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Partial Lobular Hepatectomy: A Surgical Model for Morphologic Liver Regeneration

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Cover Letter

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CPC

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11.09.2017

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Dear Indrani,

We have been contacted to submit a protocol we have developed regarding neonatal liver surgeries published in our recent study in the Proceedings of the National Academy of Sciences entitled "Localized hepatic lobular regeneration by central-vein–associated lineage-restricted progenitors." We thank you for invitation to submit a full protocol and have included it in this submission. Briefly, we have previously identified that morphologic liver lobe regeneration is able to occur through localized clonal proliferation of hepatocyte stem or progenitors (instead of global hepatocyte hypertrophy) during a transient neonatal period. We developed and optimized a protocol in neonatal mice, that has also been adapted for juvenile mice, that involves resection of a small portion of the left lobe that allow for the elucidation of the underlying mechanisms of morphologic regeneration. We believe that our protocol will be widely used for studies in regeneration and stem cell biology. We hope you find our manuscript favorable and look forwarding to hearing from you.

Sincerely,

Yuval Rinkevich

TITLE:

Partial Lobular Hepatectomy: A Surgical Model for Morphologic Liver Regeneration

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KEYWORDS:

Partial Lobular Hepatectomy, Hepatectomy, Liver, Liver Resection, Liver Amputation, Regeneration

SUMMARY:

Here, we present a new method for partial resection of the left hepatic lobe in neonatal (day 0) mice. This new protocol is suitable for studying acute liver injury and injury response in the neonatal setting.

ABSTRACT:

Morphological organ regeneration following acute tissue loss is common among lower vertebrates, but is rarely observed in mammalian postnatal life. Adult liver regeneration after 70% partial hepatectomy results in hepatocyte hypertrophy with some replication in remaining lobes with restoration of metabolic activity, but with permanent loss of the injured lobe's morphology and architecture. Here, we detail a new surgical method in the neonate that leaves a physiologic environment conducive to regeneration. This model involves amputation of the left

lobe apex and a subsequent conservative management regimen, and lacks the necessity for ligation of major liver vessels or chemical injury, leaving a physiologic environment where regeneration may occur. We extend this protocol to amputations on juvenile (P7-14) mice, during which the injured liver transitions from organ regeneration to compensatory growth by hypertrophy. The presented, brief 30 min protocol provides a framework to study the mechanisms of regeneration, its age-associated decline in mammals, and the characterization of putative hepatic stem or progenitors.

INTRODUCTION:

The ability to regenerate an organ, or to restore form and function, has been thought to be mostly lost over evolutionary time. The regenerative potential of the adult mammalian liver after acute chemical or physical injury has been found to involve the mobilization of all remaining hepatocytes resulting in waves of hypertrophy and few rounds of cell division, resulting in a functional but architecturally different organ[1–5]. Recently, studies have begun to characterize the regenerative response of neonatal mammalian organs to injury within the first week of life[6–8]. These studies have shown that when injured during neonatal development, certain mammalian organs respond with morphological regeneration instead of compensatory growth or fibrosis[7,8].

Recent studies have shown that regeneration of both global structure and function occurs during the early neonatal period[6–8]. Established liver injury protocols involve chemical injury or administration of ethanol[9–11], acetaminophen[12–15], carbon tetrachloride[16–19], 70% partial hepatectomy[4,20,21], or removal of the left and median lobes. Chemical administration leads to hepatocyte cell death, but often leaves micro- and macro-structures intact. Morphologic regeneration cannot be readily studied in this context, as the overall hepatic architecture was not obliterated. The 70% partial hepatectomy involves suture ligation of the major vessels, which is necessary to stop bleeding, but leaves a non-physiologic environment with permanent disruption of vasculature. Furthermore, this method has only been used on adult rodents, and its application to neonates is technically extremely difficult. With this in mind, we developed a method in which 20-30% of the apex of the left lobe is removed in a newborn P0 mouse (**Figure 1A-1B**). This method is surgically conservative, minimally invasive, not technically challenging, and leads to gross loss of morphology without the ligation of vasculature, leaving room for regeneration to occur. The resulting step-by-step protocol, described below, allows for any researcher to perform a partial lobular hepatectomy on neonatal mice in order to study mammalian neonatal regeneration in the early stages of post-natal life. This method also has clear applications to comparative studies in regenerative medicine and stem cell biology, as it can be used in the liver during later stages of life.

The most common acute liver injury studies are chemically-induced damage, adult liver amputation, or 70% partial hepatectomy. Chemical damage often involves intravenous, intraperitoneal or oral administration of acetaminophen, carbon tetrachloride, or ethanol, and is a relatively easy and non-invasive injury model. As previously discussed, chemical damage results in hepatocyte cell death, but often leaves stroma and parenchyma structures intact, making it difficult to make claims about morphologic regeneration. Chemical damage often centers on hepatic vessels, making it a useful technique to study site and cell-specific injury, but also makes

it difficult to interrogate, at the whole organ level, other populations that may be situated further from vessels and that may contribute to regeneration. Despite these limitations, chemical damage still remains a useful and highly physiologically relevant injury model.

Adult 70% partial hepatectomy involves the removal of the left and median lobes following ligation of hepatic vasculature. The response to hepatectomy has been well characterized: the amputated liver 14 days post 70% partial hepatectomy develops a grossly different architecture from that of the original undamaged lobe, as the hepatocytes of the remaining right and caudate lobes undergo hypertrophy and a few rounds of cell division[4,5]. This makes up lost mass and function, but fails to regenerate the two amputated lobes, and therefore does not replace gross morphology. As a result, the injury response to 70% partial hepatectomy is useful to study compensatory growth mechanisms with limited regeneration.

Here, we fully describe a protocol for a neonatal partial lobular hepatectomy. The procedure involves appropriate animal selection and preparation, surgical field preparation, surgery, and recovery. Optimization and adaptation of each of these steps may be required for different applications of the protocol.

We have extensively performed and optimized this protocol on wild type C57BL/6J pups (JAX 000664), however, to study different cell populations and mechanisms of regeneration, we also used various transgenic animals including mice harboring various Cre and CreERT2 transgenes and/or knock-ins (*Axin2*^{CreERT2} JAX 018867*,* and *Sox9*^{CreERT2} JAX 018829) in combination with fluorescent reporters, such as the Rainbow and mTmG systems (*R26*$^{VT2/GK3}$, *R26*$^{mT/mG}$)[22,23]. We found no need to change this methodology for different mouse strains, as no differences in survival outcomes or regenerative potential were observed.

