydropac. Change weekly.

Buprenex (pain injection)
Dose: 0.025mg/kg-0.05mg/kg. Dilute 0.1mL of 0.3mg/mL Buprenex to 0.9mL of sterile water to yield 0.03mg/mL solution.
For 25gm mouse at 0.05mg/kg give 0.04mL of diluted solution SQ.
Or calculate for higher dose: example; 2.5mg/kg dose for a 25mg mouse, give 0.06mg or 2mLs of the diluted solution.
Store at room temperature. Label with date of dilution. Expires on same date of the next month.
SR Buprenorphine-dose is 0.5mg/kg. For a 25gm mouse give 0.0125ml SQ.

Calcium Gluconate ***(*Use first for dystocia-see Oxytocin and dystocia guidelines)***
Dose: 100mg/kg given intraperitoneal 10 minutes prior to Oxytocin inj.
Stock Solution: 98mg/mL.
For average 25 gm mouse give 0.02mL full strength solution or 2.5mg.

Carprofen (pain injection)
Dose: 5mg/kg. Use diluted solution. Dilute 1mL of full strength Carprofen (50mg/mL) with 3mLs of Sterile Water yielding a 12.5mg/ml solution.
For 25gm mouse give 0.01mL SQ.
Keep refrigerated. Label with date of dilution. Expires on same date the next month.

Doxycycline Powder
Use gram scale to measure out 0.25g of powder per 8oz of water.

Ibuprofen (for pain; used in the water)
Mice-Stock solution is 20mg/mL = 100mg/5mL. Use diluted solution. Add 2.5ml of 20mg/mL Ibuprofen suspension to 8oz water bottle (0.2mg/mL solution.), 4.4mL to a 14oz bottle, or 4.2mL to a 13.5oz Hydropac. Change weekly or bi-weekly. **Contains sugar.
Rats-Add 7.6ml (20mg/mL) ibuprofen into 16oz water bottle, 6.7ml to a 14oz bottle, or 6.4mL to a 13.5oz Hydropac. Change weekly or bi-weekly. ** Contains sugar. Results in a dose of 32mg/kg.

Ivermectin (parasiticide in drinking water for pinworms)
Dose is 0.08% sheep drench diluted as follows:
Mice: 2.4mL/8oz of water.
Rats: 12mL/16oz water.

Updated 3/5/2018 (ELS)

Or 192mL/5 gallons of water for one mouse rack.

Ketoprofen (pain injection)
Dose: 5mg/kg. Use diluted solution. Dilute 1mL of 100mg/mL Ketoprofen injection to 9mLs of sterile water to yield 10mg/mL solution. For 25gm mouse give 0.0125mL subcutaneously.
Store at room temperature. Label with date of dilution. Expires on same date of the next month.

Mouse Mix (for anesthesia)
Dose: 0.02mL/gm IP.
Full volume: Mix 0.22mL Xylazine (20mg/mL), 0.65mL Ketamine (100mg/mL), 9.13 mL Sterile water.
Half volume: Mix 0.11mL Xylazine, 0.32mL Ketamine, 4.56mL Sterile water.
Solution expires after 10-14 days.

Meloxicam (analgesic)
Mice: 1mg/kg/day in the water
Add 0.19 mL of 5mg/ml of injectable Meloxicam to an 8oz bottle, 0.33mL to a 14oz bottle, or 0.32mL to a 13.5oz Hydropac. Change weekly.
Mice: 5 mg/kg for injection SC once daily

Rats: 1mg/kg/day in the water
Add 0.94mL of 5mg/ml of injectable Meloxicam in a 16 oz. water bottle, or 0.79mL to a 13.5oz Hydropac.
Rats: 1mg/kg SC once daily

Neomycin (Antibiotic in water for irradiation experiments in Mice and Rats)
Mice- Dose: 2mg/mL.
Prepare as follows: Use 25g bottle of powdered Neomycin (732mc/mg) to 223mL of R/O water from sink.
This yields an 82mg/mL solution. For a 2mg/mL solution, add 6mL of the prepared solution to an 8oz water bottles, 10.5mL to a 14oz bottle, or 10.1mL to a 13.5oz Hydropac. Solution should be changed weekly.
Rats-Dose 3.2mg/ml. Reconstitute powdered Neomycin as per mice instructions to a 82mg/ml solution. Add 18.5mL of this solution into a 16 oz water bottle, 16.2ml to a 14oz bottle, or 15.6mL to a 13.5oz Hydropac.

Ondansetron (anti-nausea medication)
Rats – Dose: 0.1mg/kg SQ
Dilute full strength (2mg/mL) 1mL in 9ml sterile water to make a 0.2mg/mL solution. Inject 0.25 mL for a 500g rat.

Oxytocin (Injection for dystocia- see dystocia guidelines)
Dose: 1.0IU/kg. Use diluted solution.
From 20 IU Oxytocin, use 0.1mL to 9.9 mL sterile water to yield a 0.21 IU/mL solution. Give 0.125mL SQ for an average 25 gm mouse every 30min- hour.
If mouse has not given birth after three doses, euthanasia is necessary.

Rat Mix (for anesthesia)
Dose: 0.9mL of prepared mix/kg IP.
Prepare as follows: 5mL Ketamine (100mg/mL), 1.6mL Xylazine (20mg/mL).
After calculating dose, dilute further with 2mL of sterile water before giving IP.
Full strength mix expires in approximately one week.

Sucralfate oral suspension (20mg/ml)
Rats – Dose: 0.5ml per os BID

SMZ-TMP
Dose is approximately 54mg/kg/day.
Mice: Use 1mL of full strength (Sulfa 80mg/mL & Trimeth 16mg/mL) per 8oz of drinking water, or 1.7mL per 13.5oz Hydropac.
Rats: Use 3mLs of full strength per 16oz of drinking water, or 2.5mL per 13.5oz Hydropac.
*** Diluted drug labels are filed in filing cabinet under "Diluted Drug Labels"

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Antibiotic Administration in the Drinking Water of Mice

James O Marx,[1,2,*] Daljit Vudathala,[3] Lisa Murphy,[3] Shelley Rankin,[4] and F Claire Hankenson[1,2]

Although antibiotics frequently are added to the drinking water of mice, this practice has not been tested to confirm that antibiotics reach therapeutic concentrations in the plasma of treated mice. In the current investigation, we 1) tested the stability of enrofloxacin and doxycycline in the drinking water of adult, female C57BL/6 mice; 2) measured the mice's consumption of water treated with enrofloxacin, doxycycline, amoxicillin, or trimethoprim–sulfamethoxazole; and 3) used HPLC to measure plasma antibiotic concentrations in mice that had ingested treated water for 1 wk. Plasma concentrations of antibiotic were measured 1 h after the start of both the light and dark cycle. The main findings of the study were that both enrofloxacin and nonpharmaceutical, chemical-grade doxycycline remained relatively stable in water for 1 wk. In addition, mice consumed similar volumes of antibiotic-treated and untreated water. The highest plasma antibiotic concentrations measured were: enrofloxacin, 140.1 ± 10.4 ng/mL; doxycycline, 56.6 ± 12.5 ng/mL; amoxicillin, 299.2 ± 64.1 ng/mL; and trimethoprim–sulfamethoxazole, 5.9 ± 1.2 ng/mL. Despite the stability of the antibiotics in the water and predictable water consumption by mice, the plasma antibiotic concentrations were well below the concentrations required for efficacy against bacterial pathogens, except for those pathogens that are exquisitely sensitive to the antibiotic. The findings of this investigation prompt questions regarding the rationale of the contemporary practice of adding antibiotics to the drinking water of mice for systemic antibacterial treatments.

Abbreviations: C_{max}, peak plasma concentration; MIC, minimum inhibitory concentration; TMS, trimethoprim–sulfamethoxazole.

The use of antibiotics to treat bacterial infections is a standard of care in veterinary medicine. In many species, the administration of antibiotics is a routine procedure with proven efficacy. Unfortunately, this is not the case for laboratory mice used in biomedical research, where the delivery of antibiotics may be associated with stress to animals and where confirming that drugs reach therapeutic concentrations in the blood has proven challenging.

The administration of antibiotics to mice either parenterally or bolused enterally involves handling of the mice and induces stress in the animal.[3] To ameliorate this handling-associated stress, medications—including antibiotics—frequently are added to the drinking water. This method is time-efficient for laboratory animal personnel and is thought to be of added therapeutic benefit, because it provides continuous accessibility to the medication. As with any route, there are potential limitations to this route of delivery to mice: first, the antibiotic must remain stable in the drinking water and be available for consumption by the mouse; second, mice must drink predictable volumes of treated water; and, last, sufficient concentrations of antibiotic must be maintained in the bloodstream to achieve systemic antibacterial efficacy.

To date, few studies have tested the stability of antibiotics in the drinking water of laboratory mice. For example, one study[16] that tested the stability of amoxicillin–clavulanic acid and trimethoprim–sulfamethoxazole (TMS) in acidified and reverse-osmosis (RO)–treated water found that amoxicillin was stable in RO water but had an immediate drop in concentration to approximately 50% in acidified water, whereas clavulanic acid dropped to 40% in RO over 7 d and immediately was degraded in acidified water. TMS showed variability over the course of 7 d, making reliable dosing with this drug difficult.[16] The cited study did not measure the consumption or systemic absorption of the antibiotics in the mice; therefore although these antibiotics exhibit variable stability in the drinking water, it is unknown whether these drugs reach concentrations sufficient to eliminate pathogenic bacteria.

When antibiotics are administered in drinking water, it is challenging to estimate accurately the total amount of water consumed by the mice. Many factors complicate this measurement, including: spillage of water from the bottle into the bedding; altered taste of the antibiotic-treated water, which may alter the daily water consumption by the mice; ill or unhealthy mice, which may consume less water than would clinically healthy animals, resulting in dehydration and inadequate antibiotic ingestion; and the diurnal pattern of water intake in laboratory mice, which tend to consume most of their daily water intake at the beginning of the dark cycle, creating potential circadian changes in the ingestion of the antibiotics, with the highest dosing occurring at night.[7,19] Therefore, despite the common practice of adding drugs to drinking water, using this route for dosage of antibiotics to mice is unpredictable.

Even when consumption is sufficient, it is complicated to determine whether the antibiotics reach plasma concentrations adequate to eliminate the pathogenic bacteria responsible for the infection. When inadequate antibiotic concentrations occur, there is an increased risk of selecting for drug-resistant pathogens and eliminating normal flora. The risks of failure to

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achieve adequate drug concentrations are augmented further in laboratory mice, given that many mice are immunosuppressed due to genetic manipulation, radiation, or pharmacologic immunosuppression. These laboratory mice will be almost totally dependent on the bactericidal activity of the antibiotic, with little contribution of the immune system, in the resolution of an infection. The minimum inhibitory concentration (MIC) is the lowest concentration of antibiotic that will effectively inhibit bacterial growth.[10,24] Another important parameter when discussing antimicrobial susceptibility of a given bacterial species is the MIC_{90}. The MIC_{90} represents the MIC value at which 90% of the bacterial strains within a test population containing multiple independent isolates of the same species are inhibited.

An important factor that potentially limits the efficacy of antibiotics in mice is allometric scaling (also referred to as scaling). Scaling relates to the change in physiologic parameters in species in relationship to body size. Obvious examples of scaling can be recognized as changes in heart rate, gestation, and life expectancy with body size. Factors influencing drug pharmacokinetics, such as metabolic rate, glomerular filtration rate, and hepatic blood flow also scale relative to overall body size, resulting in increased metabolic clearance and decreased drug half lives in small species, such as mice.[4,17,22] Pharmacokinetic features of both enrofloxacin and doxycycline have been demonstrated to have significant scaling effects,[6,21] and amoxicillin is reported to have potential scaling effects.[22] The drugs that comprise TMS do not demonstrate evidence of a significant scaling effect, although this drug combination has yet to be tested against scaling parameters other than body size, which may yield a more accurate representation of the effects of scaling on the metabolism of these drugs.[14,15] This increase in the metabolic clearance of antibiotics may limit their ability to achieve the necessary plasma concentrations in mice required for antibiotic efficacy.

The purpose of the current study was to analyze the limitations of the administration of 4 commonly used antibiotics in the drinking water of mice. The antibiotics studied were doxycycline, which typically is administered for the control of gene expression in genetically manipulated mice,[28] and TMS, amoxicillin, and enrofloxacin, which are broad-spectrum antibiotics that have been added to the drinking water of mice.[5,16,25] The first experiment tested the stability of 2 of the antibiotics, enrofloxacin and doxycycline, in tap and acidified water, and enrofloxacin in hyperchlorinated water, all of which are commonly used in laboratory mouse vivaria. The second experiment measured the consumption of the 4 antibiotics from the drinking water of mice and the plasma concentrations of the antibiotics that were achieved. In light of anecdotal evidence of a positive therapeutic effect of antibiotics administered in the drinking water of mice, we hypothesized that 1) the antibiotics would be stable in the drinking water, 2) the treated water would be consumed normally by the mice, and 3) therapeutic plasma concentrations would be achieved.

Materials and Methods

Experiment 1: stability of enrofloxacin and doxycycline in drinking water. The dosages and concentrations of the antibiotics in the drinking water were based on published antibacterial doses and the projected daily consumption of 5 mL by an adult mouse (Table 1). The products used were injectable enrofloxacin (Baytril 100 mg/mL, Bayer HealthCare Animal Health Division, Shawnee Mission, KS); oral pharmaceutical-grade doxycycline calcium (Vibramycin calcium 5 mg/mL, Pfizer Labs, New York, NY); and chemical, nonpharmaceutical-grade doxycycline HCl (Research Products International, Mt Prospect, IL).

Table 1. Antibiotic doses and concentration of antibiotic in the drinking water

Drug	Daily oral dose (mg/kg; [reference])	Antibiotic concentration (mg/mL) in water
Enrofloxacin	50 (5)	0.25
Doxycycline	10 (18)	0.05
Amoxicillin	50 (23)	0.25
TMS	160 (16)	0.8

Sample collection. The stability of enrofloxacin in tap, acidified, and hyperchlorinated water was tested over 7 d, and that of doxycycline in tap and acidified water was tested over 7 d (Table 2), in light of the water systems available at the University of Pennsylvania. The antibiotic-treated water was maintained in a standard, clear mouse water bottle (265 mL; Polysulfone Water Bottles, Ancare, Bellmore, NY) and was placed in a complete mouse cage setup that was empty of animals in a mouse holding room. Facility temperatures were maintained at 22.2 ± 1.1 °C (72 ± 2 °F); humidity was between 30% and 70% with 10 to 15 air changes hourly, as recommended by the *Guide*.[12] The cages were 7.5 in. × 11.5 in. × 5 in. polycarbonate, static isolation cages (Ancare, Bellmore, NY) with 1/4-in. corn cob bedding (Animal Specialties and Provisions, Quakertown, PA). All treated water bottles were shaken daily by the research staff. Samples of treated water were collected on days 0 and 7. At the time of sample collection, 10 mL of treated water was collected by syringe from the end of the sipper tube from each water bottle. Care was taken to disturb the water bottle as little as possible before the sample was collected, to obtain a representative sample of the water that would be available to the mice from the sipper tubes. The samples were stored in centrifuge tubes and frozen at −80 °C until analysis. The effect of the antibiotics on the pH of the water was tested by measuring its pH (pH 510 Benchtop Meter, Oakton Instruments, Vernon Hills, IL) before and after the addition of the antibiotic.

Analysis of antibiotic concentrations in water. Water samples were analyzed by using Shimadzu (Columbia, MD) liquid chromatography with a diode array detector. Water samples containing enrofloxacin were diluted 1:10 with 0.5% formic acid containing 10% acetonitrile. Doxycycline-containing water samples were diluted 1:1 into the same diluent. Control samples of both antibiotics were prepared by dissolving in methanol to obtain a concentration of 1 mg/mL of free drug. Standards were prepared that reflected the expected concentrations for each drug: enrofloxacin standards were 0, 5, 10, 25, and 50 µg/mL; and doxycycline standards were 0, 10, 50, 100, and 200 µg/mL. Enrofloxacin was analyzed by using water and acetonitrile (20:80, both containing 0.1% formic acid) in an isocratic run with a flow rate of 0.6 mL/min. Water samples containing doxycycline were analyzed by gradient chromatography using 0.1% formic acid with 0.005 M EDTA and acetonitrile at a flow rate of 0.8 mL/min; the gradient was as follows: 20% acetonitrile for first 2 min, ramp to 70% acetonitrile over 1 min and then held constant for 3 min, and back to original conditions over 1 min. The system was equilibrated for 5 min prior to the next injection. The diode array detector was monitored from 190 to 320 nm, with quantification done at 280 nm for enrofloxacin and at 265 nm for doxycycline. Acetonitrile, methanol, formic acid, and EDTA were purchased from Thermo-Fisher Scientific (Fair Lawn, NJ). Enrofloxacin and doxycycline hyclate standards were obtained from Sigma-Aldrich (St Louis, MO).

