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A mouse model of Lumbar Spine Instability

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Corresponding Author:	Qin Bian Fudan University Shanghai, CHINA
Corresponding Author's Institution:	Fudan University
Corresponding Author E-Mail:	bianqin213@gmail.com
Order of Authors:	Shufen Liu
	Yueli Sun
	Jingcheng Dong
	Qin Bian
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TITLE:

A Mouse Model of Lumbar Spine Instability

AUTHORS AND AFFILIATIONS:

Shufen Liu[1], Yueli Sun[1], Jingcheng Dong[2], Qin Bian[2]

[1]Spine Research Institute, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China

[2]Department of Integrative Medicine, Huashan Hospital, Fudan University, Shanghai, China

Corresponding Author:

Qin Bian (bianqin213@126.com)

Email Addresses of Co-authors:

Shufen Liu (xiaoliufenfen@163.com)

Yueli Sun (yueli_sun@foxmail.com)

Jingcheng Dong (jcdong2004@126.com)

KEYWORDS:

intervertebral disc, disc degenerative disease, lumbar, spine, spinous process, mouse model, in vivo

SUMMARY:

We developed a lumbar intervertebral disc degeneration mouse model by resection of L_3–L_5 spinous processes along with supra- and inter-spinous ligaments and detachment of paraspinous muscles.

ABSTRACT:

Intervertebral disc degeneration (IDD) is a common pathological change leading to low back pain. Appropriate animal models are desired for understanding the pathological processes and evaluating new drugs. Here, we introduced a surgically induced lumbar spine instability (LSI) mouse model that develops IDD starting from 1 week post operation. In detail, the mouse under anesthesia was operated by low back skin incision, L_3–L_5 spinous processes exposure, detachment of paraspinous muscles, resection of processes and ligaments, and skin closure. L_4–L_5 IVDs were chosen for the observation. The LSI model develops lumbar IDD by porosity and hypertrophy in endplates at an early stage, decrease in intervertebral disc volume, shrinkage in nucleus pulposus at an intermediate stage, and bone loss in lumbar vertebrae (L_5) at a later stage. The LSI mouse model has the advantages of strong operability, no requirement of special equipment, reproducibility, inexpensive, and relatively short period of IDD development. However, LSI operation is still a trauma that causes inflammation within the first week post operation. Thus, this animal model is suitable for study of lumbar IDD.

INTRODUCTION:

Intervertebral disc degeneration (IDD) is commonly seen in aging and even young people caused by many factors[1]. Surgery for patients who suffer from IDD, causing low back pain and impaired movement, is usually performed at a later stage or in severe cases and has potential risks such as nonunion or infection[2]. Ideal non-operative treatment requires comprehensive understanding of the IDD mechanism. The IDD animal model serves as a crucial tool for studies of IDD mechanism and evaluation of IDD treatment.

Larger animals have been chosen for IDD models such as primates, sheep, goats, dogs, and rabbits due to their similarity with human anatomical structure to a great extent and the strong operability in terms of size of intervertebral discs (IVDs)[3–8]. However, these animal models are time-consuming and cost-intensive[9]. Mouse IVD is a poor representation of the human IVD based on geometrical measurements of the aspect ratio, nucleus pulposus to disc area ratio, and normalized height[10]. Despite the difference in size, mouse lumbar IVD segment exhibits mechanical properties similar to human IVD such as compression and torsion stiffness[11]. In addition, mouse IDD model has the advantage of low cost, relatively short IDD development, and more options for genetically modified animals and antibodies utilized in further mechanistic studies[12–15].

Experimental-induced IDD models vary from the inducers and applications. For example, collagenase-induced extracellular matrix (ECM) degeneration is appropriate for ECM regeneration research[16]. Genetically modified phenotype are suitable for studying the gene function in the IDD process and in genetic therapies[17]. Annulus fibrosus incision and smoke models mimic trauma and non-inflammation induced IDD[12,18].

Spinal instability (SI) leads to an unstable spine that is not in an optimal state of equilibrium. It can be caused by abnormal movement of a lumbar motion segment due to the weakness of the surrounding supportive tissue such as ligaments and muscles. It is also commonly seen post spinal fusion operation[19]. SI is considered as the main cause of IDD. Therefore, we aim to develop a SI mice model (focused on lumbar spine) that mimics the human IDD process[20,21].

In the protocol, we introduced the procedure of establishing lumbar spinal instability (LSI) mouse model by the resection of lumbar third (L_3) to lumbar fifth (L_5) spinous processes along with the supraspinous and interspinous ligaments (**Figure 1A,B**). The animal model develops IDD as early as 1-week post-surgery as shown by hypertrophy and porosity in endplates (EPs). IVD volume starts to decrease 2 weeks post-surgery through 16 weeks along with increased IVD score, which indicates the degree of IDD. We believe the detailed and visualized procedure is useful for researchers to establish the LSI mouse model in their laboratory and apply to IDD research as needed.