In addition to using different animal strains, we also performed partial lobular hepatectomies on neonatal mice treated with small molecules, such as 4-hydroxy-tamoxifen and 5-ethynyl-2'-deoxyuridine (EdU). Dimethyl sulfoxide (DMSO) and ethanol were used as solvents, as it was found that corn oil was a significant cause of morbidity. We otherwise found that intraperitoneal administration of small molecules did not affect survival or regenerative outcomes. We predict that this protocol will be adapted for use with other small molecules to interrogate various aspects of regeneration.

Neonatal mouse surgeries can be technically challenging and may require special expertise in animal handling and microscopic dissection. Animal husbandry expertise is necessary to avoid maternal cannibalism following surgery and during the immediate recovery period.

PROTOCOL:

All animal experiments were carried out in strict accordance with the guidelines set forth by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Stanford University's Administrative Panel on Laboratory Animal Care (APLAC), (Protocol number #10266) and in the United States, or the European Animal Welfare Act, Directive 2010/63/EU. The protocol was approved by the Committee on the Ethics of Animal Experiments

of the Government of Bavaria, Germany, and received the permission No: 55.2-1-54-2532-150-2015.

1. Animal Preparation

1.1. Prepare an empty cage with appropriate bedding on a heating pad.

1.2. Prior to touching animals with gloves, rub the mother's bedding onto the gloves.

1.3. Remove all pups from their mother and place into the empty cage. Remove some of the mother's bedding and place it in the empty cage with the pups.

1.4. Place the mother in a separate, clean, dry cage away from the surgical field.

2. Surgical Field Preparation

2.1. Using a 10-mL pipette, add 10 mL of phosphate buffered saline (PBS) to a 10-cm Petri dish.

2.2. Pipette 2 mL of betadine or equivalent anti-septic solution in the 10-cm Petri dish.

2.3. Place a dissecting scope on the surgical field. Turn on dissecting scope lamp and adjust the level of light to the surgeon's comfort. A pup can be placed in the surgical field underneath the dissecting scope lamp. Adequate light can be confirmed by the absence of shadows on the ventral surface of the pup.

2.4. Prepare the isoflurane anesthesia chamber (see **Table of Materials**) with a nose cone. The chamber should be cleaned without evidence of urine or feces. Place the nose cone and associated tubing under the dissecting scope in the surgical field. Divert the flow of oxygen and isoflurane solely to the nose cone.

2.5. Prepare a post-operative recovery area with a heating pad set at approximately 37 °C. The post-operative recovery should consist of 4 pieces of gauze placed on top of the heating pad. If possible, rub gauze pads in the mother's bedding, feces, and urine to preserve the mother's scent.

Note: It is ill-advised to use a heat lamp, as this makes it difficult to control the temperature. Elevated temperatures will result in the death of neonatal mice.

2.6. Prepare and sterilize all surgical instruments with 70% ethanol or an equivalent anti-septic solution. The tools needed include: micro-dissecting scissors, micro-dissecting forceps, gauze, hemostat, and 6-0 silk sutures.

3. Partial Lobular Hepatectomy

3.1. Anesthetize the pup by placing it in the nose cone on its back and gently taping its feet and hands in place. The pup should be receiving 5% isoflurane in oxygen.

3.1.1. Allow the pup to sit for 5 min or until adequately anesthetized, which can be verified by a toe pinch test.

3.1.2. Inject 5 mg/kg of carprofen subcutaneously prior to incision.

NOTE: The entire surgery should take no longer than 30 min. Poorer outcomes may be observed in neonates that are under general anesthesia for over 30 min. Take precautions to minimize the length of surgery through thorough field preparation and wetting of the skin prior to closure to minimize suture induced skin tears.

3.2. Gently clean off the posterior abdominal wall with a small gauze pad wet with betadine. Allow the betadine to dry for 1 min.

3.3. Make a right mid-clavicular 0.5 cm incision immediately below the rib cage with the micro-dissecting scissors and forceps. Gently separate the skin using forceps and make a second deeper incision into the peritoneal cavity (See **Figure 1C**, left and center).

3.4. Gently apply lateral pressure from both sides of the abdomen using the back, blunt ends of the micro-dissecting scissors and forceps to force the apex of the left lobe out of the peritoneal cavity. The left apex of the left lobe should be easily visualized (**Figure 1C**, right).

3.5. From the apex, amputate and weigh the amount of tissue to be removed.

3.5.1 Using the micro-dissection scissors, gently amputate the desired amount of tissue from the apex of the left lobe.

3.5.2 Place the amputated tissue into a 1.5-mL tube filled with PBS. Weigh the amputated tissue using an analytical balance (see **Table of Materials**).

Note: Place a piece of gauze or paper on the balance and tare it. Then place the amputated tissue on the gauze or paper and measure its mass.

3.5.3 Fix the amputated area in 2% paraformaldehyde at room temperature for 1 h and place in optimal cutting temperature (OCT) compound over dry ice for frozen section analysis. Analyze frozen sections by cutting 7-10 µm sections using any standard cryostat.

3.6. Using a rolled piece of gauze, gently replace the left lobe into the peritoneal cavity. Leave the gauze in the cavity until the bleeding stops.

3.7. Remove the gauze and wet the surgical site and the surrounding area with gauze soaked with PBS.

3.8. Close the surgical site with 6-0 silk sutures with a running stitch. The peritoneum and skin can be closed separately or together.

Note: Gently wetting the skin with gauze soaked with PBS may minimize suture induced tears.

3.9. Gently but thoroughly clean the pup with gauze soaked with PBS and ensure no blood or betadine remains. Roll the soaked gauze pad and gently scrub over the wound sign to clean off any blood or betadine.

Note: This is especially important as the mother may cannibalize the pups if they are cleaned inadequately.

3.10. Remove the pup from the nose cone and place it on the recovery area that includes the gauze pads exposed to the mother's feces and urine. Once the pup recovers, replace the pup in the empty cage with the mother's bedding.

Note: Do not use a heat lamp. The use of a heat lamp can cause the neonate to overheat.

3.11. Repeat the procedure on the desired number of pups. Although all pups can be used, it is generally advisable to leave a few pups not operated on to be replaced together with the operated pups.