Table 2. Antibiotic concentrations (mg/mL± SE; n = 4 water bottles) during experiment 1

	Tap water		Acidified water		Hyperchlorinated water	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
Enrofloxacin	0.239 ± 0.006	0.237 ± 0.006	0.250 ± 0.004	0.248 ± 0.005	0.246 ± 0.002	0.155 ± 0.019
Doxycycline						
Pharmaceutical grade	0.017 ± 0.001	0.017 ± 0.003	0.048 ± 0.001	0.043 ± 0.001	not tested	not tested
Chemical grade	0.052 ± 0.004	0.032 ± 0.001	0.037 ± 0.002	0.042 ± 0.002	not tested	not tested

Experiment 2: consumption of treated water and serum antibiotic concentrations. Young adult (6 to 10 wk) female C57BL/6J mice (*Mus musculus*, Jackson Laboratory, Bar Harbor, ME) were used in this investigation. The mice were housed in polycarbonate cages with bedding, as described earlier, with free access to autoclaved food (Lab Diet 5010, Animal Specialties and Provisions, Quakertown, PA) and were maintained on a 12:12-h light:dark cycle. Prior to the start of the study, the mice were allowed at least 1 wk to acclimate to the housing facility and conditions. Sentinel mice were tested routinely and were free of pinworms by cecal exam and of fur mites by fur pluck and were antibody-negative for tested pathogens including mouse hepatitis virus, mouse parvoviruses, rotavirus, ectromelia virus, Sendai virus, pneumonia virus of mice, Theiler murine encephalomyelitis virus, reovirus, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, and polyomavirus. All aspects of the current investigation were approved by the University of Pennsylvania IACUC.

Mice were pair-housed and randomly assigned to receive 1 of the 4 antibiotics (n = 8 mice for each antibiotic) during the study. The antibiotics tested were enrofloxacin and chemical-grade doxycycline as in experiment 1, amoxicillin (50 mg/mL, Sardoz, Princeton, NJ), and TMS (48 mg/mL, Hi-Tech Pharmacal, Amityville, NY; Table 1). The mice were weighed at the start of the study, and daily water consumption was measured by weighing the water bottles for each pair of mice for 7 d. On day 7, the mice were weighed again, and the antibiotic was added to fresh tap water. The water bottles were shaken daily, and water consumption was measured over an additional 7 d. At the day 14 endpoint, the mice were weighed, and a random half of the mice (n = 4 per group) underwent blood collection into heparinized tubes at 0700; the remaining mice had blood collected at 1900. All blood collection in the study was by retroorbital bleeding under isoflurane anesthesia. Mice were induced at 3% isoflurane until they lost the righting reflex, after which they were maintained at 2.25% for 3 min. This protocol allowed sufficient anesthesia time after removing the mice from the anesthetic to safely collect the blood. Approximately 200 µL blood was collected into heparinized centrifuge tubes at each time point. The mice then were allowed to recover and were returned to their home cages. Two days later, the mice underwent a terminal blood collection at either 0700 or 1900, so that blood was collected from each mouse during both the morning and evening. The blood sample was centrifuged and the plasma separated and frozen at −80 °C until analysis.

To detect the highest possible plasma enrofloxacin concentration, 2 additional groups of 4 mice each were studied. In these mice, the blood was collected at 0100, in an effort to measure the concentration when mice are likely to recently have consumed the greatest water volume (and thus largest therapeutic dose of antibiotic). In addition, the enrofloxacin dose was increased in one group of mice to increase the plasma antibiotic concentration. Specifically, one group of 4 mice received the 50-mg/kg daily dose used in the previous mice, and remaining mice received 100 mg/kg daily.

Analysis of antibiotic concentrations in plasma. Due to the higher sensitivity required for plasma samples compared with water samples, plasma samples were analyzed by using an API 4000 (AB Sciex, Foster City, CA) liquid chromatography–tandem mass spectrometry system. The system was equipped with a Luna C18 (150 × 4.6 mm, 5-µm particle size) analytical column (Phenomenex, Torrance, CA). For each sample, 50 µL plasma was mixed with 0.1 mL acetonitrile containing 1% formic acid. The mixture was vortexed, centrifuged, and filtered through a 0.22-µm nylon filter prior to analysis. Plasma samples containing doxycycline, enrofloxacin, or TMS were analyzed by using a gradient run with 0.1% formic acid and 85% methanol containing 0.1% formic acid at a flow rate of 0.5 mL/min. The gradient conditions were as follows: methanol for the first 2 min, ramp to 95% methanol over the next 3 min, hold at 95% methanol for 4.5 min, return to the original conditions over 0.5 min, and then hold for 4 min. Samples containing amoxicillin were analyzed by using the same gradient conditions but with 0.1% formic acid and acetonitrile instead of methanol. The following ion transitions were selected to quantitate each antibiotic: doxycycline, 445.4 /154; enrofloxacin, 360/316.2; TMS, 291/261.1; and amoxicillin, 366/143.9. The test samples were quantified against curves obtained by analyzing control bovine serum spiked with antibiotic in concentrations ranging from 0.001 to 0.5 µg/mL. Methanol was purchased from Thermo-Fisher Scientific, and control bovine serum was obtained from Sigma-Aldrich.

Statistical analysis. Antibiotic concentrations in water were compared by ANOVA (SigmaPlot 12.3, Systat Software, San Jose, CA). Morning and evening plasma antibiotic concentrations were compared by repeated-measures ANOVA. Statistical significance was defined as a P value of less than 0.05.

Results

Enrofloxacin. Enrofloxacin remained stable in both tap and acidified water throughout the 7-d test period (Table 2). The addition of the injectable enrofloxacin immediately and dramatically increased the pH of both the tap and acidified water (Table 3). In the hyperchlorinated water, a precipitate rapidly formed over the first 24 h. This precipitate was absent from the untreated tap and acidified water, and once formed, the precipitate remained throughout the entire 7-d period. The time 0 sample had the expected antibiotic concentration; however at day 7, only 62% of the antibiotic remained in solution and was available to mice.

Doxycycline. The pharmaceutical-grade oral doxycycline immediately dissolved in the acidified water and remained at stable concentrations for the entire 7-d period (Table 2). However when the drug was added to tap water, a precipitate immediately formed and quickly settled to the bottom of the water bottle. HPLC analysis of the water samples revealed that the concentrations of doxycycline at days 0 and 7 were ap-

Table 3. pH of antibiotic-treated water after drug addition

	Tap water (pH = 6.99 before addition)	Acidified water (pH = 3.29 before addition)
Enrofloxacin	9.54	8.78
Doxycycline	7.02	3.26
Amoxicillin	6.89	3.50
TMS	6.92	3.39

Note the profound effect of the addition of injectable enrofloxacin on the pH of both the tap and acidified water.

proximately 30% of the expected value (Table 2). The sample then was acidified to a pH of 3.0 with hydrochloric acid and remeasured. This action resulted in a doxycycline concentration that was 90.8% of that expected, indicating that the majority of the active ingredient was present but unavailable for consumption by the mice because it was in the precipitate at the bottom of the water bottle.

The nonpharmaceutical, chemical-grade doxycycline powder was tested in both tap and acidified water. In acidified water, the chemical-grade doxycycline immediately dropped to approximately 75% of the expected concentration and then remained stable over 7-d period. There was no significant difference between the day 0 and day 7 doxycycline measurements. In tap water, the initial concentration was approximately 100% of the expected value, dropping significantly ($P < 0.05$) to 64% of the expected concentration at day 7. The addition of chemical-grade doxycycline had little effect on the pH of either the tap or the acidified water (Table 3).

Consumption of antibiotic-treated water and measurement of body weight. The pairs of mice in experiment 2 consumed 9.6 ± 0.2 mL water daily when no antibiotic was added (Table 4). Neither baseline body weight nor water consumption differed between any of the groups. Only the enrofloxacin group had a significant ($P < 0.05$) change in water consumption during the week of antibiotic administration; consumption increased from 9.7 ± 0.4 mL/d to 11.4 ± 0.2 mL/d per pair of mice. Initial body weight did not differ between any of the groups at the start of the experiments, and mice in all 4 groups gained weight over the next 2 wk.

Plasma antibiotic concentrations. Plasma concentrations (Table 4) showed no significant differences between the morning and evening sampling time points for any of the antibiotics. To maximize the measured plasma enrofloxacin concentration, 2 additional groups of mice were tested at 0100, during the dark cycle. The 0100 plasma enrofloxacin concentrations for the group receiving the 50-mg/kg dose was similar to those of the earlier time points; and the plasma antibiotic concentration of the group that received 100 mg/kg enrofloxacin was 174.8 ± 55.5 ng/mL.

Discussion

Achieving therapeutic concentrations of antibiotic in patients is critical to the efficacy of any antibiotic, independent of species. The current investigation demonstrates that, although the antibiotics tested remained stable in the drinking water and the mice consumed predictable volumes of antibiotic-treated water, plasma concentrations above the reported MIC values for most common pathogenic bacteria (Table 5) were not attained. These findings question the rationale for the common practice of antibiotic administration in the drinking water of mice.

The efficacy of antibiotics depends on the pharmacodynamics of the antibiotic–bacteria interaction. Antibiotics are commonly divided into 2 groups according to their pharmacodynamic characteristics: time-dependent, such as β-lactam drugs, in which the efficacy of the drug is determined by the total time the plasma antibiotic concentration is above the MIC of the organism being targeted, and concentration-dependent, such as fluroquinolones, in which efficacy is associated with the peak plasma concentration of the antibiotic.[1,20] Administering antibiotics in the drinking water of mice will optimize the performance of the time-dependent antibiotics, maintaining elevated concentrations of antibiotics in the blood stream whenever mice drink water. In the current study, the amoxicillin plasma concentrations were similar at the start of both the light and dark cycles. However, dosing by water resulted in plasma concentrations that were well below the MIC of most common bacterial pathogens, so that only exquisitely sensitive organisms would be effectively killed by this route of dosing (Table 5).[2,20] The results of the current study are similar to those reported previously[16] regarding plasma levels achieved after antibiotic administration in animals' food.

Providing enrofloxacin in the drinking water failed to achieve effective plasma concentrations. Enrofloxacin is a concentration-dependent drug, which means that the peak serum concentration (C_{max}) achieved has been shown to be a critical factor in the efficacy of bactericidal activity. An C_{max}:MIC value greater than 10 has been shown to predict efficacy.[10,24] Oral bolus dosing of enrofloxacin in dogs has been shown to achieve C_{max} values of 2.1 to 5.2 µg/mL, whereas the plasma concentration measured in the current murine experiment were only 112.2 ± 11.7 ng/mL at 0700 and 140.1 ± 10.4 ng/mL at 1900. We hypothesized that the peak plasma concentration would occur in the middle of the dark cycle, when mice tend to drink the most water,[7,13] so we measured plasma enrofloxacin concentrations in mice in the middle of the dark cycle and found that, surprisingly, this value (117.5 ± 16.9) was lower than the 1900 value. In an effort to maximize C_{max}, a second group of mice for which the enrofloxacin dose was doubled were tested in the middle of the dark cycle, but this adjustment resulted in an average plasma concentration of only 174.8 ± 55.5 ng/mL. Considering that the goal is to achieve a C_{max}:MIC ratio of 10 or greater, these findings indicate that providing enrofloxacin in the drinking water of mice likely will be ineffective against most pathogenic bacteria.[20]

The plasma concentrations of both TMS and doxycycline were well below the MIC_{90} values (Table 5) for common pathogenic bacteria, indicating that the administration of these antibiotics by this route for the treatment of systemic infections in mice should be discouraged. The doses reported in the literature for mice are similar to those used in other species. This dosing regimen fails to take into account the effects of allometric scaling on drug metabolism, which as discussed earlier, will result in an increase in drug metabolism and a subsequent decrease in plasma concentration. Both doxycycline and TMS are used frequently with success in laboratory mice for purposes other than systemic bacterial infections. Doxycycline is used most often in genetically manipulated mice in the control of 'Tet-on' gene expression, by using a tetracycline-sensitive promoter gene to control either the expression or inhibition of gene expression.[28] TMS frequently is added to the drinking water of mice after ionizing irradiation to prevent bacterial sepsis by reducing the number of potential pathologic bacteria within the gastrointestinal tract.[8]

Increasing the amount of antibiotic consumed by the mice can be accomplished by either increasing the concentration of antibiotic in the drinking water or by increasing the amount of water consumed by the mice.[11] However, according to our

Table 4. Water consumption (mL; mean ± SE; n = 4 cages) and plasma antibiotic concentration (ng/mL; mean ± SE)

	Consumption[a]		Plasma antibiotic concentration[b] at		
Antibiotic	Control water	Antibiotic water	0700	1900	0100
Enrofloxacin	9.7 ± 0.8	11.4 ± 0.3[c]	112.2 ± 11.7	140.1 ± 10.4	117.5 ± 16.9
Doxycycline	9.3 ± 0.3	10.1 ± 0.9	56.6 ± 12.5	42.9 ± 7.8	not tested
Amoxicillin	9.3 ± 0.4	8.8 ± 0.8	299.2 ± 64.1	275.2 ± 50.2	not tested
TMS	10.1 ± 1.3	11.2 ± 1.5	5.7 ± 2.3	5.9 ± 1.2	not tested

[a]Consumption data represent 2 mice per cage.
[b]n = 8 mice per antibiotic, except for the 0100 enrofloxacin sample (n = 4).
[c]Value significantly ($P < 0.05$) different from that for consumption of control water.

Table 5. MIC of various antibiotics for common bacteria

	Bacteria	MIC_{90} (ng/mL; [reference])
Enrofloxacin	*E. coli*	30-125 (20)
	S. aureus	120-250 (20)
	Enterococcus spp.	1000-2000 (20)
	Pseudomonas aeruginosa	1000-8000 (20)
	P. multocida	500 (27)
Doxycycline	*M. pneumonia*	500 (26)
	Pasteurella spp.	125 (27)
Amoxicillin	***S. aureus***	**50 (20)**
	E. coli	5000 (20)
	S. pseudintermedius	2000 (20)
	C. perfringens	**50 (20)**
	P. multocida	**250 (27)**
TMS	*S. xylosus*	>2000 (25)
	K. pneumonia	<500 (20)
	E. coli	<500 (20)
	β-hemolytic streptococci	2000 (20)
	Pasteurella spp.	250 (27)

The bacteria–antibiotic combinations represent common pathogenic bacteria in veterinary medicine. MIC_{90} values for ampicillin were used interchangeably with those for amoxicillin. Systemic infections with bacteria in bold can be treated reasonably effectively with the corresponding antibiotic. Note that most of the bacteria isolated during common murine infections lack published MIC_{90} values for various antibiotics.

findings, plasma concentrations would need to be increased by 10-fold to achieve effective plasma concentrations through the drinking water or those that are achieved with oral bolus dosing in other species. Further compounding these difficulties are that the plasma concentrations may not increase linearly with increasing doses, meaning it may take more than a 10-fold increase in the amount of antibiotic consumed to achieve the desired increase in plasma concentration.

A potential use of administration of antibiotics in the drinking water of mice involves the treatment of localized infections in mice. Both amoxicillin and enrofloxacin are concentrated in the urine due to renal excretion.[18] This concentration may enable these antibiotics to achieve sufficient urinary concentrations to be effective for the treatment of cystitis and renal infections in mice. The results of the first experiment indicate care must be taken to ensure the stability of the antibiotic–water combination. Enrofloxacin is stable in both tap and acidified water but radically alters hyperchlorinated water, making the addition of this drug to hyperchlorinated water a poor option. When in either tap or acidified water, this antibiotic appeared to be minimally affected by light over the brief time period studied, given that the water concentrations remained stable over the 7-d period. Ultimately, however, bolus dosing of some antibiotics, particularly enrofloxacin (which works in a concentration-dependent fashion), is more likely to achieve effective plasma concentrations.