PROTOCOL:

The investigations described conform to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by Shanghai University of Traditional Chinese Medicine Animal Care and Use Committee. All surgical manipulations were

performed under deep anesthesia and the animals did not experience pain at any stage during the procedure.

1. Pre-operation preparation

1.1. Instrument sterilization: Steam-sterilize surgical instruments in an autoclave (121 °C for 15 min) prior to the surgery. Pack instruments in a metal container and maintain them until they are used in the surgery.

1.2. Surgery platform setup: Assign a bench area of at least 60 cm x 60 cm for the operation. Clean the surface of area with 75% alcohol and cover with a disposable medical towel. Place a sterile surgical instruments pack, reagents, surgical items onto a disposable medical towel within the upper 1/3 of area. Leave the remaining 2/3 of area clean for surgical operation.

1.3. Animal preparation

1.3.1. Place the animal (C57BL/6J mice, male, 8-week old) into the induction chamber. Turn on the vaporizer at an induction level of 4% for isoflurane and 4 L/min for oxygen. After the animal is fully anesthetized, maintain the anesthetic with the nose cone and the anesthetic delivery at a level of 1.5% for isoflurane and 0.4 L/min for oxygen during surgery. Monitor the animal for respiration.

1.3.2. Apply chlortetracycline hydrochloride eye ointment to prevent corneal dryness during the surgery.

1.3.3. Shave the surgical area on the dorsal surface from the lower thoracic region to the top of the sacral region using a small animal trimmer. Remove the shaved fur with tissue wipes.

1.3.4. Apply depilatory cream onto the shaved area and leave it there no longer than 3 min. Remove the cream with gauze and flush with 2 mL of 0.9% sterile saline.

1.3.5. Place a custom-made surgical cylindrical pad (**Figure 2A**) under the abdomen of the mouse to raise up the lumbar spine and facilitate the surgical operation.

2. Exposure of the lumbar third to lumbar fifth (L_3–L_5) spinous processes

2.1. Use the index finger to touch the subcutaneous spinous processes of the lumbar vertebrae, which are more outward, and compare with thoracic vertebrae and sacral vertebrae to identify the lumbar region.

2.2. Rinse the skin using 75% alcohol. Perform a 3–4 cm midline skin incision over the lumber region from the mid-thoracic region to the hip using a scalpel blade to expose the fascia.

2.3. Identify the lumbar spine by the morphology of the posterior fascia inserted onto the tips of the spinous processes. In detail, the third lumbar (L_3) to the first sacral (S_1) fasciae are distinct from other fasciae by their "V" shapes. The last "V" tip connects to the first sacral (S_1) fascia and the first "V" tip corresponds to the L_3 spinous process (**Figure 2B**).

2.4. Make the posterior paraspinous muscle incisions along the spinous processes from L_3 to L_5 on both sides laterally with a scalpel blade (**Figure 2C**). Control the incision depth towards the facets to reduce hemorrhage.

2.5. Separate the muscle layers using two ophthalmic forceps to expose L_3 to L_5 spinous processes and supraspinous ligaments.

3. Resection of L_3–L_5 spinous processes along with the ligaments

3.1. Separate individual spinous processes by cutting off interspinous ligaments using Venus shears (**Figure 2D**).

3.2. Resect the L_3–L_5 spinous processes along with the interspinous ligaments with Venus shears (**Figure 2E**).

3.3. Suture the skin incision with sterile silk braided (suture size 5.0) without reattachment of the paravertebral muscles.

3.4. Apply chlortetracycline hydrochloride ointment to the surgical site.

3.5. Place the animals in a warm chamber and monitor during recovery from the anesthesia. Monitor food and water intake before returning the animals to the home cage.

3.6. Monitor the animal once daily for the first 3 days after operation. The animal should be able to have a normal appetite and should heal with no sign of pus, hemorrhage, or swelling. They may have minor impairment in locomotion.

3.7. Carry out sham operations only by the detachment of the posterior paravertebral muscles from the L_3–L_5 vertebrae.

REPRESENTATIVE RESULTS:

The LSI mouse model is applied in the studies of IDD mechanism, IDD treatment, endplate (EP) degeneration such as sclerosis, and sensory innervation in EP[20–23]. The LSI mouse develops IDD and EP degenerative changes, as identified, by decreased IVD volume and height, increased EP volume, and increased IVD and EP scores.

The dissected and fixed lower thoracic and lumbar spine were examined by high-resolution Micro Computed Tomography (μCT) as previously described[20,21]. The lower thoracic lumbar with ribs were included for the identification of L_3–L_5 vertebrae (**Figure 3A**). X-rays of the L_3–L_5 spine on a

lateral view indicate existence and inexistence of spinous processes in Sham and LSI groups (**Figure 3B**). The results are clearer by 3D-reconstruction of L_3–L_5 spine on a left anterior oblique view (**Figure 3C**) and by transverse image of a L_3–L_5 vertebrae (**Figure 3D**).