3.12. Replace all pups simultaneously with their mother's bedding in the mother's cage.

4. Recovery and Analysis

4.1. Follow up on the mice daily.

4.1.1. Check that the wound remains closed and remove any dead pups, if present.

4.1.2. If the wound reopens, prepare the surgical site and recovery area as previously described, and repeat steps 3.8 to 3.12.

4.1.3. Inject 5 mg/kg of carpofen subcutaneously 24 and 48 h following the procedure.

4.1.4. Follow Pups for 56 days or more.

4.2. Euthanize the animals after the desired amount of post-operative days by carbon dioxide (CO_2) exposure and cervical dislocation.

4.2.1. Place mice in the induction / euthanasia chamber and turn on the CO_2 until mice stop

breathing.

4.2.2. Ensure euthanasia by cervical dislocation. Push down on the dorsal neck of the mouse using the fore-finger and thumb and using the other hand, pull down on tail.

4.3. Remove the entire liver *en bloc* and weigh it.

4.3.1. Carefully separate each lobe and weigh separately.

Note: Remove the liver by careful dissection of the diaphragm and hepatic and portal vessels. Separate the lobes from each other by carefully dissecting each lobe at its proximal attachment.

4.4. Determine the extent of regeneration by comparing the mass of the amputated left lobe to the mass of the whole liver. An uninjured left lobe is approximately 30% of the whole liver.

REPRESENTATIVE RESULTS:

Figure 1A details a general timeline of the neonatal partial lobular hepatectomy (schematic in **Figure 1B**), and the expected length of time to wait until regeneration is observed. Subtle regeneration of the left lobe can be observed 7-14 days post surgery. Full regeneration was often observed after 56 days post surgery. Mice should show no signs of physiologic abnormalities after surgery.

Mice undergoing partial lobular hepatectomies were allowed to recover for 2, 7, 14, 35, and 56 days. Hematoxylin and eosin (H&E) of injured left lobes from these mice after recovery are shown in **Figure 2**. Notably, after 56 days, the amputated left lobe may look indistinguishable from control, uninjured lobes. Surgeries done on P14 juvenile mice were done for comparison and allowed to recover for 7, 14, and 56 days post surgery (**Figure S1**).

To characterize neonatal regeneration, 45 mice underwent partial lobular hepatectomy at day 0 and the masses of all their lobes were taken 56 days post surgery. The mass of the injured left lobe underwent an increased change in mass when compared to the other uninjured median, right, and caudate lobes (**Figure 3A**) and uninjured controls, nearing the mass of an uninjured left lobe at P56. This indicates that regeneration following neonatal liver injury is localized to the left lobe. Surgeries done on P14 juvenile mice were done for comparison, which showed decreased regeneration in the left lobe and increased compensation from the uninjured lobes (**Figure 3B**), indicating that by 14 days, the injury response to acute resection switched from lobe specific regeneration to global compensation. Further characterization was done by staining areas of the left lobe from injured mice at post-operative day 56 with filamentous actin (f-actin) to visualize cell membranes (**Figure 4A**). Areas distal and proximal to the area of injury were compared to uninjured controls and adult lobes 14 days following 70% partial hepatectomies. Hepatocytes were found to have similar areas as uninjured controls, about 1.5-2x less than adult mice undergoing regeneration following classical 70% partial hepatectomy (**Figure 4B**). This suggests that hypertrophy does not play a role in regeneration. Finally, neonatal mice were injected with 0.025 mg of 5-ethynyl-2'-deoxyuridine (EdU) in 90% PBS and 10% ethanol and 1, 3, 5, 7, and 14

days following surgery. The number of EdU positive cells were counted from mice allowed to recover for 7 days following surgery (**Figure 4C**). A significant increase in the number of EdU positive cells were found in the injured/regenerating left lobe when compared to uninjured controls, indicating that cell proliferation contributes to neonatal regeneration.

FIGURE AND TABLE LEGENDS:

Figure 1: Partial Lobular Hepatectomy Overview. (**A**) A general schematic and timeline of the partial lobular hepatectomy is shown with neonatal liver resection done at P0. Analyses were done at P7, P14, P35, or P56. Resections were also tried at P7 and P14. (**B**) A schematic of the extent of resection of the left lobe is shown, demarcating 20 and 30% resections. (This figure has been modified from Tsai *et al.*)[6]. (**C**) Images from neonatal surgeries showing: right sided mid-clavicular incision (left, center) and exposure of the left lobe apex (right).

Figure 2: Regeneration Following Partial Lobular Hepatectomy. Mice undergoing partial lobular hepatectomies at P0 were followed for 2, 7, 14, 35, and 56 days. Livers were fixed and stained with H&E, and the extent of regeneration of the left apex was noted. Arrows denote areas where regeneration occurred in P0 mice. Scale bar is 1 cm. This figure was modified from Tsai *et al.*[6]

Figure 3: Regeneration per Lobe Following Partial Lobular Hepatectomy. (**A**) Mice undergoing partial lobular hepatectomies at P0 were analyzed at 7, 35, and 56 days post-operatively. Mice were euthanized and masses of all lobes from injured mice (red) were taken and compared to age matched masses of uninjured control (red). (**B**) Mice undergoing partial lobular hepatectomies at P14 were analyzed at 7, 35, and 56 days post-operatively. Masses of all lobes from injured mice (red) were taken and compared to masses of uninjured control (red). * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$, NS = not significant. This figured has been modified from Tsai *et al.*[6]

Figure 4: Characterization of Regeneration Post Hepatectomy. (**A**) Mice undergoing partial lobular hepatectomies at P0 were analyzed 56 days after resection and stained for F-actin. Images are shown of stains from areas proximal and distal to the area of amputation, as well as from age-matched uninjured controls, and from adult mice 14 days after 70% partial hepatectomy. Scale bars are 100 µm. (**B**) Areas of hepatocytes following injury at areas proximal or distal to the resection site were compared to areas of hepatocytes from uninjured controls and adult 70% partial hepatectomies. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$, **** = $p < 0.00005$, NS = not significant. (**C**) Mice undergoing lobular hepatectomies at P0 were treated with EdU and were analyzed 7 days following resection. EdU^+ cells in the left lobe are shown. (Scale bar, 100 µm). (**D**) Quantification of EdU^+ cells in mice treated with EdU 7 and 14 days following partial lobular hepatectomy compared to controls. Values are means ± SEM. This figured been has modified from Tsai *et al.*[6]