Several different water treatments are used to prevent the exposure of immunosuppressed mice to bacterial pathogens, particularly *Pseudomonas aeruginosa*. These include acidification, hyperchlorination, and reverse-osmosis.[9] The stability and solubility of the antibiotics we tested was dependent on the type of water and the formulation of the antibiotic. Preliminary experiments used a pharmaceutical-grade, oral doxycycline suspension and showed that it dissolved into and was stable in acidified water but remained as a suspension in tap water. We then tested a chemical nonpharmaceutical-grade doxycycline powder that is used by many research laboratories for control of gene expression using the Tet promoter, and the drug demonstrated mild degradation over the 7-d observation period. This distinction is an important one to make for institutional committees that review the use of doxycycline for research purposes, because investigators typically are expected to justify the use of nonpharmaceutical chemical-grade products for research animals. This difference in solubility would be a scientific justification for investigators to choose the chemical-grade product over the pharmaceutical grade product. Similar findings occurred with enrofloxacin, which was soluble in both acidified and tap water but precipitated in hyperchlorinated water. Finally, injectable enrofloxacin had a profound effect on the pH of the water, both acidified and tap, whereas the other antibiotics had little effect on this parameter. The effects of drugs on the drinking water's pH is an important consideration, particularly when adding drugs to acidified water, given that a loss of acidification may favor the growth of *Pseudomonas* spp. in the water of vulnerable immunosuppressed mice. The finding that the mice drank more of the enrofloxacin-treated water when compared with the untreated control was surprising, considering that enrofloxacin is reported to have a bitter taste. It is possible that the novel taste of the water appealed to the mice and promoted increased drinking during the week of treatment. Future work examining the taste preferences of mice will be valuable in an effort to increase their consumption of medicated water.

The findings of the current study demonstrate that the administration of antibiotics in the drinking water of mice does not result in plasma antibiotic concentrations that are effective against most pathogenic bacteria. Although this oral administration route may be adequate for treatment of some bacterial infections, such as when the antibiotic is concentrated at the site

of infection, it is inappropriate for general systemic bacterial infections in which the sensitivity of the pathogenic bacteria has not been identified.

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References

1. **Aarestrup FM.** 2006. Antimicrobial resistance in bacteria of animal origin. Washington (DC): American Society for Microbiology Press.
2. **Adams HR.** 2001. Veterinary pharmacology and therapeutics. Ames (IA): Iowa State University Press.
3. **Balcombe JP, Barnard ND, Sandusky C.** 2004. Laboratory routines cause animal stress. Contemp Top Lab Anim Sci **43:**42–51.
4. **Boxenbaum H.** 1984. Interspecies pharmacokinetic scaling and the evolutionary–comparative paradigm. Drug Metab Rev **15:**1071–1121.
5. **Brook I, Elliott TB.** 1991. Quinolone therapy in the prevention of mortality after irradiation. Radiat Res **128:**100–103.
6. **Cox SK, Cottrell MB, Smith L, Papich MG, Frazier DL, Bartges J.** 2004. Allometric analysis of ciprofloxacin and enrofloxacin pharmacokinetics across species. J Vet Pharmacol Ther **27:**139–146.
7. **Dole VP, Ho A, Gentry RT.** 1983. An improved technique for monitoring the drinking behavior of mice. Physiol Behav **30:**971–974.
8. **Duran-Struuck R, Dysko RC.** 2009. Principles of bone marrow transplantation (BMT): providing optimal veterinary and husbandry care to irradiated mice in BMT studies. J Am Assoc Lab Anim Sci **48:**11–22.
9. **Fox JG.** 2002. Laboratory animal medicine. New York (NY): Academic Press.
10. **Gillespie EL, Kuti JL, Nicolau DP.** 2005. Pharmacodynamics of antimicrobials: treatment optimisation. Expert Opin Drug Metab Toxicol **1:**351–361.
11. **Hojman P, Eriksen J, Gehl J.** 2007. Tet-On induction with doxycycline after gene transfer in mice: sweetening of drinking water is not a good idea. Anim Biotechnol **18:**183–188.
12. **Institute for Laboratory Animal Research.** 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Pres.
13. **Kutscher CL.** 1974. Strain differences in drinking in inbred mice during ad libitum feeding and food deprivation. Physiol Behav **13:**63–70.
14. **Mahmood I.** 1998. Interspecies scaling of renally secreted drugs. Life Sci **63:**2365–2371.
15. **Mahmood I, Sahajwalla C.** 2002. Interspecies scaling of biliary excreted drugs. J Pharm Sci **91:**1908–1914.
16. **McIntyre AR, Lipman NS.** 2007. Amoxicillin–clavulanic acid and trimethoprim– sulfamethoxazole in rodent feed and water: effects of compounding on antibiotic stability. J Am Assoc Lab Anim Sci **46:**26–32.
17. **Mordenti J.** 1986. Man versus beast: pharmacokinetic scaling in mammals. J Pharm Sci **75:**1028–1040.
18. **Plumb DC.** 2011. Plumb's veterinary drug handbook. Ames (IA): Wiley.
19. **Possidente B, Hegmann JP, Elder B, Carlson L.** 1980. Dissociation of circadian rhythms for food and water consumption in mice. Physiol Behav **25:**279–281.
20. **Prescott JF, Baggot JD, Walker RD.** 2000. Antimicrobial therapy in veterinary medicine. Ames (IA): Iowa State University Press.
21. **Riond JL, Riviere JE.** 1990. Allometric analysis of doxycycline pharmacokinetic parameters. J Vet Pharmacol Ther **13:**404–407.
22. **Riviere JE, Martin-Jimenez T, Sundlof SF, Craigmill AL.** 1997. Interspecies allometric analysis of the comparative pharmacokinetics of 44 drugs across veterinary and laboratory animal species. J Vet Pharmacol Ther **20:**453–463.
23. **Russell RJ, Haines DC, Anver MR, Battles JK, Gorelick PL, Blumenauer LL, Gonda MA, Ward JM.** 1995. Use of antibiotics to prevent hepatitis and typhlitis in male SCID mice spontaneously infected with *Helicobacter hepaticus*. Lab Anim Sci **45:**373–378.
24. **Rybak MJ.** 2006. Pharmacodynamics: relation to antimicrobial resistance. Am J Med 119:S37–44; discussion S62–70.
25. **Sader HS, Jones RN, Dowzicky MJ, Fritsche TR.** 2005. Antimicrobial activity of tigecycline tested against nosocomial bacterial pathogens from patients hospitalized in the intensive care unit. Diagn Microbiol Infect Dis **52:**203–208.
26. **Waites KB, Crabb DM, Duffy LB.** 2003. Inhibitory and bactericidal activities of gemifloxacin and other antimicrobials against *Mycoplasma pneumoniae*. Int J Antimicrob Agents **21:**574–577.
27. **Yu VL, Merigan TC, Barriere SL, editors.** 1999. Antimicrobial therapy and vaccines. Baltimore (MD): Williams and Wilkins.
28. **Zhu Z, Zheng T, Lee CG, Homer RJ, Elias JA.** 2002. Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. Semin Cell Dev Biol **13:**121–128.

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> Aubrey
>
> **AUBREY SCHOENLEBEN, PhD, CPIA**
> Scientific Liaison & Review Scientist
> Office of Animal Welfare

Health Sciences Building, Box 357160
1705 NE Pacific Street, Seattle, WA 98195-7160
vm: 206.685.6923 / fax: 206.616.5664
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<Outlook-1471462127.png>

FERPA
RCW 42.56.070(1)

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Monday, August 10, 2020 3:05 PM
To: Molly K. Lucas
Subject: Re: Additional Vet Questions for Protocol 4174-03

I know - these new ones have been surprisingly complicated lately! I think Zhang would be better to start with since it's the second round of revisions. It also sounds like [redacted] the grad student) will not be at UW for much longer due to visa + pandemic issues, so I know they are anxious to get started.

Aubrey

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Monday, August 10, 2020 2:58 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Subject: Re: Additional Vet Questions for Protocol 4174-03

Thanks for the heads up Aubrey.

Since Abuzeid and Zhang are both you and me, and are both in my Inbox, do you have an opinion on which I should do first? It sounds like Zhang might be faster since it's round two?

I (and you too it seems) have been busy with a lot of fairly complicated new protocols lately!

Molly

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Monday, August 10, 2020 2:02 PM
To: Molly K. Lucas <mklucas@uw.edu>
Subject: Fw: Additional Vet Questions for Protocol 4174-03

Hi Molly,

How are you? I (finally) received the revision for Dr. Zhang's protocol last week, and was able to finish looking through it this afternoon. Overall, I think it's looking good. They incorporated most (if not all) of your suggestions. I just sent the protocol back to you in HoverBoard. Attached are the additional questions that you sent via email, along with their responses. Let me know if you have additional follow-up for the group.

Thanks,
Aubrey

From: [redacted]
Sent: Tuesday, August 4, 2020 10:25 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: Additional Vet Questions for Protocol 4174-03

FERPA
RCW 42.56.070(1)

Hi Dr. Schoenleben,
Here are the responses to the questions you sent me by email. Thanks for your efforts and time to help us with the protocol! Hope the revised protocol is good enough to perform this time:-)
Cheers,

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年7月31日 18:12
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: Additional Vet Questions for Protocol 4174-03

Hi

Good question! I would recommend adding your response to each question in the document that I sent you, and then emailing that document back to me. Please also incorporate the response/any needed edits in to the protocol itself.

Hope this helps! Any other questions, let me know.

Cheers,
Aubrey

From:
Sent: Friday, July 31, 2020 5:04 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: Additional Vet Questions for Protocol 4174-03

Hi Dr. Schoenleben,
Thanks for your prompt respond! Your answers are very helpful.
You sent me the additional comments raised by the vets. But I am not sure where to attach these questions/suggestions and our replies in the IACUC system. Would you please send me an instruction on that? Thanks a lot!

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年7月31日 16:40
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: Additional Vet Questions for Protocol 4174-03

Hi

Its good to hear from you! I hope you've been doing well. In response to your questions:

- That sounds reasonable. I would recommend including that information in response to the vet question, and asking for alternate anesthesia options if ketamine/xylazine is not recommended for that procedure.
- Absolutely - the surgery training only needs to be completed prior to performing surgeries (not prior to approval of the protocol).

Take care,
Aubrey

From: [redacted]
Sent: Friday, July 31, 2020 11:45 AM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: Additional Vet Questions for Protocol 4174-03

FERPA
RCW 42.56.070(1)

Hi Dr. Schoenleben,
Hope you are doing well!
Thank you for all the support you provided. The vets helped me on the analgesic dosing, so I can answer most of the questions now, except for 2 issues:

1. We haven't found a micro CT that equiped with inhalation anesthesia apparatus. So we are not sure that we can use isoflurane at imaging procedures. As the vets didn't totally deny the ketamine/xylazine, can we still adopt this kind of anesthesia?
2. Because of the COVID pandemic and other ongoing study, it seems that I won't have enough time to accomplish the socket preservation surgery. I only have 2 months to be here, and we want to start on the blood collection experiment as soon as possible. The reviewer kindly reminded me to contact the training group, to reserve a vet's observation over my surgery procedure. In this case, can I only receive a training on blood collection, rather than the tooth extraction surgery?

Thank you very much!
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年6月27日 15:37
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: Additional Vet Questions for Protocol 4174-03

Hi [redacted]

Absolutely- the best email address for the vets is vsreview@uw.edu.

Hope you're having a good weekend,
Aubrey

Sent from my iPhone

On Jun 27, 2020, at 2:33 PM, [redacted] wrote:

Hi Dr. Schoenleben,
I am doing well, I hope you are doing good too.
Thank you for informing me of the update. I appreciate the suggestions from the vets. I have some questions about the dose of the recommended medicine. May I know how to contact the vets who gave the comments?
Thank you!
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年6月26日 17:51
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Additional Vet Questions for Protocol 4174-03

Hi [redacted]

How are you? The vets have a few additional questions/suggestions for your protocol. Since the protocol is currently in your court, we're not able to add these questions as Reviewer Notes in HoverBoard, so I am attaching them here. Please be sure to address these questions along with the 8 questions that the vets and I sent to you on 6/3/20. If you have any questions or if I can help with anything, let me know.

Take care,
Aubrey

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From: Daniel Eldridge <deldrid@uw.edu>
Sent: Wednesday, June 17, 2020 6:12 PM
To: Molly K. Lucas
Subject: Re: assignment for first protocol review on 6/18/20
Attachments: 6.18.20 Zhang protocol questions_DE.docx

Hey Molly,

Sorry, I was without internet until this evening. See attached.

Thanks,
Daniel

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Wednesday, June 17, 2020 11:53 AM
To: Daniel Eldridge <deldrid@uw.edu>; Jourdan E. Brune <jourdi@uw.edu>; Kristin Zabrecky <zabrecky@uw.edu>
Cc: Leandra Mosca <lmosca@uw.edu>
Subject: Re: assignment for first protocol review on 6/18/20

Just a reminder - questions due to me by 8pm.

Thanks,
Molly

From: Molly K. Lucas
Sent: Thursday, June 11, 2020 5:31 PM
To: Daniel Eldridge <deldrid@uw.edu>; Jourdan E. Brune <jourdi@uw.edu>; Kristin Zabrecky <zabrecky@uw.edu>
Cc: Leandra Mosca <lmosca@uw.edu>
Subject: assignment for first protocol review on 6/18/20

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than 8pm on Wednes 6/17 (earlier is fine!), so I have some time to look over what you came up with before we meet via Zoom on Thurs AM. Remember to address your questions/comments to the group (not to me). And please be prepared to discuss, it will be small and informal and I don't want to do all of the talking. If you can use video I think it would be nice.

Let me know if you have questions and have fun ☺
Molly

- Pg. 19 bioluminescent imaging: Is it possible to administer anesthesia with isoflurane during scans rather than injectable ketamine/xylazine?

- Pg. 22 Cyclosporin A: Would it be worth monitoring effective plasma cyclosporin levels in the rat to ensure you are reaching effective therapeutic levels?

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- Pg. 56 Micro CT imaging: Is it possible to administer anesthesia with isoflurane during CT scans rather than injectable ketamine/xylazine?

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Thursday, June 18, 2020 8:59 AM
To: Leandra Mosca
Subject: Re: assignment for first protocol review on 6/18/20

Hi again,

I was looking at your question re: whether the tie-2 agonist (I50) should be BSL-2. I don't think so. All the protocols go to the IBC (institutional biosafety committee) as well as the IACUC, and they are the ones who make those final decisions.

This is another institution's info, but I thought it was informative (esp the earlier slides):

https://www.uthsc.edu/research/safety/documents/principles-of-biosafety-bsl2-self-study.pdf

> Principles of Biosafety (BSL2 Training)
>
> Principles of Biosafety (BSL2 Training) Department of Research Safety September 2017. Objectives This course is intended to provide researchers with information necessary to comply with the requirements of the CDC Biosafety in Medical and Biological Laboratory
>
> www.uthsc.edu

Molly

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Wednesday, June 17, 2020 8:51 PM
To: Leandra Mosca <lmosca@uw.edu>
Subject: Re: assignment for first protocol review on 6/18/20

Hi Leandra,

I was thinking if we have time at the end of class we can go over what I've asked so far. As usual, you guys found some things I missed ☺

Or if we don't have time you can look at it on your own.

Molly

From: Leandra Mosca <lmosca@uw.edu>
Sent: Wednesday, June 17, 2020 3:00 PM
To: Molly K. Lucas <mklucas@uw.edu>
Subject: RE: assignment for first protocol review on 6/18/20

Hi Molly,

My comments are attached. Is there a way to see what your comments were once we've had class? I'm interested in reading them.

Thank you,
Leandra

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Sent: Wednesday, June 17, 2020 11:53 AM
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Subject: Re: assignment for first protocol review on 6/18/20

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Sent: Wednesday, June 17, 2020 3:00 PM
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Subject: RE: assignment for first protocol review on 6/18/20

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Thank you,
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Molly

From: Jourdan E. Brune <jourdi@uw.edu>
Sent: Wednesday, June 17, 2020 4:24 PM
To: Molly K. Lucas
Subject: Re: assignment for first protocol review on 6/18/20
Attachments: Jourdan_Zhang 6.18.20.docx

Hi Molly,

My homework is attached.