Coronal images of the L_4–L_5 IVD were used to perform 3D histomorphometric analyses of IVD[20] (**Figure 4A**). IVD volume is defined as the region of interest (ROI) covering the whole invisible space between L_4 and L_5 vertebrae. Parameter: TV (total tissue volume) was used for 3D structural analysis (**Figure 4B**). IVD volume significantly increased 1 week post-surgery and started to decrease from 2 weeks to 16 weeks post operation as observed in **Figure 4C**.

The height of IVD space varied from the anterior to the posterior (**Figure 4E,G**). LSI had a significant effect on the rear site. Thus, the posterior one-third coronal plane of IVD space was chosen for IVD height measurement (**Figure 4D,E**). IVD height decreased from 2 weeks to 16 weeks post-surgery (**Figure 4F**), which was consistent with the findings in IVD volume (**Figure 4C**).

Coronal images of the L_4–L_5 IVD space were applied to 3D histomorphometric analyses of both cranial and caudal endplates (Eps) (**Figure 5A**). EP volume is defined as the ROI covering the whole bony plate adjacent to the vertebrae (**Figure 5A,B**)[21]. The anterior one-fourth coronal plane of five consecutive images of cranial EP were used for 3D reconstruction (**Figure 5C**), which showed increased cavities within cranial EP in LSI mice (**Figure 5D**). The results were also indicated by an increased percentage of trabecular separation values which were greater or equal to 0.089 (**Figure 5E**). Meanwhile, EP volumes significantly increased post-surgery (**Figure 5F**). Caudal EPs exhibit similar phenotype by LSI (**Figure 5G,H**), indicating LSI leads to EP hypertrophy and an increase in cavities.

L_5 vertebral bodies were reconstructed by drawing the outline of all transverse sections of each L_5 vertebral body without accessories and converting all 2D images to a 3D model. The construction and analysis were done with commercial software (e.g., NRecon v1.6 and CTAn v1.9, respectively). The volumes of L_5 vertebra slightly increase post-surgery but only have statistic difference between sham group and 16-week LSI group (**Figure 6B**). A significant decrease in BV/TV was also present 16 weeks post-surgery, indicating that LSI causes vertebral bone loss at a later stage (**Figure 6A,C**).

LSI induces IVD degeneration and EP degeneration as indicated by increased IVD and EP scores[24] (**Figure 7A,C**). A reduction in intracellular vacuoles of nucleus pulposus cells was accelerated in LSI groups (**Figure 7B**). Cavities increase in LSI EPs (**Figure 7D**) accompanied with increased number of osteoclasts as indicated by Trap staining (**Figure 7E,F**).

The data was shown as mean ± s.d. Statistical significance was determined by a *Student's t-test*. The level of significance was defined as $p < 0.05$. All data analyses were performed using SPSS 15.0.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of LSI mouse model. (**A**) Anatomy of L_3–L_5 vertebrae in the lower back area of the mouse. (**B**) Resection of spinous processes along with interspinous ligaments and supraspinous ligaments (marked pale). A red dotted line indicates a section plane.

Figure 2: Exposure of L_3–L_5 spinous processes and LSI operation. (**A**) A custom-made cylindrical pad is placed under the mouse's abdomen. (**B**) Exposure of the lumbar fasciae and identification of L_3 to S_1 spinous processes by "V" shapes. (**C**) Lateral paraspinous muscle incisions on both sides of L_3 to L_5 spinous processes. (**D**) Exposure of individual spinous processes by cutting off interspinous ligaments. (**E**) Resection of L_3–L_5 spinous processes with inter- and supra-spinous ligaments.

Figure 3: LSI identification by µCT. (**A**) Localization of L_3–L_5 vertebrae by ribs with thoracic vertebrae in X-rays. (**B**) X-rays on a lateral view and (**C**) 3D reconstruction on a left anterior oblique view of L_3–L_5 vertebrae in Sham and LSI groups. (**D**) Transverse plane of lumbar vertebra with the resection of the spinous process. (**D**) has been modified from Bian et al.[21].

Figure 4: LSI reduces IVD volume. (**A**) Consecutive images of the invisible space (Red) between L_4 and L_5 EPs are used for 3D reconstruction. (**B**) IVD volume is defined by the TV of (**A**). (**C**) Quantification of L_4–L_5 IVD volume at five timepoints post-operation. N = 8 per group. Data is shown as mean ± s.d. $*p < 0.05$, $**p < 0.01$ versus Sham. (**D**) Transverse plane and (**E**) mid-sagittal plane of lumbar vertebral bodies. Blue double-arrows indicate anteroposterior diameter. Yellow line indicates posterior 1/3 plane. (**F**) Reconstruction of cranial and caudal EPs using five consecutive images of posterior 1/3 coronal plane of L_4–L_5. Red indicates IVD space. (**G**) Mid-sagittal plane of L_4–L_5. (**C**) has been modified from Bian et al.[20]. (**F,G**) have been modified from Bian et al.[21].