Figure S1: Incomplete Regeneration of Juvenile Mice. Mice undergoing partial lobular hepatectomies at P14 were followed for 7, 35, and 56 days. Livers were fixed and stained with H&E and the extent of regeneration of the left apex was noted. Arrows denote areas where regeneration occurred in P0 mice. Scale bar is 1 cm. This figured was modified from Tsai *et al.*[6]

DISCUSSION:

Acute hepatic injury has traditionally been studied using chemical (acetaminophen, ethanol, carbon tetrachloride), or surgical models (70% partial hepatectomy). The regenerative response after 70% partial hepatectomy has been characterized to involve global hepatocyte hypertrophy and multiple rounds of cell division[4,5]. To stop hemorrhaging, however, this model is limited, as the major vessels must be ligated leaving an abnormal environment for regeneration. Many studies have therefore employed other less invasive models of acute injury through chemical damage, leaving the gross architecture in place for regeneration to occur. Recently, Porrello *et al.* and Chang *et al.* have demonstrated a markedly different neonatal regenerative response after acute injury in the heart, digit tips, and ears[7,8]. Their results parallel presented conclusions that the liver also undergoes a distinct regenerative phenomenon in neonatal life[6]. With multiple similar findings in major organs, regeneration in the early stages of post-natal development is an emerging field with potential implications for stem cell biology.

Early mortality from neonatal partial lobular hepatectomies often comes from inadequate recovery, major hemorrhage, or maternal neglect. As stated previously, the use of a higher intensity heat source such as a heat lamp for recovery, may lead to death following surgery. Neonatal mice are dependent on their mother for at least the first two weeks of life. At the same time, the mother will often neglect and or cannibalize her young if she senses an abnormality (such as the scent of blood or other chemicals)[24,25]. It is therefore highly important that the neonate is cleaned thoroughly post-operatively and rubbed with maternal bedding to mask any offensive scents. If these issues are adequately addressed, survival can reach up to 100%. If maternal cannibalism becomes an issue, the pups can be placed into a cage with a surrogate mother with some of her own pups. If this is the case, use the surrogate mother's bedding in the previous steps.

The resection of 20-30% of the neonatal left lobe and subsequent regeneration is likely not inherent to only the left lobe. Currently, this method has only been tested on the left, as exposing the median and more posterior right and caudate lobes would necessitate a larger laparotomy, resulting in a higher risk of hemorrhage and, indirectly, a higher risk of maternal cannibalism for the neonate. However, whether the mechanisms of neonatal regeneration are heterogeneous within the liver is an important question to be addressed, and therefore surgical adjustments to this protocol should be made to interrogate the other hepatic lobes.

The results from these neonatal hepatectomy studies have shown a time period (P0-P7) during which regeneration is able to occur. Similar hepatic resections have been done on juvenile mice (P7, P10, P14) and do not result in full regeneration with demonstrated scar and fibrosis, marking a clear area where the amputation occurred. Although the injury response in juvenile mice following partial lobular hepatectomy was not the focus of an initial study, the discrepancy in regenerative potential between neonatal and juvenile mice, and the loss of the ability to reconstitute organ and tissue architecture, will be essential to understanding by what mechanism stem or progenitor cell neonatal regeneration occurs.

We have previously demonstrated that neonatal regenerated livers not only appear the same in architecture and structure, but are also indistinguishable by function. Immunofluorescence stains for functional hepatic enzymes such as glutamine synthetase (GS), carbamoylphosphate synthetase (CPS), and cytochrome p450 2E1 show a similar distribution within regenerated areas when compared to uninjured lobes. However, the secondary regenerative potential of a regenerated neonate has not been tested. As neonatal mice allowed to recovery for 56 days are physiologically indistinguishable from uninjured controls, it is likely that the classical regenerative response following adult 70% partial hepatectomy would occur. However, this liver regeneration is often limited by hepatocyte exhaustion, and therefore serial hepatectomies following partial lobular hepatectomies would be an important study.

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DISCLOSURES:

The authors have no disclosures.