Best,
Jourdan

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Wednesday, June 17, 2020 11:53 AM
To: Daniel Eldridge <deldrid@uw.edu>; Jourdan E. Brune <jourdi@uw.edu>; Kristin Zabrecky <zabrecky@uw.edu>
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Molly

Jourdan Brune
Protocol Review
Assignment #1 Zhang 4173-03

General

1. Wei He and Hai Zhang are the only team members listed on this protocol but neither are certified to perform independent surgery in rodents. Until certified, they must perform surgery under the supervision of a veterinarian from Animal Use and Training or under the supervision of a team member certified in independent rodent surgery who will need to be added to the protocol. What are your plans for implementing supervision of surgery or adding a certified team member to the protocol?
2. Under animal use location, the protocol describes that the researcher may leave to get equipment essential for surgery. Please plan to move animals from their housing room to the surgery/procedure room after all supplies have been acquired and brought to the procedure room. In general, animals should not be left unattended in procedure rooms in the vivarium. This section is meant to describe animal procedure spaces within the PIs laboratory outside of the vivarium. If you intend to do all work in the vivarium, this section may not be relevant for your protocol.

Procedures

1. Cyclosporine is a potent immunosuppressant that poses a risk for toxicity at high doses. The regime in this protocol, cites a xenograft transplantation study which may necessitate robust immunosuppression. As this protocol proposes an allograft transplantation a tapered or reduced cyclosporine dosing protocol may be more appropriate for the studies proposed. Additionally, cyclosporine can be administered in the water and after an initial period of administration by injection, transitioning to oral administration by placing cyclosporine in the cage water bottle may improve animal welfare and reduce stress that could be associated with daily injections. Please reconsider the proposed administration route and dose/duration of cyclosporine administration based on this feedback.
2. In the 7-day post-operative monitoring period, will the rats be weighed daily or will only body condition scores be taken? Weighing the rats daily will provide a more objective measure of weight loss potentially related to discomfort from tooth extraction. Additionally, depending on if significant weight loss is seen, soft food may need to be offered for longer than 2 days.
3. I recommend that you plan to use a smaller gauge needle (20-25G) over the 15G needle described in your protocol. Additionally, if you enter the thorax with only the needle, without opening the chest, you are likely to get larger blood volumes by maintaining the negative pressure of the chest cavity.
4. Daily IP injections of antibiotics may cause additional stress to your rats in the post-operative period and negatively impact their recovery. Amoxicillin administration in the water would be a refinement and provide slightly enhanced antibacterial activity. Penicillin is mainly effective against only gram-positive aerobic organisms. Enrofloxacin is another antibiotic with even broader antibiotic activity than amoxicillin and it also penetrates bone well. I recommend either amoxicillin or enrofloxacin administered through the cage water bottle over penicillin given by IP injection.

5. Would the use of an opioid analgesic be contraindicated for your study? Dental extractions and surgery are considered to be category 3 (invasive, moderate to severe pain, longer duration expected) by the UW IACUC and multimodal analgesia is recommended. I recommend adding at least one dose of slow-release buprenorphine to your post-operative analgesics for the tooth extraction procedure. Additional doses may be necessary post-operative observations of rat if signs of discomfort and weight loss from reduced food intake are apparent.
6. Intraperitoneal injections should be performed with a 25G needle or smaller. (Larger number indicates smaller gauge)
7. Please double check the volume and units of bone mineralization matrix you plan to administer. 16 mL is likely a typo.
8. Please clarify for consistency where dental defects will be made. The CT imaging procedure describes a mandibular defect while maxillary extraction and defect are described elsewhere.
9. Bioluminescence imaging typically requires cells to be transfected with luciferase enzyme and for D-luciferin substrate to be administer to animals immediately prior to imaging. Please add D-luciferin as a substance administration to your protocol if you intend to use this. Please describe if all cells will be transfected to express luciferase enzyme or if cells used in Experiment 3 "Socket Preservation-Full Study" will not be transfected.
10. Bioluminescence imaging is also typically of short duration and isoflurane anesthesia is sufficient and available in IVIS machines. Please clarify if you have access to an IVIS machine with isoflurane anesthesia available as this is preferred over ketamine/xylazine anesthesia for this procedure.
11. Similarly, please clarify if you have access to a CT machine with isoflurane anesthesia available as this is preferred over ketamine/xylazine anesthesia for this short procedure.

Experiments

1. Please see above comment regarding cardiac blood draw technique. With the recommended technique, you can expect the blood volume of the draw to be approximately 3% of body weight and are likely to draw more than 4ml based on weight of the rat you intend to use. Avoid specifying the volume you intend to draw and simply state "blood will be collected from the heart; the rat will be euthanized by exsanguination".
2. In the total animal number justification for experiment 3, please edit your justification to reflect that the pilot study has not been conducted and that the effect size for the power justification is an estimation at this time.

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Subject: Re: assignment for first protocol review on 6/18/20
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- Pg. 56 Micro CT imaging: Is it possible to administer anesthesia with isoflurane during CT scans rather than injectable ketamine/xylazine?

From: Leandra Mosca <lmosca@uw.edu>
Sent: Friday, August 14, 2020 1:01 PM
To: Molly K. Lucas
Subject: RE: [dcmresidents] follow up on Hai Zhang protocol (from 6/18 protocol review class)

Hi Molly,

I agree with the sentiment below that any orally-administered antibiotic would be ideal especially since they'd also probably want to avoid any sort of oral gavaging after the procedure. There are formulations of Clavamox that are flavored and I can't imagine they're cost prohibitive since they're routinely dosed for small animals (cats/dogs).

I can't remember if we discussed this, but I was working with Nick for the new Liu pig protocol and something I saw in Swindle is doing a betadine rinse before any oral surgery. I see that they'll be scrubbing the site with 1% chlorhex but maybe for future oral surgeries we could discuss full-mouth rinses.

Thank you,
Leandra

From: dcmresidents <dcmresidents-bounces@mailman11.u.washington.edu> **On Behalf Of** Molly K. Lucas
Sent: Thursday, August 13, 2020 12:19 PM
To: dcmresidents@uw.edu
Subject: Re: [dcmresidents] follow up on Hai Zhang protocol (from 6/18 protocol review class)

Hi everyone,

I thought you all did a good job with your comments on the protocol Gerry gave us and it was a good discussion. Although I miss having class in person, I enjoyed our sessions this summer, and hopefully you did, too.

I haven't heard back from anyone yet re: the rat antibiotic question below. I really should respond with something by tomorrow at the latest, and although I have an idea or two, it would be great to know what you think as well.

Thanks!
Molly

From: Molly K. Lucas
Sent: Tuesday, August 11, 2020 5:06 PM
To: dcmresidents@uw.edu <dcmresidents@uw.edu>
Subject: follow up on Hai Zhang protocol (from 6/18 protocol review class)

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From: Daniel Eldridge <deldrid@uw.edu>
Sent: Thursday, August 13, 2020 1:36 PM
To: Molly K. Lucas; dcmresidents@uw.edu
Subject: Re: [dcmresidents] follow up on Hai Zhang protocol (from 6/18 protocol review class)

Molly thanks for much for putting the class together this Summer. I also enjoyed the sessions and topics covered.

I agree that avoiding IM injections would be ideal. I feel like amoxicillin is commonly administered in the drinking water. I've also heard I think before of people putting clavamox in the drinking water?

-Daniel

From: dcmresidents <dcmresidents-bounces@mailman11.u.washington.edu> on behalf of Molly K. Lucas <mklucas@uw.edu>
Sent: Thursday, August 13, 2020 12:18 PM
To: dcmresidents@uw.edu <dcmresidents@uw.edu>
Subject: Re: [dcmresidents] follow up on Hai Zhang protocol (from 6/18 protocol review class)

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To: Molly K. Lucas; Nicholas L. Reyes
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Umm don't really like PenG IM – ouch. I would argue that it may have good action on some anaerobes, but not all. It is not very broad spectrum. Generally for something in the mouth, we see gram negative as well as gram positive organisms in rats and recommend a broader class of drug.

Finally, IM injections would need strong justification in my opinion.

I don't know if the rats would be as forgiving with metronidazole in the water as mice. They are more taste sensitive and I just have no experience. There might be stuff in the literature though.

Thea

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Sent: Tuesday, August 11, 2020 5:06 PM
To: dcmresidents@uw.edu <dcmresidents@uw.edu>
Subject: follow up on Hai Zhang protocol (from 6/18 protocol review class)

Hi everyone,

I am just getting the responses back for this protocol that we discussed back in mid-June (rat maxillary tooth extraction). The group made many, but not all, of the modifications we suggested. Most comments are in Hoverboard but I also emailed a few follow-ups that Aubrey shared with the group after our discussion.

One thing I specifically wanted to run by you all for feedback on is the response about the antibiotics. That Q&A is pasted below.

I think their dose is too high if they stick with IM penicillin, but the primary question is - is there a better option we can suggest that does not involve IM injections for 5 days in a row? They have a point about anaerobes. The Carpenter formulary has an entry for Clavamox PO 20 mg/kg PO q 12 h for mice and rats. I don't have experience with Clavamox in rodents. There's also regular amoxicillin. Then there's metronidazole potentially in combo with something...

Please let me know what you think, you can reply all or just to me if you prefer. There's not a "right answer" I'm looking for, as I am mulling this over as well. They did take the suggestion to do 7 days of cyclosporine A injection followed by CsA water, based on the reference Jourdan found, so if we suggest a post-op water-based medication for 5 d postop we don't need to worry about mixing with another drug in the water.

Thanks,

Molly

https://hoverboard.washington.edu/Hoverboard/sd/Rooms/DisplayPages/LayoutInitial?Container=com.webridge.entity.Entity[OID[95829C76DE43184F9C017A96ED19BBB5]]

Veterinarian Change Request (response required)
Jump To: Experiments
Substance Administration: Zhang: Administration of Ampicillin (Team):

The title states ampicillin, but the text inside lists sodium penicillin G. These are related but different antibiotics. I appreciate your intention to reduce pain by administering the penicillin G IP rather than IM; however, unless there is a reference that recommends a specific dose/route/frequency for a given species, I don't recommend making changes to the route as different routes exhibit different pharmacokinetics/pharmacodynamics.

An alternative you may want to consider is administration of enrofloxacin (Baytril) in the drinking water. Baytril is a broad-spectrum antibiotic, and Baytril water is a relatively common way to administer post-op antibiotics to rats at UW and avoids the need for handling and injection.

We should be able to share a procedure that describes administration of Baytril water to rats if you are interested in switching to that option. Or if you specifically want to use a penicillin, I can provide some dosing information from laboratory animal formularies (generally IM or SC injections).

Please comment/edit as needed.

FERPA
RCW 42.56.070(1)

Change Request Completed - [redacted] - 8/4/2020 5:54 PM
Thanks for your advice. Certain proportion of oral infections are induced by anaerobic bacteria. However, enrofloxacin seems not as effective as penicillin G against anaerobic bacteria, so we think penicillin G may be a better choice for our study. Thanks for pointing out the mistake in the title and content of the procedures. We corrected the drug name as penicillin G sodium, and corrected the route into IM, as it was successfully applied by this route in our previous study.

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Friday, June 26, 2020 2:45 PM
To: Aubrey Schoenleben
Subject: Re: Hai Zhang new protocol
Attachments: HZhang_6_26_20.docx

Hi Aubrey,

Here are some additional questions for the vet review. If they need help with any of the vet questions, they can contact vsreview@uw.edu - the residents are familiar with the protocol.

Thanks,
Molly

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Friday, June 26, 2020 9:20 AM
To: Molly K. Lucas <mklucas@uw.edu>
Subject: Re: Hai Zhang new protocol

Sounds good!
Aubrey

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Friday, June 26, 2020 9:01 AM
To: Aubrey Schoenleben <aubreys@uw.edu>
Subject: Re: Hai Zhang new protocol

Hi Aubrey,

I will work on writing them up today (I need a little time to check a few things) and then email them to you. I think it would be easier for the group to deal with them all at once if possible.

Thanks,
Molly

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Thursday, June 25, 2020 4:36 PM
To: Molly K. Lucas <mklucas@uw.edu>
Subject: Re: Hai Zhang new protocol

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Thanks,
Molly

1. Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team): Since tooth extractions are known to be moderately painful, multimodal analgesia following this surgery is recommended unless scientifically contraindicated. The 72 hr meloxicam procedure that is already here is a good choice for one type of analgesic. Other types of analgesics that can be added on to the NSAID already in place are local anesthesia and an opioid:

-Local anesthesia (e.g., lidocaine/bupivacaine): Could be injected in a specific location as a maxillary nerve block (similar to what is done in human dentistry), or could be applied as a "splash block" (dripped onto the extraction site). The former is probably more technically challenging, and I'm not sure if there are any concerns about the latter interfering with the implant? If you are interested in pursuing the nerve block, vet services and AUTS could likely work with you to practice/develop the technique, and you can contact vsreview@uw.edu for more information/resources.

-Addition of an opioid at the time of surgery, so that the rats receive an opioid and an NSAID. There is a slow release form of buprenorphine for which one injection (as the animal is recovering from anesthesia when ket/xyl is used for anesthesia) lasts approx. 72 hr. There is a 48 hr standard procedure for buprenorphine that includes options for both the slow release formulation (one dose) and the regular formulation (dosed every 8-12 hr for 48 hr).

Please comment/edit as needed.

2. Imaging: Zhang: Micro CT Imaging (Team), Q #6: Re: the statement, "Within experiment assessment of bone formation in mandible defect." I believe this should be maxillary not mandibular?

Please comment/edit as needed.

3. Tissue/Blood Collection: Zhang: Intracardiac Blood Collection Under Anesthesia (Team): I recommend editing so that needle size and blood volume are not specified, to give you more flexibility. Another thing to consider is that you will likely be able to collect a larger volume if you enter the thorax with the needle only (not opening the chest first to access the heart) because the negative pressure of the chest cavity will be maintained.

Please comment/edit as needed.

4. Substance Administration: Zhang: Administration of Cyclosporine (Team): There are references supporting successful administration of cyclosporine A to rats in the drinking water. It would be a refinement to this procedure if cyclosporine could be administered via the water following an initial period of injections (e.g., perhaps something like 7 days of injections followed by water administration). Is this something that would work for your project? (E.g., see this reference, note that these rats received human cells so likely more immunosuppression was required compared to rats receiving rat cells. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3995133/).

Please comment/edit as needed.

5. Exp 02 and 03, Q #7: Re: the statement, "The surgical site will be monitored daily for 3 days post-surgery for bleeding, dislodgement of suture and any signs of infection such as redness, swelling and pus." I think it would be difficult to monitor the surgical site in this model (at minimum it would require restraint and manipulation to open the mouth, which would be stressful for the rat). I think it would be

acceptable to monitor weight and behavior as proxies for wound healing in the mouth since visualization of the surgical site would not be straightforward.

Please comment/edit as needed.

6. Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team):

-The surgery time is listed as up to 40 min. Ket/xyl anesthesia typically lasts 25-30 min, so you may need to re-dose rats once during surgery in order to maintain an appropriate anesthetic plane. Vet services often recommends re-dosing with half of the starting dose of ketamine only (no xylazine). Some groups use xylazine at half the starting dose or less (re-dosing xylazine carries a risk of marked respiratory depression that can sometimes lead to death under anesthesia). Please edit to indicate how K/X will be re-dosed, if it is necessary to re-dose based on monitoring of anesthetic depth.

-Q #3: Re: the statement, "…using a sterilized round bur and copious amount of sterilized saline for cooling." How will aspiration of the saline be prevented?

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Friday, June 26, 2020 9:01 AM
To: Aubrey Schoenleben
Subject: Re: Hai Zhang new protocol

Hi Aubrey,

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Subject: Re: Hai Zhang new protocol

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Thanks,
Molly

From: stephh26 <stephh26@uw.edu>
Sent: Thursday, June 25, 2020 4:26 PM
To: Aubrey Schoenleben
Subject: RE: Hai Zhang new protocol

Hi again Aubrey,

Just did a quick test and confirmed new questions are not available to study staff until the state change. Let me know how you would like for me to proceed.

Steph

From: stephh26
Sent: Thursday, June 25, 2020 4:20 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Subject: RE: Hai Zhang new protocol

Hi Aubrey,

Doing well! Hope you're keeping cool today. HoverBoard won't let me submit responses back to pre-review with the previous reviewer notes unanswered. We have a few options:

1. If you pass me the new reviewer notes, I can go ahead and enter them with an admin account. The group may not be able to see them until there's a state change though. So they will need to respond, submit responses, new notes will be vieawable, you can send them back.
    a. Occasionally, I've heard groups CAN see these notes if they go into the protocol, just not on the reviewer notes tab. I can do some tests real quick to confirm.
2. I could enter placeholder text into each unanswered reviewer note so they're answered, then submit. However, those reviewer notes will need to be recreated if they need to be answered again (So there would be duplicates).
3. You or I could copy all unanswered reviewer notes outside of HB, then I would delete all unanswered notes and Submit responses back to pre-review. We'd then have to enter the previous reviewer notes again.