Figure 5: LSI induces EP hypertrophy and porosity. (**A**) Coronal plane of L_4–L_5. Red dotted line indicates image of caudal EP used for 3D construction. (**B**) Reconstruction of caudal L_4 and cranial L_5. Blue cartoon indicates caudal EP of L_4–L_5. (**C**) Mid-sagittal plane of L_4–L_5. Blue double-arrows indicate anteroposterior diameter. Yellow line indicates anterior 1/4 plane. (**D**) Reconstruction of cranial EPs by five consecutive images of anterior 1/4 plane of L_4–L_5. (**E,G**) Percentage of trabecular separation distribution of cranial (**E**) and caudal (**G**) EPs obtained from µCT analyses. (**F,H**) Quantification of cranial (**F**) and caudal (**H**). L_4–L_5 EP volume in indicated timepoints. N = 8 per group. Data is shown as mean ± s.d.* $p < 0.05$ versus. Sham. (**D–H**) have been modified from Bian et al.[21].

Figure 6: LSI causes vertebral bone loss at late stage. (**A**) Reconstruction of L_5 vertebral bodies in 16-week Sham and LSI groups. (**B,C**) Quantification of L_5 vertebra TV (**B**) and BV/TV (**C**). N = 8 per group. Data is shown as mean ± s.d.* $p < 0.05$, ** $p < 0.01$ versus. Sham. (**B**) has been modified from Bian et al.[21].

Figure 7: LSI leads to IVD and EP degeneration. (**A**) IVD score in LSI or sham mice as an indication of IVD degeneration. (**B**) Representative images of Safranin O staining for NPs in L_4–L_5 IVD. White indicates vacuoles. Red indicates proteoglycan. (**C**) EP score in LSI or sham group as an indication

of EP degeneration. (**D**) Representative images of Safranin O-Fast green staining for caudal L_4–L_5 EPs. Green/blue stains calcified cavities. (**E**) Representative images of trap staining for caudal L_4–L_5 EPs. Purple indicates $Trap^+$. N = 6 per group. Data is shown as mean ± s.d.* $p < 0.05$, ** $p < 0.01$ versus. Sham. (**F**) Quantification of $Trap^+$ osteoclasts in (**E**). (**A,B**) have been modified from Bian et al.[20]. (**C–F**) have been modified from Bian et al.[21].

DISCUSSION:

We developed the lumbar spine instability mouse model based on the cervical spondylosis mouse model in which the posterior paravertebral muscles from the vertebrae were detached and the spinous processes along with the supraspinous and interspinous ligaments were resected[25]. We performed a similar operation onto the lumbar spine, which has more prominent spinous processes. The LSI mouse model developed similar IDD in the lumbar spine.

The advantages of the LSI model include strong operability, no requirement of a special equipment, reproducibility, and a relatively short period of IDD development.

Some key points are presented here to help improve the success rate during the operation. These are also the critical steps. First, remove the hair on the lower back portion, as clear as possible, because any shaved hair left in the wound may cause anaphylactic reaction. Second, a cylindrical pad or any other pad is recommended to raise up the lumbar vertebrae. Third, use micro scissors to control the incision depth and hemorrhage. When hematocoelia is noticed during the operation, stop the operation and sacrifice the mouse since the mouse will not survive during or post-surgery. Fourth, reattachment of paraspinous muscle is not recommended because the reattachment may make up the instability. Fifth, a full resection of the whole L_3–L_5 spinous processes reduces variability in individual model. Sixth, avoid injuring surrounding nerves and blood vessels, otherwise, the mouse may develop non-canonic pathological changes. If the models do not exhibit the typical phenotype as shown in the results, check the above six points.

The success of this LSI model can be assessed by two golden standards including decreased IVD volume measured by small animal MRI or by μCT, and an IVD score based on the histological observation. The LSI model develops IDD as early as 2 weeks post LSI surgery, but develops porosity in endplate as early as 1 week, as observed. It is suitable for study on nucleus pulpous shrinkage, endplate sclerosis, IDD related to osteoclast-induced cytokines, IDD-induced osteoporosis (16 weeks post LSI) etc.

There are some limitations in the LSI model. LSI operation is a relatively great trauma for the mouse. The inflammation is inevitable and usually seen within 7 days post operation. Thus, this model is not suitable for observing early pathological changes of IDD, especially within 7 days caused by mechanical loading changes.

The model can be modified by targeting on different lumbar vertebrae such as L_5 only or from L_1 to L_5. Healthy control is also recommended in addition to sham groups.