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Figure 1

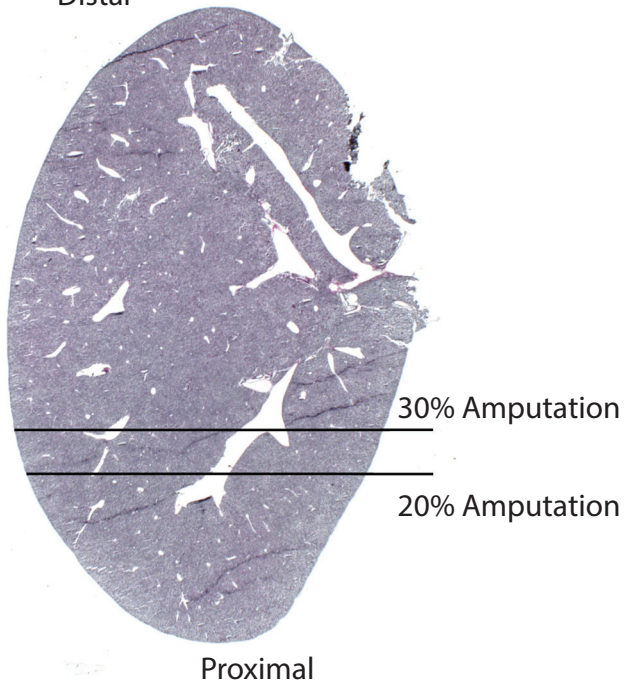
A

Day 0

Partial lobular hepatectomy

Day 56

B



C

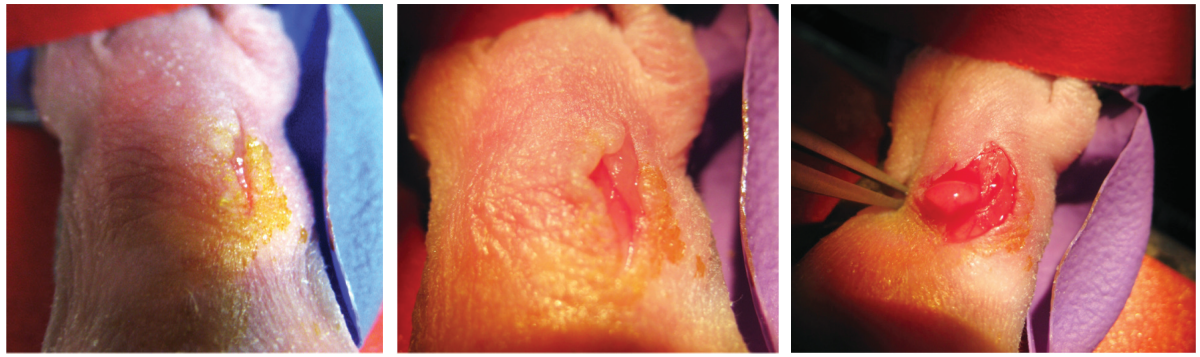


Figure 2

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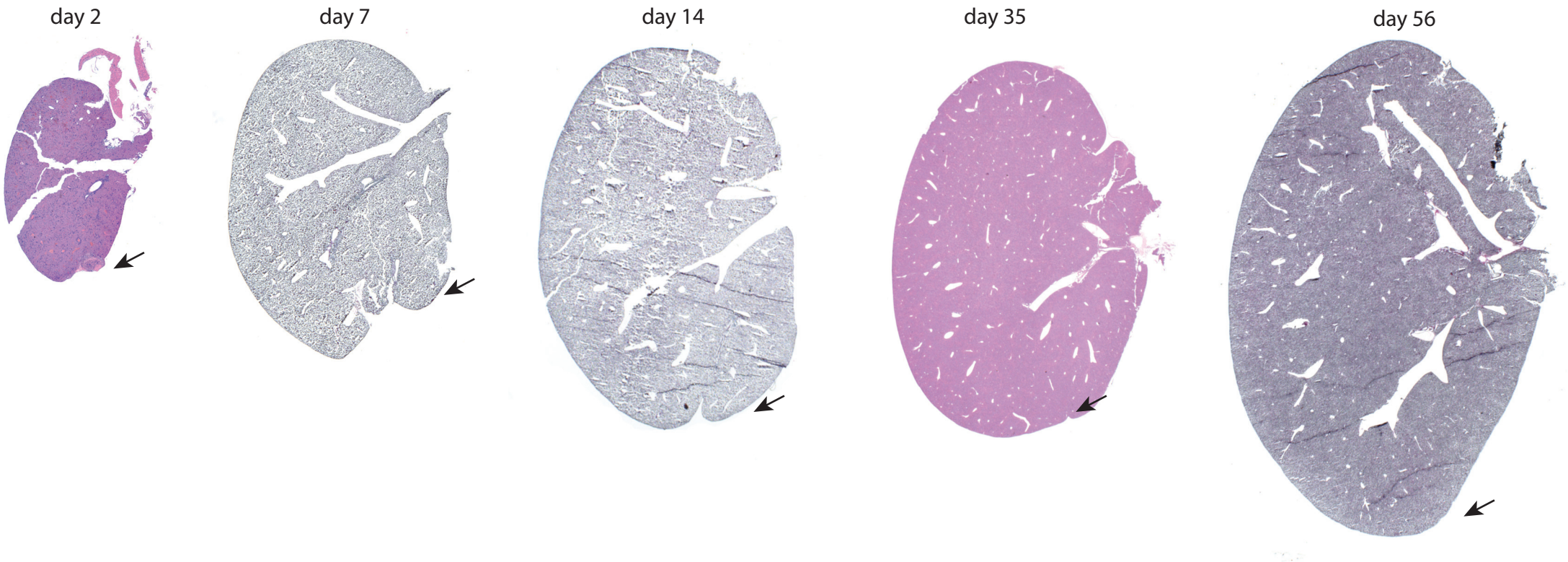