I would recommend option 1 so we don't mess too much with existing notes.

Steph

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Thursday, June 25, 2020 4:03 PM
To: stephh26 <stephh26@uw.edu>
Subject: Fw: Hai Zhang new protocol

Hi Steph,

How are you? Could you please push the Zhang protocol back to pre-review? Molly recently reviewed this protocol with the residents and they have a few additional vet suggestions to add.

Thanks!

Aubrey

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Thanks,
Molly

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Friday, June 26, 2020 5:47 PM
To: Molly K. Lucas
Subject: Re: Hai Zhang new protocol

Thanks, Molly! I will pass these along to the group.

Have a great weekend,
Aubrey

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Friday, June 26, 2020 2:44 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Subject: Re: Hai Zhang new protocol

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Here are some additional questions for the vet review. If they need help with any of the vet questions, they can contact vsreview@uw.edu - the residents are familiar with the protocol.

Thanks,
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Thanks,
Molly

From: Emily W. Clark <ewilkins@uw.edu>
Sent: Tuesday, June 2, 2020 3:34 PM
To: Molly K. Lucas
Subject: RE: hoverboard question

Hi Molly,

Thanks for your message. I agree that the Printer Version is the way to go. When you click Print at the top, it should take you to a print pop-up with the option to “Save ad PDF.” I know this is what we do when we need to send protocols to sponsors, e.g., DoD. Not sure why you’re only seeing the option to save web pages. Maybe try it again, and if you’re still having trouble, I can see if it works for me. Just let me know!

Thanks again, and hope all is well!
Emily

From: Molly K. Lucas <mklucas@uw.edu>
Sent: Tuesday, June 2, 2020 3:07 PM
To: Emily W. Clark <ewilkins@uw.edu>
Subject: hoverboard question

Hi Emily,

I'm currently doing the vet review for a new protocol (Hai Zhang) that I think would be great for an upcoming protocol review class with the residents. But that isn't starting for a few weeks. What do you think is the best way to save it so I can use it in a month or so? E.g., I want a way for the residents to look at it, without any of my vet review comments OR the groups' edits/responses to them, i.e., so it would look to them like it does to me today.

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Molly

From: Emily W. Clark <ewilkins@uw.edu>
Sent: Tuesday, June 2, 2020 4:32 PM
To: Molly K. Lucas
Subject: Re: hoverboard question

Oh, good! Glad it worked for you. Actually, with the next HoverBoard upgrade in September, there will be "print packets" so you can more easily save sections of the protocol, e.g., procedures, experiments, etc. Maybe that will be more helpful for these types of situations.

Take care,
Emily

On Jun 2, 2020, at 3:44 PM, Molly K. Lucas <mklucas@uw.edu> wrote:

Thanks Emily. I just saved a pdf of the printer version. It worked for me like you described - I think I was having a senior moment and forgot that you hit "print" to save as a pdf rather than "save as." Also I am always wondering if Hoverboard will change in some way that will allow to save something in a different way, but that's probably wishful thinking and might defeat the purpose of having an up-to-date living document ☺

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FERPA
RCW 42.56.070(1)

From: Emily Patridge <ep001@uw.edu>
Sent: Thursday, May 7, 2020 12:18 PM
To: [redacted]
Cc: Aubrey Schoenleben; Hai Zhang
Subject: RE: 回复: Can you help me with the search strategy

Hello-

This is a great question! Diana Louden, dknl@uw.edu, can answer your question in detail since she created the Libuide and I recommend reaching out to her.

Emily Patridge, MLS, AHIP
Assistant Director of Clinical Research & Data Services (CRDS)
Project Co-lead of NNLM NEO
HSL Collections Lead
School of Dentistry Liaison Librarian

University of Washington's Health Sciences Library
ep001@uw.edu
206-221-3489

From: [redacted]
Sent: Thursday, May 7, 2020 12:03 PM
To: Emily Patridge <ep001@uw.edu>
Cc: Aubrey Schoenleben <aubreys@uw.edu>; Hai Zhang <haizhang@uw.edu>
Subject: 回复: Can you help me with the search strategy

Hi Emily,
Thank you for your kind help! I followed the instruction on this website to build up my strategy:
https://guides.lib.uw.edu/c.php?g=451064&p=3966559
It suggests to include the possible alternatives in my strategy, so I didn't write animal related terms. I think the purpose of this part in IACUC is to ensure that our animal experiments can not be replaced by any other non-animal research, and we are not going to repeat a study that has already been done. Is that means I need to conduct 2 different searches? One includes animal terms and one includes non-animal terms. Am I right?
Thank you!

General Search Tips - Animal Welfare & Laboratory Animal Alternatives (IACUC Searches) - Library Guides at University of Washington Libraries

Conducting literature searches required by the Animal Welfare Act and the Institutional Animal Care and Use Committee (IACUC). Identifying ways to reduce pain or distress in laboratory animals.

guides.lib.uw.edu

[redacted]

发件人: Emily Patridge <ep001@uw.edu>
发送时间: 2020年5月7日 10:44
[redacted]
主题: RE: Can you help me with the search strategy

It might help your search if you include animal experimentation, below is a search hedge for MEDLINE:

AND (("animal experimentation"[MeSH Terms] OR ("animal"[All Fields] AND "experimentation"[All Fields]) OR "animal experimentation"[All Fields] OR ("animal"[All Fields] AND "research"[All Fields]) OR "animal research"[All Fields]))

In your search you said “non-animal”, should you include rats?

Here is a thesaurus that may help your search -
https://pubs.nal.usda.gov/sites/pubs.nal.usda.gov/files/alternativeanimalusethesaurus.pdf

Emily Patridge, MLS, AHIP
Assistant Director of Clinical Research & Data Services (CRDS)
Project Co-lead of NNLM NEO
HSL Collections Lead
School of Dentistry Liaison Librarian

University of Washington’s Health Sciences Library
ep001@uw.edu
206-221-3489

From: [redacted]
Sent: Tuesday, May 5, 2020 4:54 PM
To: Emily Patridge <ep001@uw.edu>
Cc: Aubrey Schoenleben <aubreys@uw.edu>; Hai Zhang <haizhang@uw.edu>
Subject: Can you help me with the search strategy

Hi Emily,
I am a student in [redacted] and I am working on a IACUC protocol. Now I need to build up a search strategy for the Alternatives and Duplication Searches part. Would you please help me with it? I have a procedure that may cause pain or distress (Survival Surgery: Zhang: Rat Tooth Extraction and Implantation, ver. 1 (Team)), and need to conduct a research to find out whether there are previous duplicates or alternatives. This procedure includes tooth extraction in rats and filling the tooth sockets with riPSCs, magnesium chloride or I50 (a newly engineered compound). I write the search strategy like this: ((tooth OR teeth OR dental OR incisor OR *molar) AND (extract* OR remov*) AND (implant* OR transplant* OR fill*) AND (model* OR assay*)) AND ((simulat* OR silico OR artificial* OR digital* OR virtual* OR cadaver* OR "non animal" OR "in vitro")). When I set out a search on Web of Science, it yields 593 results. I think that's too many! Do I need to add all the reagents and cell line into my strategy? And how?
Thank you!
[redacted]

From: Selesteen Jimenez
To: Hai Zhang; [redacted]
Cc: stephh26
Subject: RE: Action Required for Training #4174-03
Date: Monday, March 2, 2020 2:00:00 PM

FERPA
RCW 42.56.070(1)

Hello Dr. Zhang,

We appreciate the training update, please note that as the P.I. on the protocol UW policy requires that you complete all online courses in reference to your project. In your case please complete the Rats Online Course only.

For future reference please see our Policies and Exemptions website.

Best,
Selesteen

Selesteen Jimenez
IACUC Program Coordinator
Box 357160
(206) 616-7486
sjimenez@uw.edu
http://depts.washington.edu/oawhome/

NOTE: Pre-review of all protocol submissions is now *required*. More information at https://uwnetid.sharepoint.com/sites/OAWRSS/OAWRSSWebsite

Into the Future ... Explore UW's eIACUC Solution

From: Hai Zhang <haizhang@uw.edu>
Sent: Monday, March 2, 2020 12:19 PM
To: Selesteen Jimenez <sjimenez@uw.edu>; [redacted]
Cc: stephh26 <stephh26@uw.edu>
Subject: RE: Action Required for Training #4174-03

Hi Selesteen,
Thank you very much for your kind suggestion. To make the process move faster, we decided to take my name off from the surgery procedure but I will still supervise the project. [redacted] will attend the hands on training first. If [redacted] needs assistance in the procedure, I (or another student) will attend the training.
Please let us know if that would satisfy the requirement.

Thanks.

Hai Zhang, DMD, PhD.
Associate Professor
Vice Chair, Department of Restorative Dentistry
Assistant Director, Graduate Prosthodontics
School of Dentistry
University of Washington
1959 NE Pacific St. Box 357456 Seattle, WA 98195-7456
Tel: 206.543.5948, Fax: 206.543.7783
Email: haizhang@uw.edu

From: Selesteen Jimenez <sjimenez@uw.edu>
Sent: Monday, March 2, 2020 8:25 AM
To: [redacted]; Hai Zhang <haizhang@uw.edu>
Cc: stephh26 <stephh26@uw.edu>
Subject: Action Required for Training #4174-03

FERPA
RCW 42.56.070(1)

Dear Dr. Zhang,

I'm previewing the new project, "Magnesium Stem Cell" IACUC #4174-03. I noticed that your training requirements are incomplete. In your case as the protocol PI, we require that your training be completed prior to approval, or we will have to **hold** the approval of your new project. Please refer to the instructions provided below.

- Dr. Zhang needs to complete Rat Hands-on Laboratory. Click here for course schedules and registration.
- [redacted] needs to complete Rat Hands-on Laboratory. Click here for course schedules and registration.

Please feel free to contact me if you have any questions regarding this process.

Best,
Selesteen

Selesteen Jimenez
IACUC Program Coordinator
Box 357160
(206) 616-7486
sjimenez@uw.edu

http://depts.washington.edu/oawhome/

NOTE: Pre-review of all protocol submissions is now *required*. More information at https://uwnetid.sharepoint.com/sites/OAWRSS/OAWRSSWebsite

Into the Future … Explore UW's eIACUC Solution

UNIVERSITY of WASHINGTON

APPROVAL OF NEW PROTOCOL SUBMISSION

September 23, 2020

Dear Dr. Zhang,

This email serves as written notice of animal use approval by the Institutional Animal Care and Use Committee (IACUC).

*To help us better serve you, please take this **3 question survey** about your experience with the review process.*

Type of Review:	Designated Member Review
Short Title of Protocol:	4174-03: Magnesium Stem Cell
Investigator:	Hai Zhang
HoverBoard ID:	PROTO202000003

Please note the approval and expiration date listed. All animal use protocols that include USDA regulated species or receive support from the Department of Defense must be renewed annually from the date of IACUC approval, independent of project or funding dates. Please refer to the assigned protocol number for all animal orders and future correspondence with the IACUC.

Protocol Approval Dates: 9/23/2020 to 9/22/2023

Next Annual Expiration Date: N/A

Next Triennial Expiration Date: 9/22/2023

If you have any questions, contact OAWRSS at oawrss@uw.edu.

Sincerely,

Office of Animal Welfare

OFFICE OF ANIMAL WELFARE

Zhang Protocol 4174-03

Zhang 4174-03 Dosages for amoxicillin and SR buprenorphine injection

[redacted]

Sun 9/13/2020 10:37 PM

FERPA
RCW 42.56.070(1)

To:

- VET SERVICES PROTOCOL REVIEW

Cc:

- Hai Zhang

Hi Dr. Ellis,
Thanks for your help! I have modified our protocol accordingly. We appreciate your efforts to make our protocol better!
Best regards,
[redacted]

发件人: VET SERVICES PROTOCOL REVIEW <vsreview@uw.edu>
发送时间: 2020年9月8日 10:58
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>; VET SERVICES PROTOCOL REVIEW <vsreview@uw.edu>
主题: RE: Zhang 4174-03 Dosages for amoxicillin and SR buprenorphine injection

Hi [redacted]

Rats will often readily take antibiotic orally by syringe. Many compounding pharmacies provide flavored Baytril (enrofloxacin) in palatable flavors, such as fruit flavors.

Amoxicillin can be given IM or SQ at a dose of 150mg/kg.

The dose of SR buprenorphine is 1-1.2 mg/kg, given subcutaneously.

Best,
Megan Ellis, DVM
Veterinary Resident

FERPA
RCW 42.56.070(1)

From: [redacted]
Sent: Monday, September 7, 2020 10:38 AM
To: VET SERVICES PROTOCOL REVIEW <vsreview@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: Dosages for amoxicillin and SR buprenorphine injection

Dear doctor,
Thank you for your detailed and helpful comments on our protocol "4174-03: Magnesium Stem Cell"!
I have a question about the first comment: will the rats voluntarily take the medicine given orally by syringe? And I also doubt if the rats will take adequate Baytril in water, because the rats will experience pain in mouth for the first 3 days post-operation. If I choose giving amoxicillin injection, could you please provide dosage of that?
Also, could you provide me the dosage of SR buprenorphine as you mentioned in the second comment?
Thank you very much!
[redacted]

Exp 2, Q #7: I agree that since the surgery is in the oral cavity, body weight and body condition are important things to monitor post-op (and they are included). Just to be sure I understand, is the plan to weigh the rats every day for 7 days post-op, and then 3x/week until euthanasia?

Please comment/edit as needed.

Imaging: Zhang: Bioluminescence Imaging (Team): Isoflurane is typically used for short anesthetic sessions in the IVIS machine. Was ketamine/xylazine chosen because administration of isoflurane would interfere with imaging of the mouth, or could isoflurane be used? If isoflurane can be used, I recommend it over K/X as the rats recover from it more quickly, and thus more quickly resume normal activities such as eating and drinking.

The same question applies to Imaging: Zhang: Micro CT Imaging (Team)

Please comment/edit as needed.

Substance Administration: Zhang: Administration of Ampicillin (Team): The title states ampicillin, but the text inside lists sodium penicillin G. These are related but different antibiotics. I appreciate your intention to reduce pain by administering the penicillin G IP rather than IM; however, unless there is a reference that recommends a specific dose/route/frequency for a given species, I don't recommend making changes to the route as different routes exhibit different pharmacokinetics/pharmacodynamics.

An alternative you may want to consider is administration of enrofloxacin (Baytril) in the drinking water. Baytril is a broad-spectrum antibiotic, and Baytril water is a relatively common way to administer post-op antibiotics to rats at UW and avoids the need for handling and injection.

We should be able to share a procedure that describes administration of Baytril water to rats if you are interested in switching to that option. Or if you specifically want to use a penicillin, I can provide some dosing information from laboratory animal formularies (generally IM or SC injections).

Please comment/edit as needed.

The surgery procedure contains the following statement, "If prominent reduction of iPOB longevity is proved in the pilot study, immunosuppressant (10mg/kg cyclosporine A daily) will be administered in iPOB-transplanted groups in formal experiment beginning three days before grafting and continuously until sacrifice." The reference that the proposed immunosuppression regimen is based upon involved administration of human cells to rats, while this study involves administration of rat cells. I am guessing that rejection will be less likely with rat cells.

-Are the riPOBs derived from Sprague Dawley rats? Matching the cell/donor strain to the recipient strain typically reduces the likelihood of rejection.

-You may want to consider including ranges for the cyclosporine dose (e.g., 5-10 mg/kg/day) and duration (e.g., 10 days- 6 weeks) to increase flexibility. For example, I am aware of one study involving administration of human cells to immunosuppressed rats, and rats are treated with 5 mg/kg/day CsA for 10 days post-transplant. Once you know what is required for this study (if immunosuppression is required), you could then make it more specific.

Please comment/edit as needed.

Experiment 2: I think you may need to add a Substance Administration procedure for administration of luciferin-D to rats prior to bioluminescent imaging?

Please comment/edit as needed.

Survival Surgery: Zhang: Rat Tooth Extraction and Implantation (Team), Q #3: Re: the statement, "Soft food will be provided during this period." - Offering soft food sounds like a very good idea following this surgery. In addition to regular moistened chow, soft dietary supplement (e.g., Nutrical), and/or meat-flavored baby food post-op, can be used in rats to support nutrition.

Please comment/edit as needed.