In summary, we developed a surgical-induced lumbar IDD mouse model and have the procedure visualized to help others reproduce the animal model and apply it in IDD studies.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

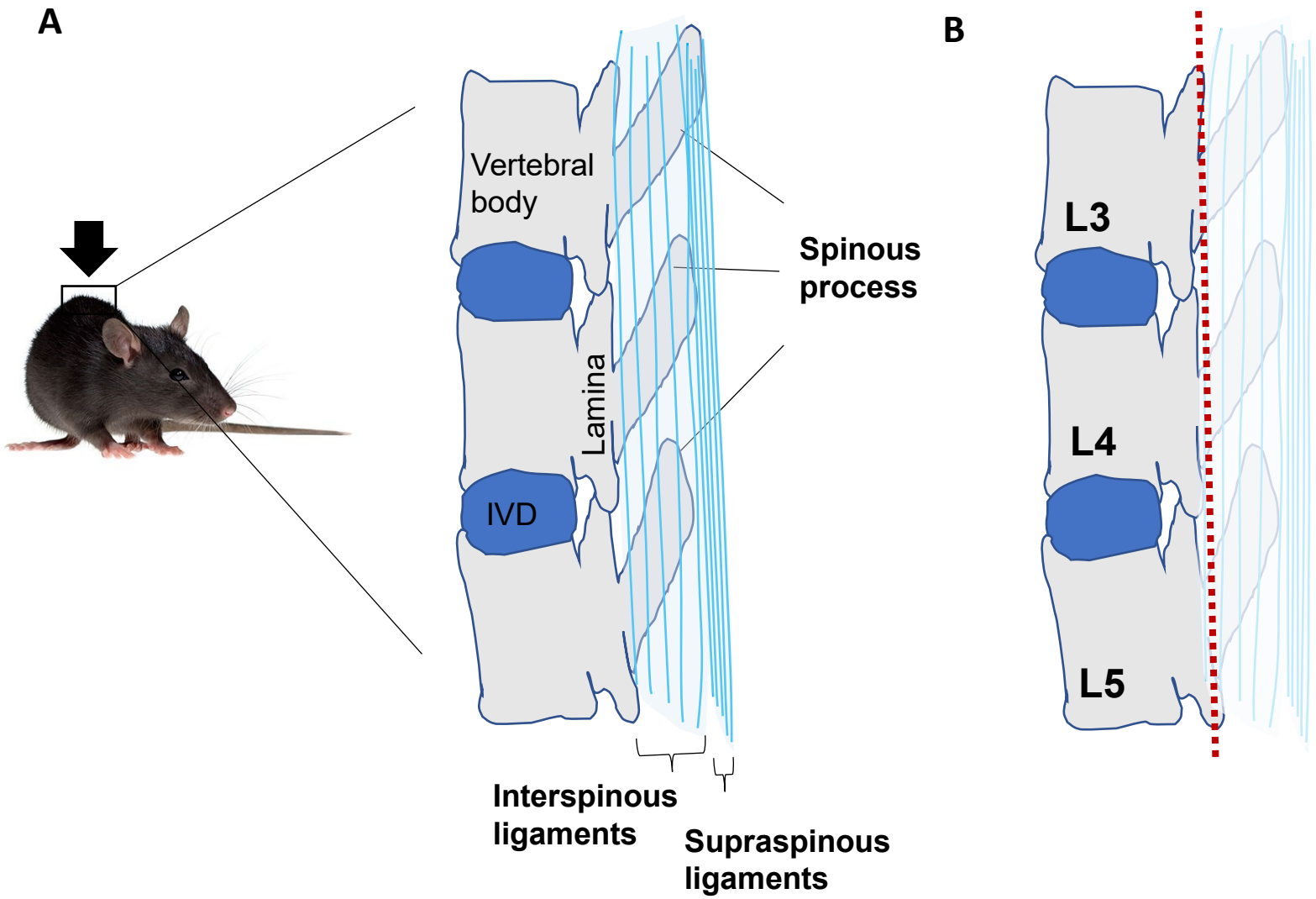
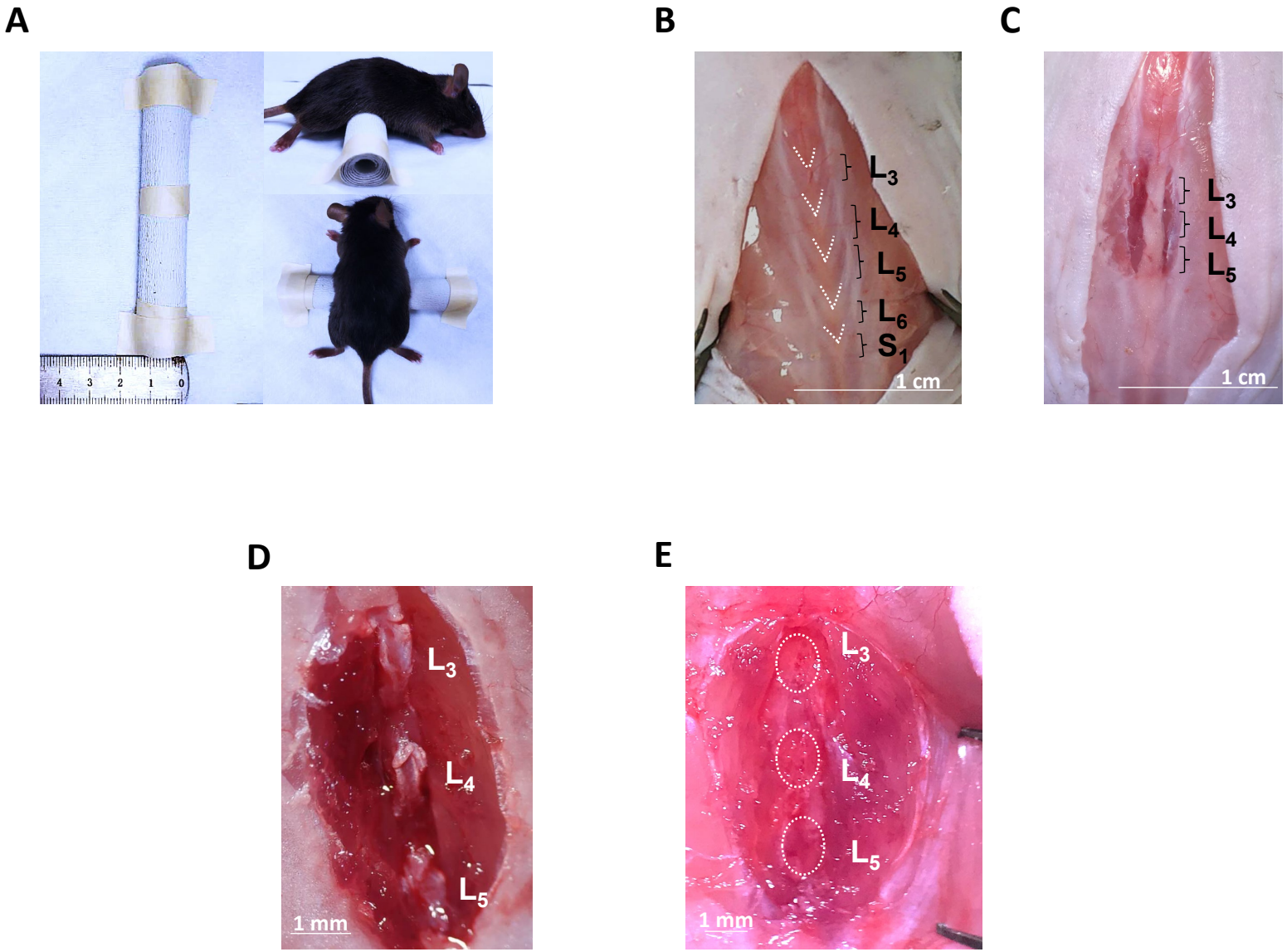