Figure 3

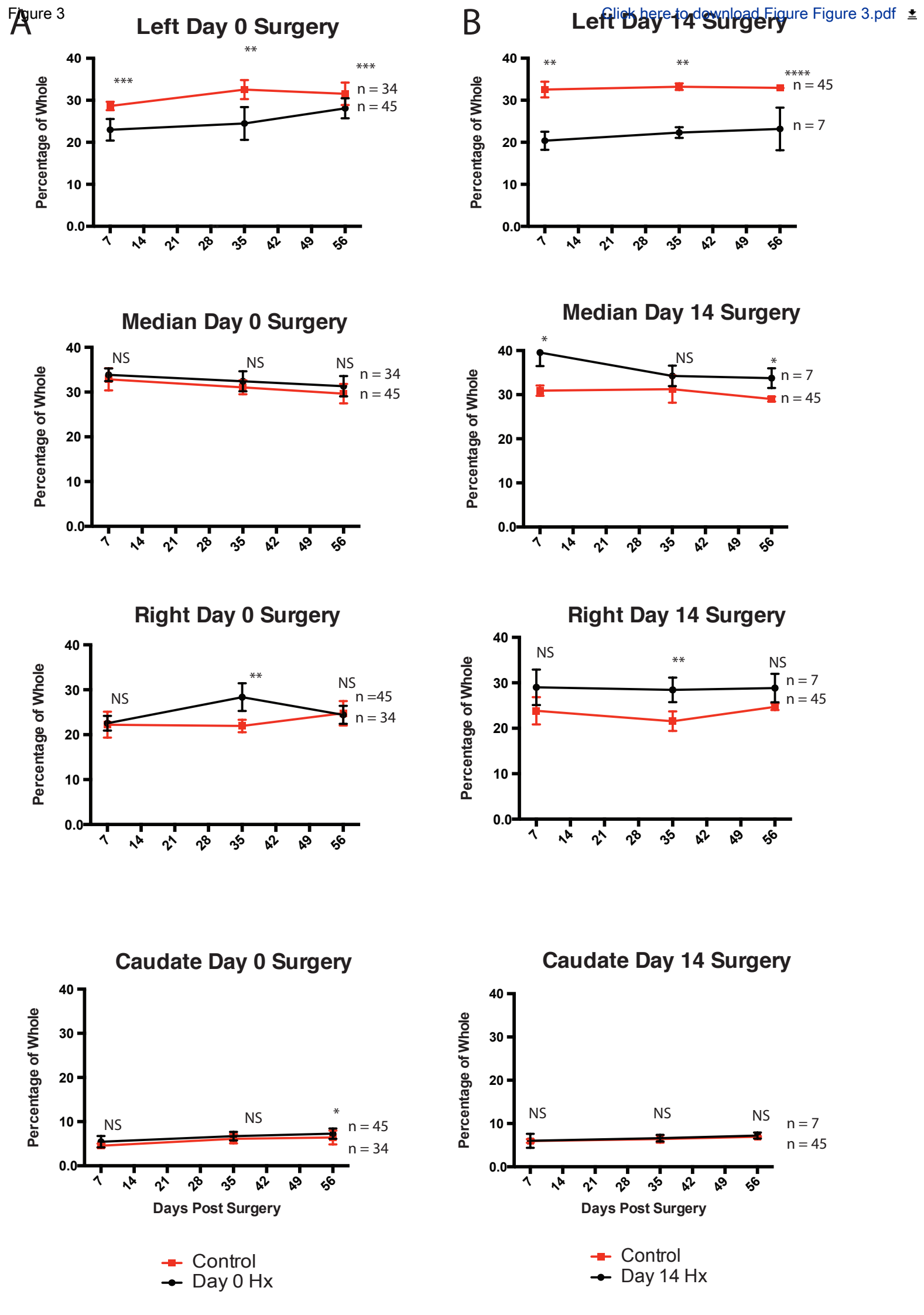

Figure 4

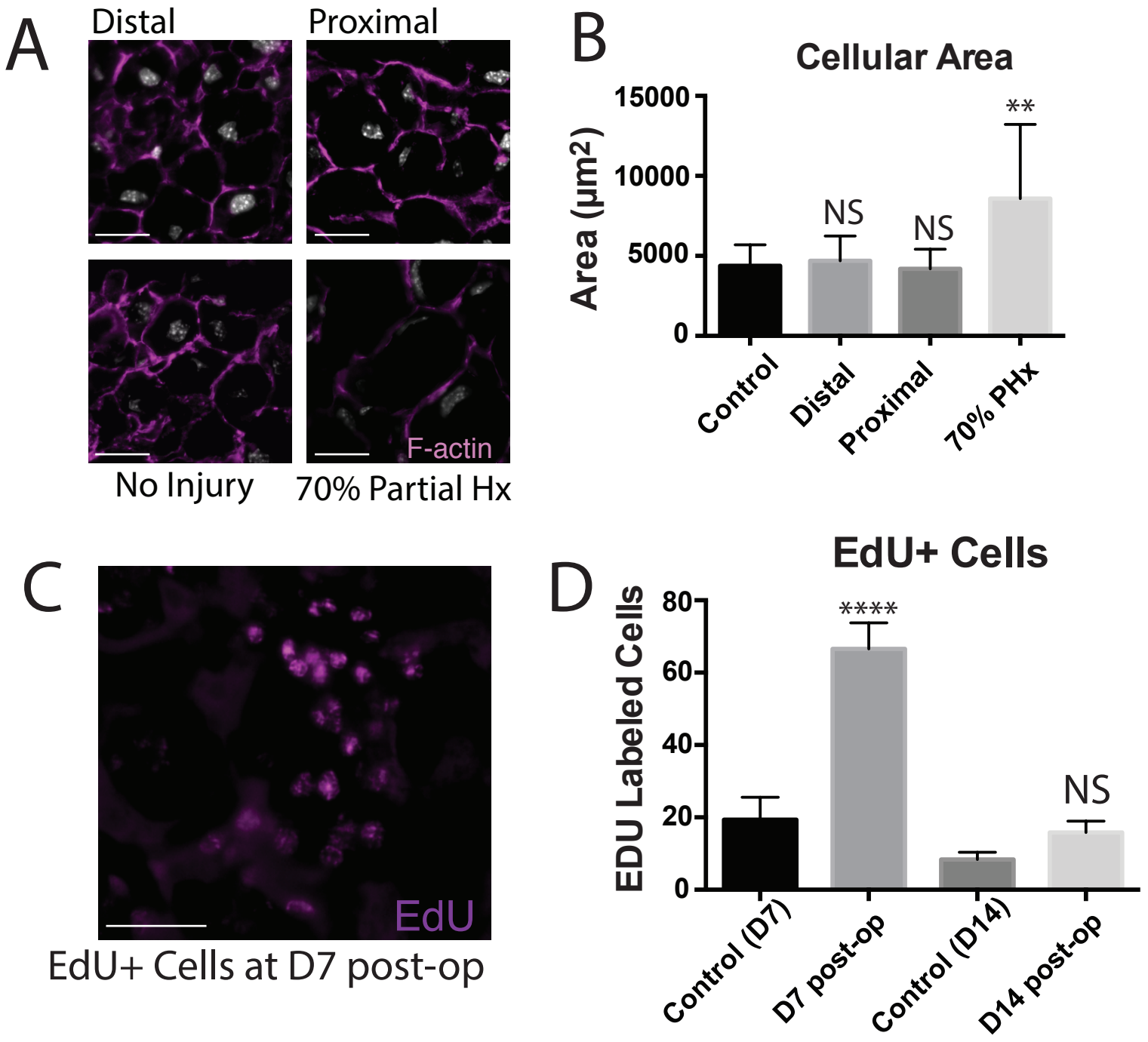

Figure S1

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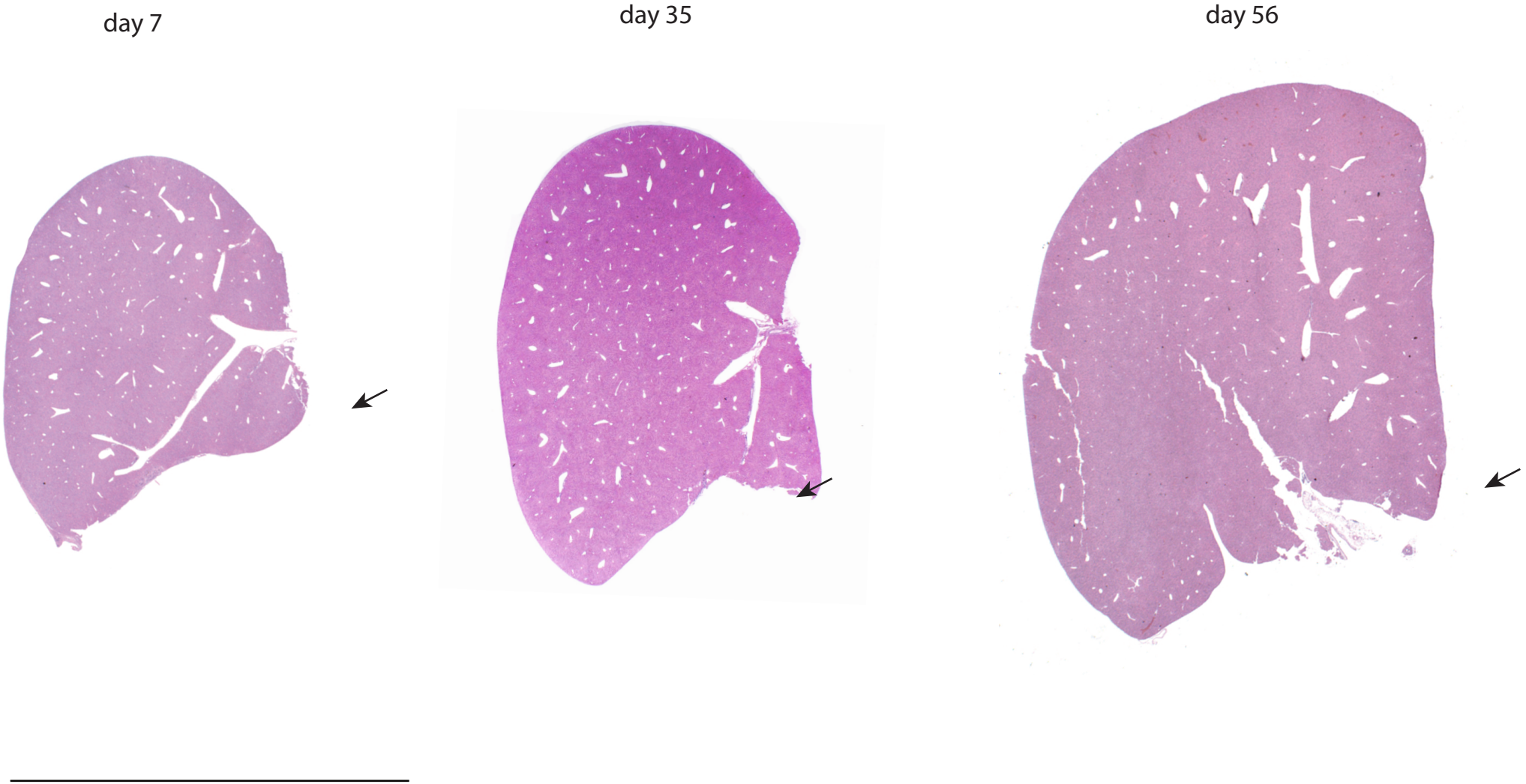

Materials and Reagents	Company
Animals	
Mother with litter of day 0 neonatal pups (any strain)	
Surrogate mother and surrogate litter (optional)	
Standard Reagents	
Phosphate Buffered Serum (PBS)	
Providine-iodine or equivalent antiseptic solution	
Surgical Equipment	
Dissecting microscope	Zeiss
3mm straight spring micro scissors	Vannas
5SF Forceps	Dumont
Straight Kelly forceps	Grainger
Heating pad	Sunbeam
Isoflurane	Abbott Labs
Rodent Anesthesia System	Kent Scientific
Gauze, 10.16 x 10.16cm	Fisher Scientific
Standard Equipment	
1.5ml microcentrifuge tube	Eppendorf
6-0 monocryl sutures	Ethicon
Petri dish	Fisher Scientific
Pipet-Aid, Plain, 110V	Drummond
Mettler Toledo NewClassic ME Analytical Balances	Fisher Scientific
Low Cost Induction Chamber	Kent Scientific

Catalog Number

ZEMSDV4L MFR # 435421-9901-000
72932-01
11252-00
17-050G
000771-810-000
0044-5260-05
1205S
13-761-52

22363204
MCP489G
S35839
4-000-110
01-912-402
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Author(s): Jonathan M. Tsai, Irving L. Weissman, Yuval Rinkevich

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CORRESPONDING AUTHOR:

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Department:	Comprehensive Pneumology Center
Institution:	Helmholtz Zentrum München
Article Title:	Partial Lobular Hepatectomy: A Surgical Model for Morphologic Liver Regeneration
Signature:	
Date:	September 11, 2017

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Dear Dr. Alaghemandi,

We would like to thank you for considering our manuscript for publication. We have gone through the editor and reviewer comments and addressed them to the best of our ability, and have submitted a rebuttal as seen below. We hope that you will view our manuscript favorably as we believe that our novel method will be of interest to those in the stem cell and liver regeneration fields and hope to hear from you soon,

Sincerely,

Yuval Rinkevich
Jonathan Tsai
Irving L Weissman

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2. Please provide at least 6 keywords or phrases.

3. Please define all abbreviations before use.

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5. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