From: [redacted]
Sent: Wednesday, September 23, 2020 11:07 AM
To: Aubrey Schoenleben
Cc: Hai Zhang
Subject: 回复: Protocol Update

FERPA
RCW 42.56.070(1)

Hi Dr. Schoenleben,
Thank you very much for letting me know this great news! It is a precious experience working with you- I learnt a lot from you and the IACUC group, and I really enjoyed the process! I will try to explore another opportunity to work on this project.
Your new baby is coming soon, a happy while challenging time is waiting for you. Please take care and wish you all the best!😊
Sincerely,
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年9月23日 9:48
收件人: Hai Zhang <haizhang@uw.edu>
[redacted]
主题: Re: Protocol Update

You're welcome, Hai! The protocol is approved for three years. If [redacted] can come back and work on the project, there might be some minor training to update, but otherwise, [redacted] should be good to go to start on things. Alternately, if another student/scholar will be working on the project, we'll just need to add him/her to the protocol before they can start animal work. Please feel free to reach out once you're ready to get started and we can double check that everything is in order.

[redacted]- So sorry to hear that you'll have to leave for your home university so soon! I've really enjoyed working with you these past few months. Safe travels!

Take care,
Aubrey

From: Hai Zhang <haizhang@uw.edu>
Sent: Wednesday, September 23, 2020 9:08 AM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: [redacted]
Subject: RE: Protocol Update

Hi Aubrey,
Thank you so much for the good news and great support during the process! I've also received an approval email from the IACUC in this morning. We don't need any additional letter for the funding purpose. Thank you for asking though.
Unfortunately [redacted] will have to leave for [redacted] home university in next week and I will have to put this project on hold till [redacted] comes back or I have another visiting student/scholar coming in the future. It might take half to one year given the current COVID situation. What should I do with this situation?
Thanks again?

-Hai

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Wednesday, September 23, 2020 8:58 AM
To: [redacted] Hai Zhang <haizhang@uw.edu>
Subject: Protocol Update

FERPA
RCW 42.56.070(1)

Good morning [redacted] and Hai,

How are you? Just a quick note to let you know that I was able to approve your new protocol this morning! Am I remembering correctly that the protocol is associated with internal funding? If so, were there any forms/approval letters needed to confirm IACUC approval?

Thank you again for all your hard work,
Aubrey

AUBREY SCHOENLEBEN, PhD, CPIA
Scientific Liaison & Review Scientist
Office of Animal Welfare

Health Sciences Building, Box 357160
1705 NE Pacific Street, Seattle, WA 98195-7160
vm: 206.685.6923 / fax: 206.616.5664
aubreys@uw.edu / oaw.washington.edu

OFFICE OF ANIMAL WELFARE
Research Support Services

FERPA
RCW 42.56.070(1)

From: [redacted]
Sent: Monday, May 11, 2020 3:00 AM
To: Diana Louden
Cc: Hai Zhang; Aubrey Schoenleben
Subject: 回复: Would you please help me with the search strategy

Hi Dr. Louden,
Thanks for your suggestion. It is very helpful! This search strategy can ease my workload to a large extent. I will try this first. Thank you!
Best wishes,
[redacted]

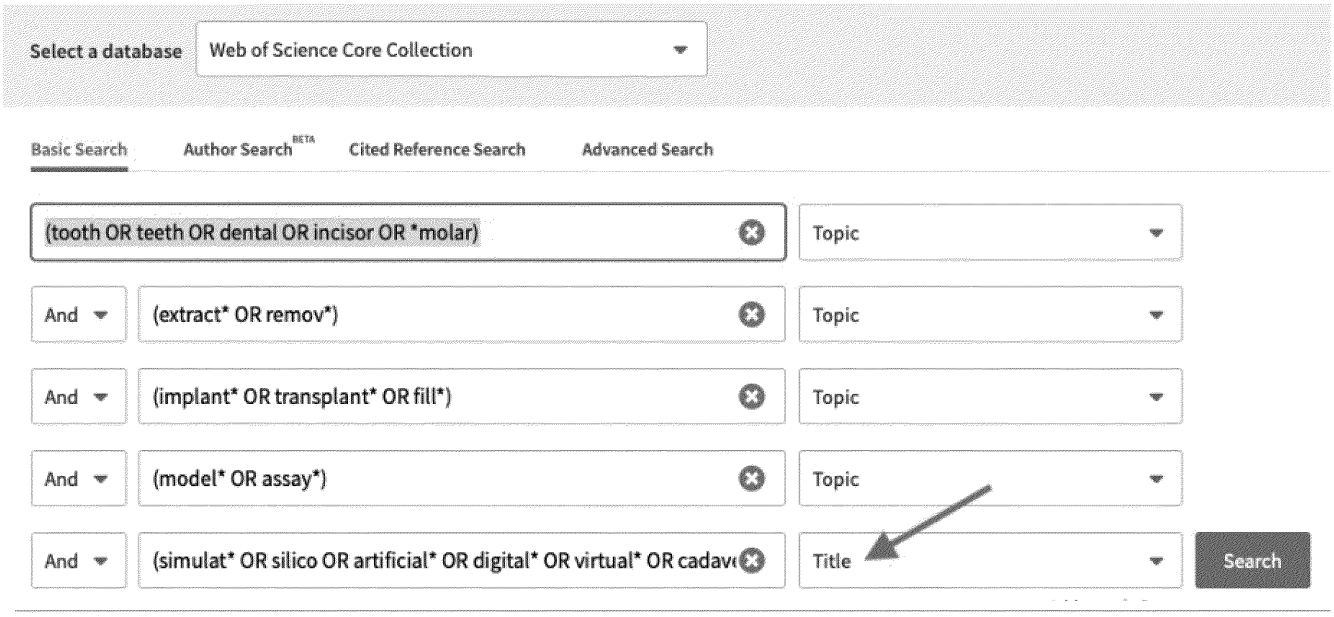
发件人: Diana Louden <dknl@uw.edu>
发送时间: 2020年5月9日 22:49
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>; Aubrey Schoenleben <aubreys@uw.edu>
主题: Re: Would you please help me with the search strategy

Dear [redacted]

I meant to mention this last night, so I wanted to send a quick follow-up email.

The search strategy you came up looks good! Because the focus of the search is to try to find articles describing non-animal models, you could consider searching for those words in the title. (I'm attaching a screen shot below.). That reduces the number of references to 151. If it's a protocol renewal, you could also limit the search to documents that have published since the time the protocol was first developed.

Best wishes,
Diana



From: Diana Louden <dknl@uw.edu>
Sent: Friday, May 8, 2020 11:06 PM
To: [redacted]
Cc: Hai Zhang <haizhang@uw.edu>; Aubrey Schoenleben <aubreys@uw.edu>
Subject: Re: Would you please help me with the search strategy

Dear [redacted]

Thank you for writing. I'll send some initial thoughts, but then I'm happy to help you refine your search strategies if you'd like. I'm afraid I can't access your protocol on Hoverboard, but you could send me a copy if you'd like.

You're exactly right about the approach. You want to search for studies like the one you're doing in rats, and you want to do a separate search to see if there are any reasonable alternatives to the animal studies. From what I learned from instructors from the Animal Welfare Information Center, it's best if you address the different questions separately. When you do more focused searches, it's easier to identify useful references.

Ideally, you'd search for:
1) papers describing the type of study you're planning in rats (pluripotent stem cells implanted in teeth, etc.) - to see if anyone has done what you're proposing or if anyone has described improvements in the model you're using.
2) papers describing ways of reducing pain or distress in tooth extraction or implantation in rats/rodents - whether that has to do with restraints or anesthetic agents, for example.

3) papers describing alternative in vitro, non-mammalian, or computer simulated methods of studying the processes you're studying by implanting materials in teeth.

Please let me know if you'd like any assistance refining your search strategies.

Best wishes,
Diana

Diana Nelson Louden
Scientific Research Librarian
Allen North Library, Room 281D
University of Washington
dknl@uw.edu | 206.685.8734
https://guides.lib.uw.edu/research/diana

FERPA
RCW 42.56.070(1)

From: [redacted]
Sent: Thursday, May 7, 2020 6:41 PM
To: Diana Louden <dknl@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>; Aubrey Schoenleben <aubreys@uw.edu>
Subject: Would you please help me with the search strategy

Hello Dr. Louden,
I am a student in [redacted] and I am working on a IACUC protocol. I encountered a problem when I try to complete the Alternatives and Duplication Searches part. I resorted to Emily Patridge first, and she came up with a search strategy that is different with the instruction in this website: https://guides.lib.uw.edu/c.php?g=451064&p=3966559. Then she suggest me to ask you for help. The details of my questions are in the following forwarded emails. Would you please help me to decide how to build up a proper search strategy for my procedure ((Survival Surgery: Zhang: Rat Tooth Extraction and Implantation, ver. 1 (Team)))? Thank you very much!
Best wishes,
-Wei He

> General Search Tips - Animal Welfare & Laboratory Animal Alternatives (IACUC Searches) - Library Guides at University of Washington Libraries
>
> Conducting literature searches required by the Animal Welfare Act and the Institutional Animal Care and Use Committee (IACUC). Identifying ways to reduce pain or distress in laboratory animals.
>
> guides.lib.uw.edu

发件人: Emily Patridge <ep001@uw.edu>
发送时间: 2020年5月7日 12:18
[redacted]uw.edu>
抄送: Aubrey Schoenleben <aubreys@uw.edu>; Hai Zhang <haizhang@uw.edu>
主题: RE: 回复: Can you help me with the search strategy

Hello-

This is a great question! Diana Louden, dknl@uw.edu, can answer your question in detail since she created the Libuide and I recommend reaching out to her.

Emily Patridge, MLS, AHIP
Assistant Director of Clinical Research & Data Services (CRDS)
Project Co-lead of NNLM NEO
HSL Collections Lead
School of Dentistry Liaison Librarian

University of Washington's Health Sciences Library
ep001@uw.edu
206-221-3489

From: [redacted]uw.edu>
Sent: Thursday, May 7, 2020 12:03 PM
To: Emily Patridge <ep001@uw.edu>
Cc: Aubrey Schoenleben <aubreys@uw.edu>; Hai Zhang <haizhang@uw.edu>
Subject: 回复: Can you help me with the search strategy

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Thank you!

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guides.lib.uw.edu

FERPA

RCW 42.56.070(1)

[redacted]

发件人: Emily Patridge <ep001@uw.edu>
发送时间: 2020年5月7日 10:44
[redacted]
主题: RE: Can you help me with the search strategy

It might help your search if you include animal experimentation, below is a search hedge for MEDLINE:

AND (("animal experimentation"[MeSH Terms] OR ("animal"[All Fields] AND "experimentation"[All Fields]) OR "animal experimentation"[All Fields] OR ("animal"[All Fields] AND "research"[All Fields]) OR "animal research"[All Fields]))

In your search you said “non-animal”, should you include rats?

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Emily Patridge, MLS, AHIP
Assistant Director of Clinical Research & Data Services (CRDS)
Project Co-lead of NNLM NEO
HSL Collections Lead
School of Dentistry Liaison Librarian

University of Washington’s Health Sciences Library
ep001@uw.edu
206-221-3489

From: [redacted]
Sent: Tuesday, May 5, 2020 4:54 PM
To: Emily Patridge <ep001@uw.edu>
Cc: Aubrey Schoenleben <aubreys@uw.edu>; Hai Zhang <haizhang@uw.edu>
Subject: Can you help me with the search strategy

Hi Emily,
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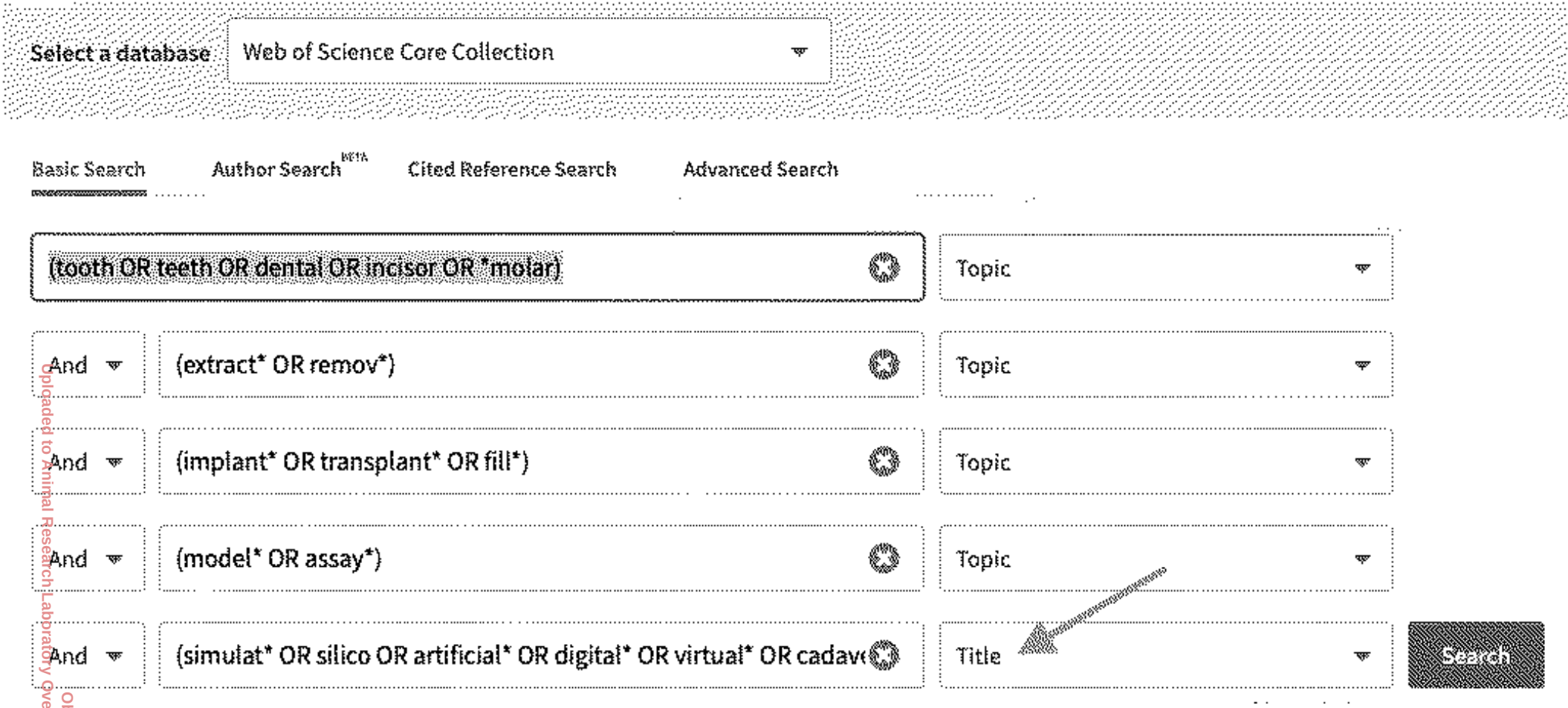
I have a procedure that may cause pain or distress (Survival Surgery: Zhang: Rat Tooth Extraction and Implantation, ver. 1 (Team)), and need to conduct a research to find out whether there are previous duplicates or alternatives. This procedure includes tooth extraction in rats and filling the tooth sockets with riPSCs, magnesium chloride or I50 (a newly engineered compound). I write the search strategy like this: ((tooth OR teeth OR dental OR incisor OR *molar) AND (extract* OR remov*) AND (implant* OR transplant* OR fill*) AND (model* OR assay*)) AND ((simulat* OR silico OR artificial* OR digital* OR virtual* OR cadaver* OR "non animal" OR "in vitro")). When I set out a search on Web of Science, it yields 593 results. I think that's too many! Do I need to add all the reagents and cell line into my strategy? And how?
Thank you!
[redacted]



From: [redacted]
Sent: Friday, February 28, 2020 2:02 PM
To: Aubrey Schoenleben
Cc: Hai Zhang
Subject: 回复: 回复: 回复: 回复: 回复: 回复: Three Year Renewal

FERPA
RCW 42.56.070(1)