Figure 2



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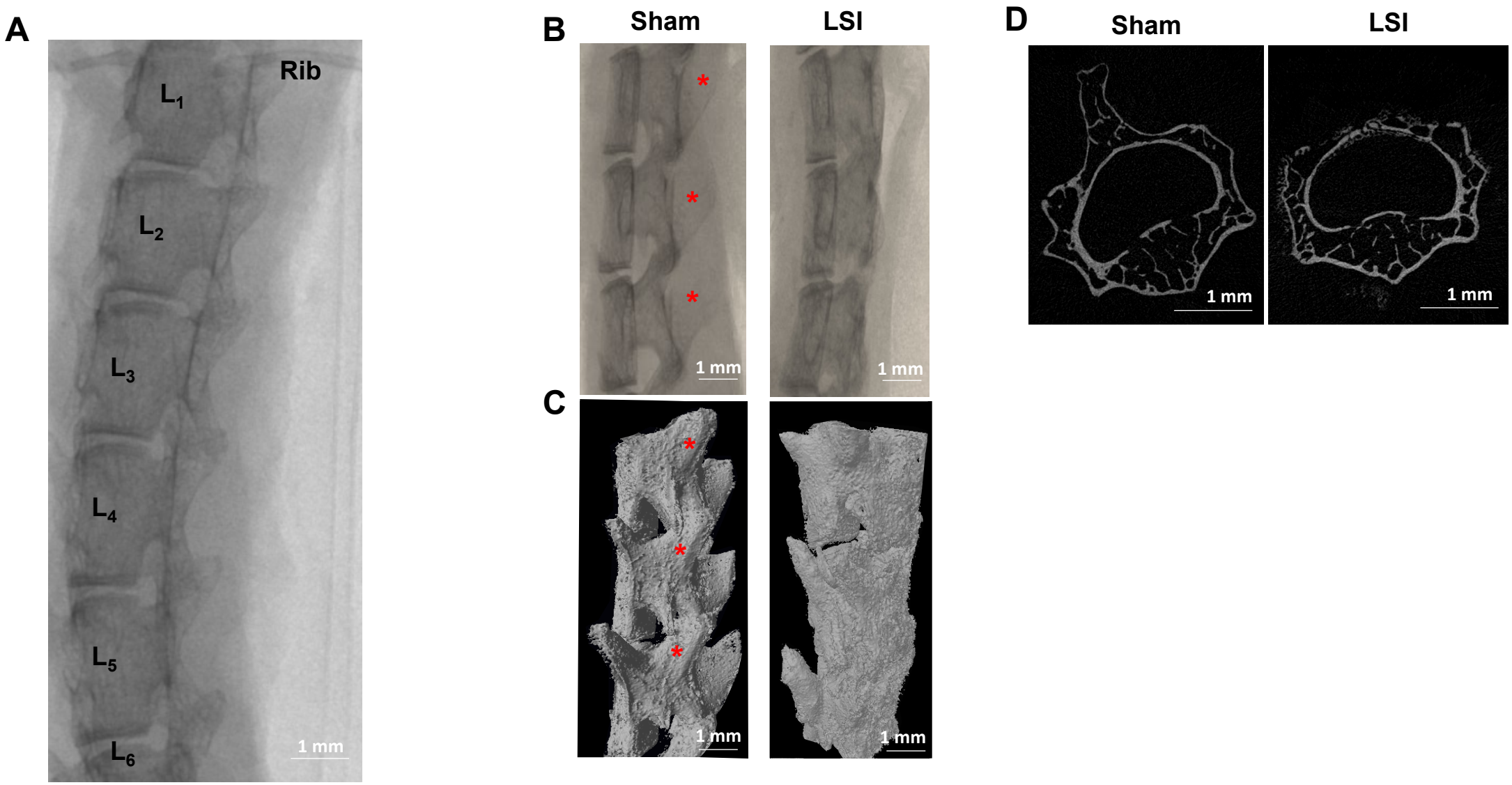