7. Protocol: Please remove the Regents and Equipment from the beginning of the protocol. Please list them in the Material Table.

8. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

9. Protocol: 1.4 What is the appropriate environment? Please provide a quantitative measure.

10. Protocol: 2.3 What is the standard dissecting scope? How much light is adequate?

11. Protocol: 2.4 What is the standard isoflurane anesthesia chamber?

12. Protocol: 2.5 What are the temperature settings used?

13. After revising the protocol, please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

14. Please include a title of each figure legend.

15. Figure 3: Please make sure that the vertical axis is in percent not a fraction of 1. Please provide more information in the figure legend.

16. Figure 4: Please label all the panels and use SI units (i.e. m, mm). Also, please describe all the panels in the figure legend. In the bottom right, is that part of panel B or C or panel D? Please use the Greek symbol mu in the micron abbreviation.

17. Figure S1 Should arrows for “day 7” and “day 56” point to a white space?

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We thank the editor for these comments and have made all the corresponding changes in the text.

Reviewers' comments:
Reviewer #1: Manuscript Summary:

This is a well written protocol on an important model of liver regeneration in neonatal mice. This protocol adds to the recent body of work on the important regenerative window in neonatal mice. The protocol is clear, and should be a very useful method for investigators.

Major Concerns: None

Minor Concerns: The current figures do not show crucial steps of the surgery, which would make it easier for investigators to perform the surgery (in addition to the video). It would be important for the authors to discuss the reproducibility of the protocol, as well as potential pitfalls and percentages of survival.

Due to the microscope nature of this surgery, we have found it difficult to obtain high quality images of the intermediate steps of the surgery, and hope that the video will be able to adequately show these steps. We have added text regarding the reproducibility, pitfalls, and survival rates in the manuscript.

Thus, I recommend it for publication in JOVE after addressing these minor points.