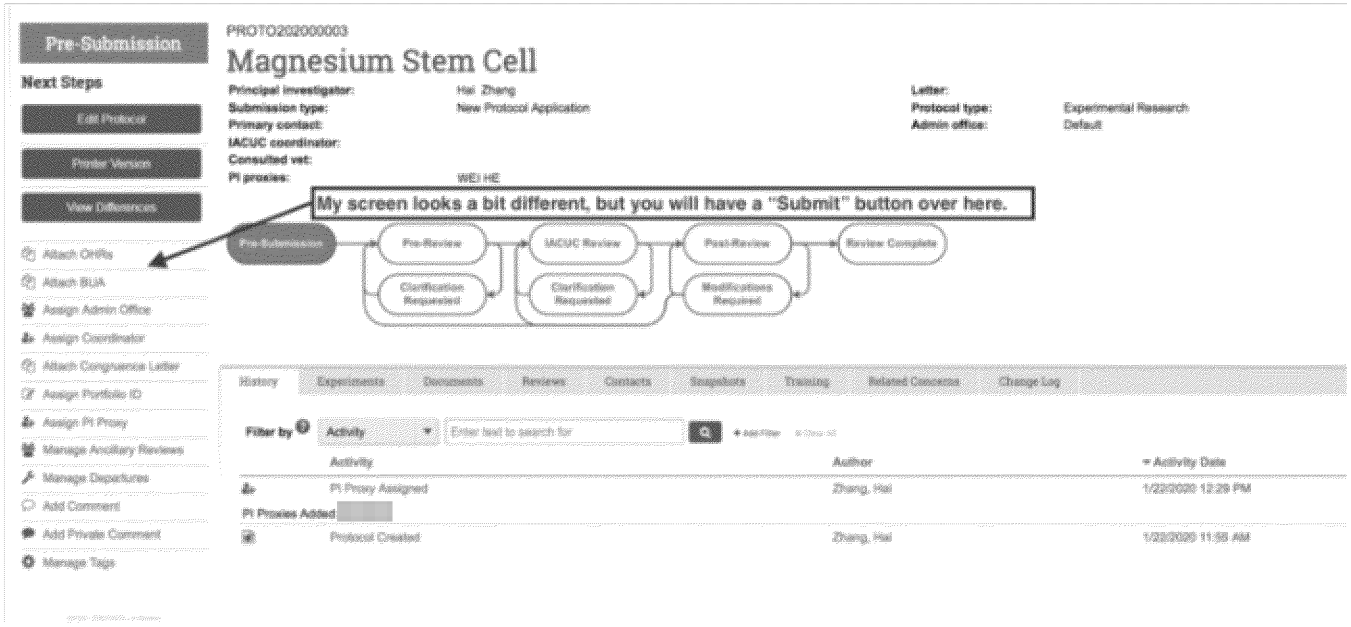
Hello Dr. Schoenleben:
Thanks for your direction. I have submitted the protocol, and hopefully you can see it now. In the tooth extraction procedure, regarding the analgesia I just selected the template of meloxicam. But I am not sure if it is the most suitable one. Can you give us some advice about pain control template? Thank you!
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年2月28日 12:51
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: 回复: 回复: 回复: 回复: 回复: Three Year Renewal

Thanks for the update, [redacted] and no need to apologize for the questions - that's what I'm here for! I'd be happy to provide feedback on the protocol. To do so, please submit the protocol so that it moves in to the "pre-review" state. Once in pre-review, I can then review the protocol and add questions/comments. The submit button is on the left hand side of the protocol workspace (see screenshot).

With regards to the Sendai virus, please reach out to our Environmental Health and Safety (EH&S) group. They can provide guidance on working with viruses in vitro/in vivo, and what may/may not be allowed on campus. The best email for them is ehsbio@uw.edu. Regarding safety testing of cells that will be transplanted in to animals, the Rodent Health Monitoring Program should be able to provide recommendations (rhmp@uw.edu).

Cheers,
Aubrey



On 2/27/20, 1:03 PM, [redacted] > wrote:

Hello Dr. Schoenleben:

We filled in the forms online. Can you please go through them and find out whether they meet the requirement? Still, there may be some details to be modified, for example, we are planning to use the Sendai Virus to reprogram the cells which will be transplanted into the rat tooth socket. The manufacturer has provide methods to remove the virus, but we haven't tried that right now. Will this kind of virus be strictly forbidden in the rodent experiment? How do you determine the safety of the biologicals applied to animals?

Sorry for so many questions. Thank you very much!

[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年2月20日 18:38
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: 回复: 回复: 回复: 回复: Three Year Renewal

FERPA
RCW 42.56.070(1)

You're welcome! Have a great evening!
Aubrey

From: [redacted]
Sent: Thursday, February 20, 2020 6:06 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: 回复: 回复: 回复: Three Year Renewal

OK! I will fill in like this. Thanks for your help Dr. Schoenleben!

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年2月20日 11:50
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: 回复: 回复: 回复: Three Year Renewal

You're welcome, [redacted] Yes, you can list the same location for animal use.
Aubrey

On 2/19/20, 4:23 PM, "weihe87" <weihe87@uw.edu> wrote:

Hello Dr. Schoenleben:
Thanks for your response. Can I list the same place for animal use? Thank you!

[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年2月19日 16:02
[redacted]
主题: Re: 回复: 回复: Three Year Renewal

Hi [redacted]

I think its okay to list the ARCF/Foege facility on the protocol for now (note: in HoverBoard, ARCF and Foege are considered a single vivarium, so select ARCF ABSL1 (and/or ABSL2 as appropriate); no need to list the specific room number). While the protocol is under review, reach out to James Mendoza (amespodi@uw.edu), the facility

supervisor for the ARCF, to discuss your housing needs. James can work with you to determine if there is available housing space in the ARCF or if we need to look at another facility.

Don't hesitate to get in touch with any other questions.

Thanks,
Aubrey

From: [redacted]
Sent: Wednesday, February 19, 2020 2:48 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: 回复: Three Year Renewal

FERPA
RCW 42.56.070(1)

Hello Dr. Schoenleben:
Thanks for your help with the animal protocol. We don't know how to fill in the Animal Housing and Use part. Do we need to discuss with Dr. James Mendoza to determine which room to use?
Thank you!
[redacted]

发件人: Hai Zhang <haizhang@uw.edu>
发送时间: 2020年1月28日 12:55
收件人: Aubrey Schoenleben <aubreys@uw.edu>; [redacted]>
主题: RE: 回复: Three Year Renewal

Thanks Aubrey for your detailed advice. We will get those started asap.
-Hai

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Tuesday, January 28, 2020 11:26 AM
To: [redacted]>; Hai Zhang <haizhang@uw.edu>
Subject: Re: 回复: Three Year Renewal

Hi Hai and [redacted]

It was really good meeting with you last week! Following up on a couple of items from our conversation:

- Here are links to the basic training courses that you will need to complete:
  - UW Animal Use Laws and Regulations. Click here to take the course.
  - Animal Use Medical Screening (AUMS). Click here for AUMS Steps and FAQs, and electronic enrollment.

- Since you will be working with rats, you will also be required to take the online "Working with Rats at UW" course and the related hands-on lab. You can find a schedule of upcoming rat classes and the registration form on the AUTS website (https://depts.washington.edu/auts/).

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- The Department of Comparative Medicine also recently implemented a new animal operations system (AOps) that is used for ordering, billing, special services requests, etc. I would strongly recommend signing up for one of the AOps

training courses as well. You can find more information about the new system and upcoming courses here.

Hope this helps! Please let me know if you have any other questions.

Cheers,
Aubrey

On 1/21/20, 5:24 PM, [redacted] wrote:

Hello Dr. Zhang and Dr. Schoenleben:
I am OK for the meeting time. Thank you!
[redacted]

FERPA
RCW 42.56.070(1)

发件人: Hai Zhang <haizhang@uw.edu>
发送时间: 2020年1月21日 17:16
收件人: Aubrey Schoenleben <aubreys@uw.edu>
[redacted]
主题: RE: Three Year Renewal

Hi Aubrey,
How about tomorrow at 11am in D751?
Thanks for your help!
-Hai

Hai Zhang, DMD, PhD.
Associate Professor
Assistant Director, Graduate Prosthodontics
Vice Chair, Department of Restorative Dentistry
School of Dentistry
University of Washington
1959 NE Pacific St. Box 357456 Seattle, WA 98195-7456
Tel: 206.543.5948, Fax: 206.543.7783
Email: haizhang@uw.edu

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Tuesday, January 21, 2020 2:28 PM
To: Hai Zhang <haizhang@uw.edu>
Cc: [redacted]
Subject: Re: Three Year Renewal

Let's try for tomorrow (1/22) or 1/29 – both days I am free any time after 11am. What time would work best for the two of you? Also I'm happy to come to your neck of the woods. Could you remind me where you are located?
Cheers,
Aubrey

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Thanks Aubrey. We are available to meet on 1/22 (Wed) am, 1/27 (Mon) pm and 1/29 (Wed) am. Please let us know your earliest available time.

Thank you and have a great weekend!~
-Hai

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Tel: 206.543.5948, Fax: 206.543.7783
Email: haizhang@uw.edu

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Friday, January 17, 2020 5:18 PM
To: Hai Zhang <haizhang@uw.edu>
Cc: [redacted]
Subject: Re: Three Year Renewal

FERPA
RCW 42.56.070(1)

Hi Hai,

I do still work in OAW – it's good to hear from you! I would be happy to help you and [redacted] get an animal protocol started. Since it's been almost three years since we closed your last protocol, it would be best to just start a new one. We now manage all of our protocols electronically through a system called HoverBoard. I would recommend that we meet so that I can give you (and [redacted] an introduction to the new system. Do you have availability over the next week or two?

With regards to training, it looks like your basic training (UW Animal Use Laws & Regulations and AUMS clearance) is up to date. Since you will be working with rats, you will need to take the online rat course and the rat hands-on class. You can find the online course and a schedule of upcoming hands-on classes here. It looks like you will also need to update your facility orientation/access. We can update that once we've sorted out where your animals will be housed.

Have a lovely weekend,
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[redacted] will work with you to get the paperwork started. I have a few trainings that I need to do.
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Associate Professor
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Tel: 206.543.5948, Fax: 206.543.7783
Email: haizhang@uw.edu

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Monday, June 12, 2017 12:05 PM
To: Hai Zhang <haizhang@uw.edu>
Subject: Re: Three Year Renewal

Hi Hai,

Thanks for the quick reply! We can archive the protocol since you don't plan to renew it - your email below is all that we need.

Take care,
Aubrey

From: Hai Zhang <haizhang@uw.edu>
Date: Friday, June 9, 2017 at 5:06 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Subject: RE: Three Year Renewal

Hi Aubrey,
I am not planning to renew that project any more. What should I do? Thank you!

Hai Zhang, DMD, PhD.
Associate Professor
Assistant Director, Graduate Prosthodontics
Department of Restorative Dentistry
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Tel: 206.543.5948, Fax: 206.543.7783
Email: haizhang@uw.edu

From: Aubrey Schoenleben [mailto:aubreys@uw.edu]
Sent: Friday, June 9, 2017 4:52 PM
To: Hai Zhang <haizhang@uw.edu>
Subject: Three Year Renewal

Hi Hai,

How have you been? I see that the three-year renewal of your protocol is coming up (expires 7/10/17). Just thought I'd check in and

see if you needed any help getting the renewal together now that the protocol is in HoverBoard. To avoid a lapse in approval, we should get the renewal submitted soon. We typically ask for three-year renewals to be submitted to our office 6-8 weeks in advance, and we are currently about 4 weeks out.

Anything I can do to help, please let me know.

Cheers,
Aubrey

AUBREY SCHOENLEBEN, PhD, CPIA
Scientific Liaison & Review Scientist
Office of Animal Welfare

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aubreys@uw.edu / oaw.washington.edu

OFFICE OF ANIMAL WELFARE
Research Support Services

HOVERBOARD ... Into the Future ... ExploreUW's eIACUC Solution

Pre-Submission

Next Steps

Edit Protocol

Printer Version

View Differences

- Attach OHRs
- Attach BUA
- Assign Admin Office
- Assign Coordinator
- Attach Congruence Letter
- Assign Portfolio ID
- Assign PI Proxy
- Manage Ancillary Reviews
- Manage Departures
- Add Comment
- Add Private Comment
- Manage Tags

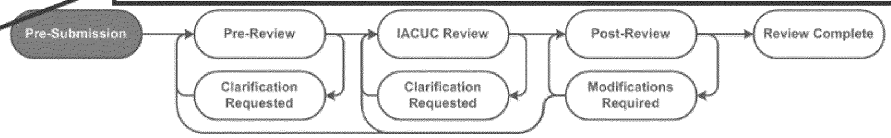
PROTO202000003

Magnesium Stem Cell

Principal investigator: Hai Zhang
Submission type: New Protocol Application
Primary contact:
IACUC coordinator:
Consulted vet:
PI proxies: WEI HE

Letter:
Protocol type: Experimental Research
Admin office: Default

My screen looks a bit different, but you will have a "Submit" button over here.



History | Experiments | Documents | Reviews | Contacts | Snapshots | Training | Related Concerns | Change Log

Filter by Activity | Enter text to search for | + Add Filter | Clear All

Activity	Author	Activity Date
PI Proxy Assigned	Zhang, Hai	1/22/2020 12:29 PM
PI Proxies Added:		
Protocol Created	Zhang, Hai	1/22/2020 11:55 AM

FERPA

RCW 42.56.070(1)

FERPA
RCW 42.56.070(1)

From: weihe87 <weihe87@uw.edu>
Sent: Sunday, May 24, 2020 5:51 PM
To: Aubrey Schoenleben
Cc: Hai Zhang
Subject: 回复: 回复: animal protocol

Hi Dr. Schoenleben,
Thank you very much for your time and hard work! You helped make our protocol better and better. I made modifications according to your suggestions. I have submitted the responses, and hope you can see them now. Thank you!
Take care,

发件人: Hai Zhang <haizhang@uw.edu>
发送时间: 2020年5月19日 11:09
收件人: Aubrey Schoenleben <aubreys@uw.edu>;
主题: RE: 回复: animal protocol

Hi Aubrey,
Thank you very much for your kind help and support!
-Hai

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Tuesday, May 19, 2020 8:47 AM
To:
Cc: Hai Zhang <haizhang@uw.edu>
Subject: Re: 回复: animal protocol

You're welcome, Wei, and sounds good! Let me know if you have any questions re: the new comments.
Aubrey

On 5/18/20, 1:01 PM, ' wrote:

Hi Dr. Schoenleben,
Thanks a lot for helping us with the protocol! I saw your comments just now. Feel we are approaching completion. I will try to reply to your comments and revise our protocol in this week. Thank you!

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年5月13日 18:00
主题: Re: animal protocol

Thanks, [redacted]! I do see it now. I should be able to look the protocol over within the next day or two, and will let you know if I have any additional questions to address before we send it to vet review.

Have a great evening,
Aubrey

FERPA
RCW 42.56.070(1)

Sent from my iPhone

On May 13, 2020, at 2:44 PM, [redacted] wrote:

Hi Dr. Schoenleben,
I have finished the replies and summitted the response. Hope you can see the protocol now. Thank you very much!☺
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年5月13日 14:03
[redacted]
主题: Re: animal protocol

Sounds good - thanks, Wei!
Aubrey

Sent from my iPhone

On May 13, 2020, at 1:14 PM, [redacted] wrote:

Hi Dr. Schoenleben,
Thanks for your response! I see the "submit response" button. I will reply to the comments then submit to you. Thanks a lot!
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年5月13日 11:25
[redacted]
主题: Re: 回复: 回复: animal protocol

Hi [redacted]

If you submit your responses to me (using the "submit response" button on the left hand side of the workspace; see screenshot), I can take a look at the changes that you made to the protocol. You will need to reply to each of the comments in HoverBoard before submitting the responses back to me.

Hope you're staying well!

Aubrey

From: [redacted]
Sent: Monday, May 11, 2020 1:15 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: 回复: 回复: animal protocol

FERPA
RCW 42.56.070(1)

Hi Dr. Schoenleben,
How are you?
I made some modifications of our animal protocol following your instruction. I hope it looks much better than before. Could you check on the protocol again to see if it meet the requirement? As I have revised the protocol according to the comments, do I need to reply the comments in IACUC system one by one?
Thanks for your hard work and kind help!
Please stay safe.
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年4月28日 11:26
[redacted]
主题: Re: 回复: 回复: animal protocol

Hi [redacted]

I'm doing well - I hope you are too!

Absolutely okay to just include the rat iPSC project in the protocol for now. If those efforts prove unsuccessful, you can add the human iPSC pilot work to the protocol in the future.

Hope this helps! Any other questions, let me know.