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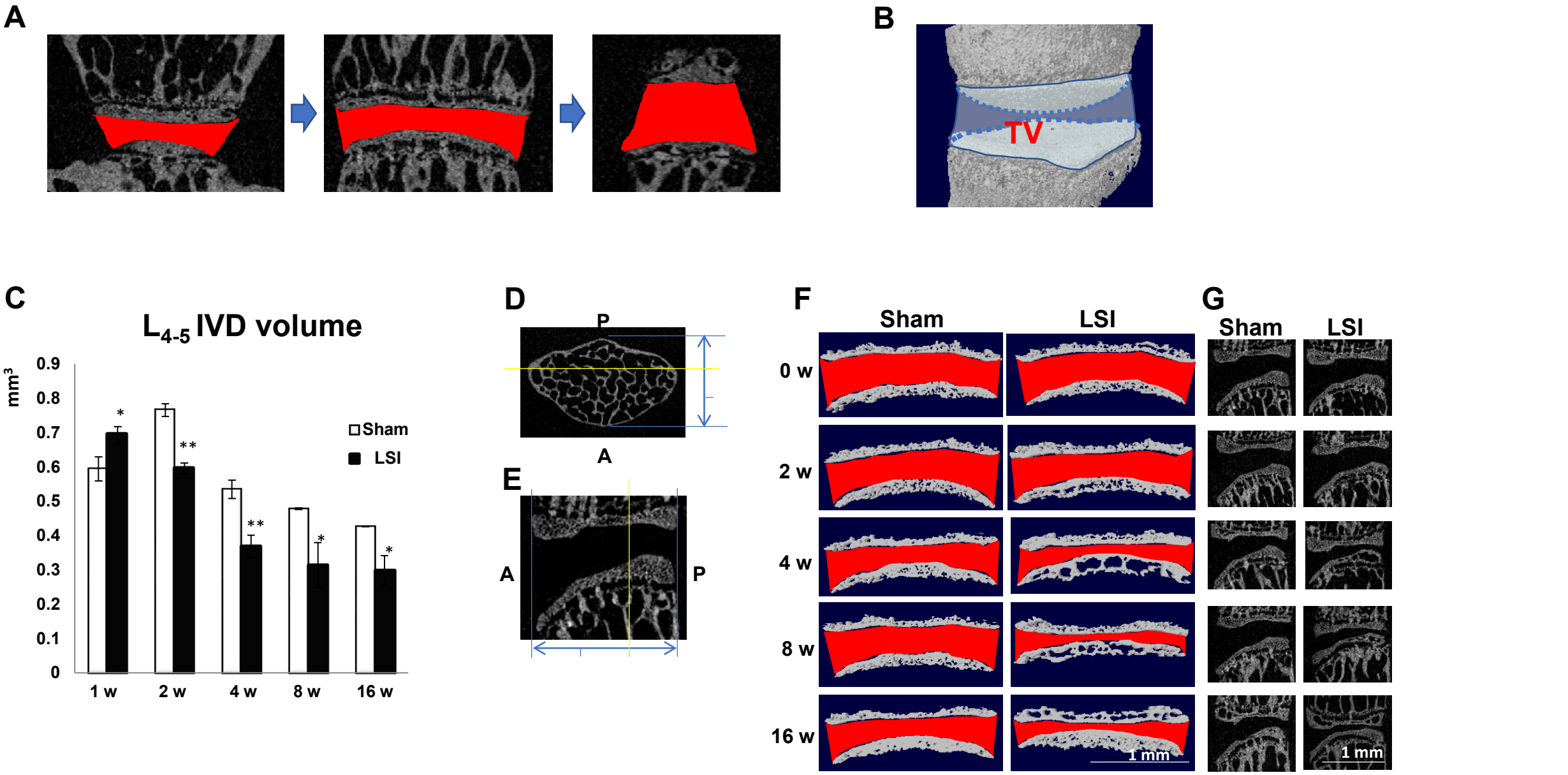