Reviewer #2: Manuscript Summary:
The authors present a surgical method to study liver regeneration in neonates. This is done to assess real liver regeneration when compared to compensatory hyperplasia of the remaining lobe which is the result of partial hepatectomy in adult mice. The authors present their protocol of resecting the apex of the liver in neonate mice and show by HE staining and EDU as a regeneration marker that there is specific regeneration at the site of resection.

The specified method woulb be of particular interest as regeneration is studied in resected lobes but not in remaining lobes as after standard two third partial hepatectomy. Therefore the physiologic environment stays intact.

Major Concerns:
- It remains unclear if the model can show specific biological processes that add information in addition to standard hepatectomy.

We acknowledge this reviewers' point and invite the reviewer to read the publication of which this method is based on for biological relevance and our pertinent findings.

- To standardize the extent of resection will be extremely difficult. First, because of the lack of anatomic landmarks and second because of the small size of livers in neonates. How did the authors ensure standardization of experiments.
- It remains unclear if the lack of liver tissue or just surgical injury leads to the differences desbried in figure 3 and 4. The authors should add other controls and also show that the effect is dose dependent: They should assess Proliferation with different extent of liver resection in neonates.
- The authors show only differences in liver weight in figure 3 but not other regeneration markers. To measure liver weight is typically not specific and precise enough because of inherent high variability and blood contents of the liver.
- Morphology: The authors need to show by the use of specific markers that that parenchymal and non-parenchymal cells (stellate cells, vasculature, infiltrating and resident inflammatory cells and others) are similar in the regenerated lobe compared to remaining tissue.

We acknowledge that all the points that the reviewer has raised are highly important but we believe are ultimately out of the scope of our methods manuscript and have been already covered in our recent publication. Please see Tsai et al, 2017 for the all the information requested.

- The authors need to state that approval for this type of experiments has been obtained.

We have added this line under an ethical statement preceding our Procedures section.

- What should be the primary read out after partial lobular hepatectomy. Proliferation marker or percentage of whole in comparison to an unresected lobe? Please specify. After defining a primary read out, the authors should suggest a power calculation in order to detect differences in regeneration.

There are multiple read outs depending on the question being tested experimentally. We have shown both proliferation in terms of EdU positivity and regeneration as a percentage of the whole.

Minor Concerns:
- What are the reasons that the authors did not use analgetic drugs?

We apologize for this omission and have added in the analgesic regimen.

- The results of individual mice should be shown with dots rather than mean and error bars.

As we have done n > 30 surgeries, we believe this data would be better represented as mean with error bars.

- The authors described that they used various genetic modified animals. The results with these mice are not described.

The results for these mice are ultimately very similar to the C57BL6 mice and were used for functional studies and were thus not included in the methods manuscript. Please see Tsai et al 2017 for these results.

Reviewer #3: Manuscript Summary:

Tsai et al have presented a methods paper on a new surgical approach for studying liver regeneration in neonatal and juvenile mice. The paper is interesting and the method presented could advance studies on liver regeneration relevant to clinical pediatric adverse events. This is in contrast to existing rodent liver regeneration methodologies, which are performed on predominantly adult rodents and are used not to model a clinical situation, but rather to study more fundamental regenerative processes. Overall, the submission is judged favorably. However, in my evaluation, some major corrections, clarifications, and additions are required before this paper can be accepted for publication, as specified below.

Major Concerns:

1) In the abstract, introduction, and elsewhere, the mechanistic presentation of liver regeneration is misleading. Thus, it is stated in the abstract (lines 33-36) and in various related ways elsewhere that "Adult liver regeneration after 70% partial hepatectomy results in hepatocyte hypertrophy in remaining lobes with restoration of metabolic activity but with permanent loss of the injured lobe's morphology and architecture." Whereas this is not untrue, it is misleading, in particular in the focus on hypertrophy and omission of cell cycle events. During regeneration there is, indeed, some hepatocyte hypertrophy, but the regenerative growth is matched by DNA replication, such that there is no change in hepatic nuclear:cytoplasmic ratios. The hypertrophy is associated with increased cell ploidy that has been shown, at least in some situations, to result from complex dysregulation of cell cycle checkpoints (Duncan, 2009; Gentric, 2015 - both citations missing from manuscript). Among adult organs, the cells of the liver (hepatocytes and others) are eccentric in their ability to reenter the cell cycle, proliferate, and occasionally escape checkpoints to allow some degree endoreduplication and thus some degree of hypertrophy. A more complete and less misleading presentation of the liver regenerative process should be used.

We thank the reviewer for this point and have changed our wording in this line to reflect this.

2) Lines 75-77. The highly speculative conjecture about non-liver organs is not supported by data in this paper nor by cited literature. This should be omitted.

We have omitted this statement as this reviewer has requested.

3) Lines 68-99 require careful exhaustive citation of the original literature to support each of the many statements.
4) Lines 111-115. All mouse strains used need citation and accession numbers (e.g., JAX stock #s or equialent). All strains reported must be available for

unrestricted use to the research community, and this should be explicitly stated.

We have included these citations as this reviewer has requested.

5) Lines 120-122. Complete scientific details of the relative juvenile hepatotoxicity of corn oil, DMSO, and alcohol this should be presented. DMSO and ethanol are generally considered far more hepatotoxic than corn oil, so this is a potentially important observation in juveniles that needs to be supported by complete detailed statistically validated data.

We believe this is out of the scope of the manuscript as we are not testing the use of chemical damage in the liver, but proposing a new injury model. We are consistent in the use of DMSO in our neonatal mice and do not use corn oil in these procedures, therefore the use of alternate chemicals should have no impact on our method.

6) Lines 128-129. The specialized animal care needs to be specified in enough detail to allow other researchers to replicate the procedure and survival rates.

We have included this as the reviewer has requested.

7) Use of analgesic, or rationale for not using analgesic, should be addressed.

We have included our analgesic regimen as the reviewer has requested.

8) Statement of institutional ethical approval of procedures is not provided.

We have included this as the reviewer has requested.

Minor Concerns:

1) Lines 59-62. Confusing sentence structure. Rewrite for clarity
2) Line 67. This is also true for other rodents, in particular rats, not just mice.
3) Lines 118-120. Specific formulations and any specialized procedures for preparing 4-OHT and 5-EdU should be specified in sufficient detail to allow other researchers to replicate the procedure and reported survival rates.

We have revised these lines as the reviewer has requested.

4) Line 234. Silk sutures are used so the protocol likely needs to include removal of suture.

We do not remove the suture and have found that they fall out from the dermis as the mice grow in size.

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