Thanks,
Aubrey

From: [redacted]
Sent: Monday, April 27, 2020 5:44 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Subject: 回复: 回复: animal protocol

Hi Dr. Schoenleben,
How are you doing?
I am still hesitating how to write the pilot study part. We are aiming to generate rat iPSC. However, this is a new technique for us. Our collaborator in Stem Cell Center only have the experience on human iPSC generation. Although he can't imagine much difference in our try, but still he can not

guarantee success. We must consider the case if we fail in generating rat iPSC, and must use human iPSC in the animal study instead. If we are to use human iPSC, then we need to add another pilot study to see whether our immune suppress therapy works well in the animal model. As there's already an existing pilot study testing the longevity of the riPSC in animal model, we don't want the protocol to be much too complicated. Can I omit the human iPSC possibility in this version first, and then make some changes if we can not successfully generate rat iPSC later?

Thank you!

[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年4月24日 13:04
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: 回复: animal protocol

FERPA
RCW 42.56.070(1)

Hi [redacted]

Thanks for the update (and I hope everything with your thesis is going well)! Please just respond to the questions directly in the IACUC system, and make any necessary edits to the protocol/procedures. The system tracks changes, so this will allow me to make sure that we have all of the required information in the protocol. Once you have everything incorporated, submit the responses back to me.

Hope you're staying healthy and safe as well,
Aubrey

From: [redacted]
Sent: Thursday, April 23, 2020 5:45 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: animal protocol

Hi Dr. Schoenleben,

I was busy with my thesis in the past month, and hardly spare time for animal protocol. I am so sorry for the delay. I learnt a lot from the animal training, and want to make some modification to our original animal protocol. I am not sure if it is proper to answer the questions directly on the IACUC system, so I summarized my replies and send it to you with this email, for you to check whether I can modify our protocol as mentioned in this file.

Thank you very much for your patience and help. Please stay safe.

[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>

发送时间: 2020年3月24日 13:37
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: animal protocol

FERPA
RCW 42.56.070(1)

Hi [redacted]

No need to apologize! We can follow whatever timeline works well for you. I just like to check in occasionally to see if there is anything that I can do to help.

Hope you and yours are staying safe and healthy,
Aubrey

From: [redacted]
Sent: Monday, March 23, 2020 5:37 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: animal protocol

Hi Dr. Schoenleben,
I have received your comments on the animal protocol. Thank you very much for your constructive advice! I attended the hands-on training, which was very helpful for both animal operation and protocol design. I conceived some modifications on the protocol according to your comments, but haven't well organized. There were several ongoing tasks in the past few weeks, that I have to accomplish them one by one. I am so sorry to keep you waiting so long. I will try to submit the modifications in this week. Thank you!
Please stay safe and sound.
Best wishes,
[redacted]

FERPA
RCW 42.56.070(1)

From: [redacted]
Sent: Wednesday, February 19, 2020 2:48 PM
To: Aubrey Schoenleben
Cc: Hai Zhang
Subject: 回复: 回复: Three Year Renewal

Hello Dr. Schoenleben:
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Thank you!
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主题: RE: 回复: Three Year Renewal

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-Hai

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Hope this helps! Please let me know if you have any other questions.

Cheers,
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RCW 42.56.070(1)

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Associate Professor
Assistant Director, Graduate Prosthodontics

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Tel: 206.543.5948, Fax: 206.543.7783
Email: haizhang@uw.edu

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Aubrey

AUBREY SCHOENLEBEN, PhD, CPIA
Scientific Liaison & Review Scientist
Office of Animal Welfare

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OFFICE OF ANIMAL WELFARE
Research Support Services

HOVERBOARD ... Into the Future ... ExploreUW's eIACUC Solution

FERPA
RCW 42.56.070(1)

From: [redacted]
Sent: Thursday, February 27, 2020 1:02 PM
To: Aubrey Schoenleben
Cc: Hai Zhang
Subject: 回复: 回复: 回复: 回复: 回复: Three Year Renewal

Hello Dr. Schoenleben:

We filled in the forms online. Can you please go through them and find out whether they meet the requirement? Still, there may be some details to be modified, for example, we are planning to use the Sendai Virus to reprogram the cells which will be transplanted into the rat tooth socket. The manufacturer has provide methods to remove the virus, but we haven't tried that right now. Will this kind of virus be strictly forbidden in the rodent experiment? How do you determine the safety of the biologicals applied to animals?

Sorry for so many questions. Thank you very much!

[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年2月20日 18:38
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: 回复: 回复: 回复: 回复: Three Year Renewal

You're welcome! Have a great evening!
Aubrey

From: [redacted]
Sent: Thursday, February 20, 2020 6:06 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: 回复: 回复: 回复: Three Year Renewal

OK! I will fill in like this. Thanks for your help Dr. Schoenleben!

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年2月20日 11:50
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: 回复: 回复: 回复: Three Year Renewal

You're welcome, [redacted] Yes, you can list the same location for animal use.
Aubrey

On 2/19/20, 4:23 PM, [redacted] wrote:

Hello Dr. Schoenleben:
Thanks for your response. Can I list the same place for animal use? Thank you!
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年2月19日 16:02
[redacted]
主题: Re: 回复: 回复: Three Year Renewal

Hi [redacted]

I think its okay to list the ARCF/Foege facility on the protocol for now (note: in HoverBoard, ARCF and Foege are considered a single vivarium, so select ARCF ABSL1 (and/or ABSL2 as appropriate); no need to list the specific room number). While the protocol is under review, reach out to James Mendoza (amespodi@uw.edu), the facility supervisor for the ARCF, to discuss your housing needs. James can work with you to determine if there is available housing space in the ARCF or if we need to look at another facility.

Don't hesitate to get in touch with any other questions.

Thanks,
Aubrey

From: [redacted]
Sent: Wednesday, February 19, 2020 2:48 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: 回复: Three Year Renewal

Hello Dr. Schoenleben:
Thanks for your help with the animal protocol. We don't know how to fill in the Animal Housing and Use part. Do we need to discuss with Dr. James Mendoza to determine which room to use?
Thank you!
[redacted]

发件人: Hai Zhang <haizhang@uw.edu>
发送时间: 2020年1月28日 12:55
收件人: Aubrey Schoenleben <aubreys@uw.edu>; [redacted]
主题: RE: 回复: Three Year Renewal

Thanks Aubrey for your detailed advice. We will get those started asap.
-Hai

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Tuesday, January 28, 2020 11:26 AM
To: [redacted]; Hai Zhang <haizhang@uw.edu>
Subject: Re: 回复: Three Year Renewal

Hi Hai and [redacted]

It was really good meeting with you last week! Following up on a couple of items from our conversation:

- Here are links to the basic training courses that you will need to complete:
  - UW Animal Use Laws and Regulations. Click here to take the course.
  - Animal Use Medical Screening (AUMS). Click here for AUMS Steps and FAQs, and electronic enrollment.

- Since you will be working with rats, you will also be required to take the online "Working with Rats at UW" course and the related hands-on lab. You can find a schedule of upcoming rat classes and the registration form on the AUTS website (https://depts.washington.edu/auts/).

- To discuss housing arrangements in our new ARC (Animal Research & Care) facility, please reach out to the facility supervisor, James Mendoza (amespodi@uw.edu).

- The Department of Comparative Medicine also recently implemented a new animal operations system (AOps) that is used for ordering, billing, special services requests, etc. I would strongly recommend signing up for one of the AOps training courses as well. You can find more information about the new system and upcoming courses here.

Hope this helps! Please let me know if you have any other questions.

Cheers,
Aubrey

On 1/21/20, 5:24 PM, [redacted] wrote:

Hello Dr. Zhang and Dr. Schoenleben:
I am OK for the meeting time. Thank you!
[redacted]

发件人: Hai Zhang <haizhang@uw.edu>
发送时间: 2020年1月21日 17:16
收件人: Aubrey Schoenleben <aubreys@uw.edu>
[redacted]
主题: RE: Three Year Renewal

Hi Aubrey,
How about tomorrow at 11am in D751?
Thanks for your help!

-Hai

Hai Zhang, DMD, PhD.
Associate Professor
Assistant Director, Graduate Prosthodontics
Vice Chair, Department of Restorative Dentistry
School of Dentistry
University of Washington
1959 NE Pacific St. Box 357456 Seattle, WA 98195-7456
Tel: 206.543.5948, Fax: 206.543.7783
Email: haizhang@uw.edu

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Tuesday, January 21, 2020 2:28 PM
To: Hai Zhang <haizhang@uw.edu>
Cc: [redacted]
Subject: Re: Three Year Renewal

FERPA
RCW 42.56.070(1)

Let's try for tomorrow (1/22) or 1/29 – both days I am free any time after 11am. What time would work best for the two of you? Also I'm happy to come to your neck of the woods. Could you remind me where you are located?
Cheers,
Aubrey

On 1/17/20, 5:41 PM, "Hai Zhang" <haizhang@uw.edu> wrote:

Thanks Aubrey. We are available to meet on 1/22 (Wed) am, 1/27 (Mon) pm and 1/29 (Wed) am. Please let us know your earliest available time.
Thank you and have a great weekend!~
-Hai

Hai Zhang, DMD, PhD.
Associate Professor
Vice Chair, Department of Restorative Dentistry
Assistant Director, Graduate Prosthodontics
School of Dentistry
University of Washington
1959 NE Pacific St. Box 357456 Seattle, WA 98195-7456
Tel: 206.543.5948, Fax: 206.543.7783
Email: haizhang@uw.edu

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Friday, January 17, 2020 5:18 PM
To: Hai Zhang <haizhang@uw.edu>
Cc: [redacted]
Subject: Re: Three Year Renewal

FERPA
RCW 42.56.070(1)

Hi Hai,

I do still work in OAW – it's good to hear from you! I would be happy to help you and [redacted] get an animal protocol started. Since it's been almost three years since we closed your last protocol, it would be best to just start a new one. We now manage all of our protocols electronically through a system called HoverBoard. I would recommend that we meet so that I can give you (and [redacted]) an introduction to the new system. Do you have availability over the next week or two?

With regards to training, it looks like your basic training (UW Animal Use Laws & Regulations and AUMS clearance) is up to date. Since you will be working with rats, you will need to take the online rat course and the rat hands-on class. You can find the online course and a schedule of upcoming hands-on classes here. It looks like you will also need to update your facility orientation/access. We can update that once we've sorted out where your animals will be housed.

Have a lovely weekend,
Aubrey

On 1/15/20, 3:21 PM, "Hai Zhang" <haizhang@uw.edu> wrote:

Hi Aubrey,
Hope you are still working in this office. I am planning to re-start a new animal study involves rats. [redacted] is the student who will handle the animals and I may work with her occasionally. Could you kindly check my status and guide us how should we re-start this process? May I re-activate my previous protocol and add this new research topic and rats related document? Right now the research proposal is under review. The grant is our departmental internal seeding grant.
[redacted] will work with you to get the paperwork started. I have a few trainings that I need to do.
Thank you very much!

Hai Zhang, DMD, PhD.
Associate Professor
Vice Chair, Department of Restorative Dentistry
Assistant Director, Graduate Prosthodontics
School of Dentistry
University of Washington
1959 NE Pacific St. Box 357456 Seattle, WA 98195-7456
Tel: 206.543.5948, Fax: 206.543.7783
Email: haizhang@uw.edu

From: Aubrey Schoenleben <aubreys@uw.edu>
Sent: Monday, June 12, 2017 12:05 PM
To: Hai Zhang <haizhang@uw.edu>
Subject: Re: Three Year Renewal

Hi Hai,

Thanks for the quick reply! We can archive the protocol since you don't plan to renew it - your email below is all that we need.

Take care,
Aubrey

From: Hai Zhang <haizhang@uw.edu>
Date: Friday, June 9, 2017 at 5:06 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Subject: RE: Three Year Renewal

Hi Aubrey,
I am not planning to renew that project any more. What should I do? Thank you!

Hai Zhang, DMD, PhD.
Associate Professor
Assistant Director, Graduate Prosthodontics
Department of Restorative Dentistry
School of Dentistry
University of Washington
1959 NE Pacific St. Box 357456 Seattle, WA 98195-7456
Tel: 206.543.5948, Fax: 206.543.7783
Email: haizhang@uw.edu

From: Aubrey Schoenleben [mailto:aubreys@uw.edu]
Sent: Friday, June 9, 2017 4:52 PM
To: Hai Zhang <haizhang@uw.edu>
Subject: Three Year Renewal

Hi Hai,

How have you been? I see that the three-year renewal of your protocol is coming up (expires 7/10/17). Just thought I'd check in and see if you needed any help getting the renewal together now that the protocol is in HoverBoard. To

avoid a lapse in approval, we should get the renewal submitted soon. We typically ask for three-year renewals to be submitted to our office 6-8 weeks in advance, and we are currently about 4 weeks out.

Anything I can do to help, please let me know.

Cheers,
Aubrey

AUBREY SCHOENLEBEN, PhD, CPIA
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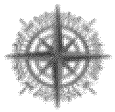

OFFICE OF ANIMAL WELFARE
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HOVERBOARD ... Into the Future ... ExploreUW's eIACUC Solution

From: [redacted]
Sent: Monday, May 11, 2020 1:15 PM
To: Aubrey Schoenleben
Cc: Hai Zhang
Subject: 回复: 回复: 回复: animal protocol

FERPA
RCW 42.56.070(1)

Hi Dr. Schoenleben,
How are you?
I made some modifications of our animal protocol following your instruction. I hope it looks much better than before. Could you check on the protocol again to see if it meet the requirement? As I have revised the protocol according to the comments, do I need to reply the comments in IACUC system one by one? Thanks for your hard work and kind help!
Please stay safe.
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年4月28日 11:26
[redacted]
主题: Re: 回复: 回复: animal protocol

Hi [redacted]

I'm doing well - I hope you are too!

Absolutely okay to just include the rat iPSC project in the protocol for now. If those efforts prove unsuccessful, you can add the human iPSC pilot work to the protocol in the future.

Hope this helps! Any other questions, let me know.

Thanks,
Aubrey

From: [redacted]
Sent: Monday, April 27, 2020 5:44 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Subject: 回复: 回复: animal protocol

Hi Dr. Schoenleben,
How are you doing?
I am still hesitating how to write the pilot study part. We are aiming to generate rat iPSC. However, this is a new technique for us. Our collaborator in Stem Cell Center only have the experience on human iPSC generation. Although he can't imagine much difference in our try, but still he can not guarantee success. We must consider the case if we fail in generating rat iPSC, and must use human iPSC in the animal study instead. If we are to use human iPSC, then we need to add another pilot study to see whether our immune suppress therapy works well in the animal model. As there's already an existing pilot study testing the longevity of the riPSC in animal model, we don't want the protocol to be much too complicated. Can I omit the human iPSC possibility in this version first, and then make some

changes if we can not successfully generate rat iPSC later?
Thank you!
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年4月24日 13:04
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: 回复: animal protocol

Hi [redacted]

Thanks for the update (and I hope everything with your thesis is going well)! Please just respond to the questions directly in the IACUC system, and make any necessary edits to the protocol/procedures. The system tracks changes, so this will allow me to make sure that we have all of the required information in the protocol. Once you have everything incorporated, submit the responses back to me.

Hope you're staying healthy and safe as well,
Aubrey

From: [redacted]
Sent: Thursday, April 23, 2020 5:45 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: 回复: animal protocol

Hi Dr. Schoenleben,
I was busy with my thesis in the past month, and hardly spare time for animal protocol. I am so sorry for the delay. I learnt a lot from the animal training, and want to make some modification to our original animal protocol. I am not sure if it is proper to answer the questions directly on the IACUC system, so I summarized my replies and send it to you with this email, for you to check whether I can modify our protocol as mentioned in this file.
Thank you very much for your patience and help. Please stay safe.
[redacted]

发件人: Aubrey Schoenleben <aubreys@uw.edu>
发送时间: 2020年3月24日 13:37
[redacted]
抄送: Hai Zhang <haizhang@uw.edu>
主题: Re: animal protocol

Hi [redacted]

No need to apologize! We can follow whatever timeline works well for you. I just like to check in occasionally to see if there is anything that I can do to help.

Hope you and yours are staying safe and healthy,
Aubrey

From: [redacted]
Sent: Monday, March 23, 2020 5:37 PM
To: Aubrey Schoenleben <aubreys@uw.edu>
Cc: Hai Zhang <haizhang@uw.edu>
Subject: animal protocol

FERPA
RCW 42.56.070(1)

Hi Dr. Schoenleben,
I have received your comments on the animal protocol. Thank you very much for your constructive advice! I attended the hands-on training, which was very helpful for both animal operation and protocol design. I conceived some modifications on the protocol according to your comments, but haven't well organized. There were several ongoing tasks in the past few weeks, that I have to accomplish them one by one. I am so sorry to keep you waiting so long. I will try to submit the modifications in this week. Thank you!
Please stay safe and sound.
Best wishes,
[redacted]