Figure 5

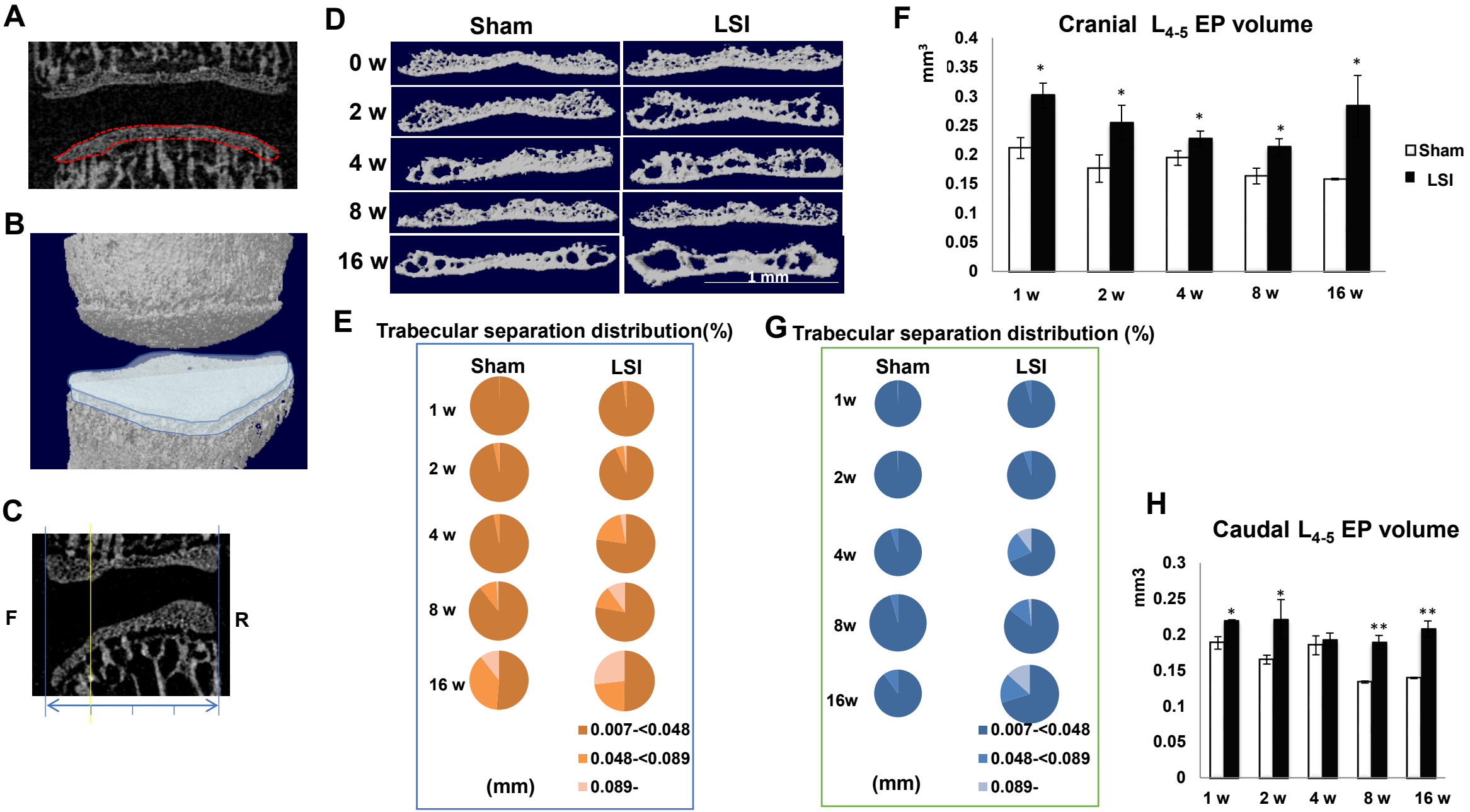


Figure 6

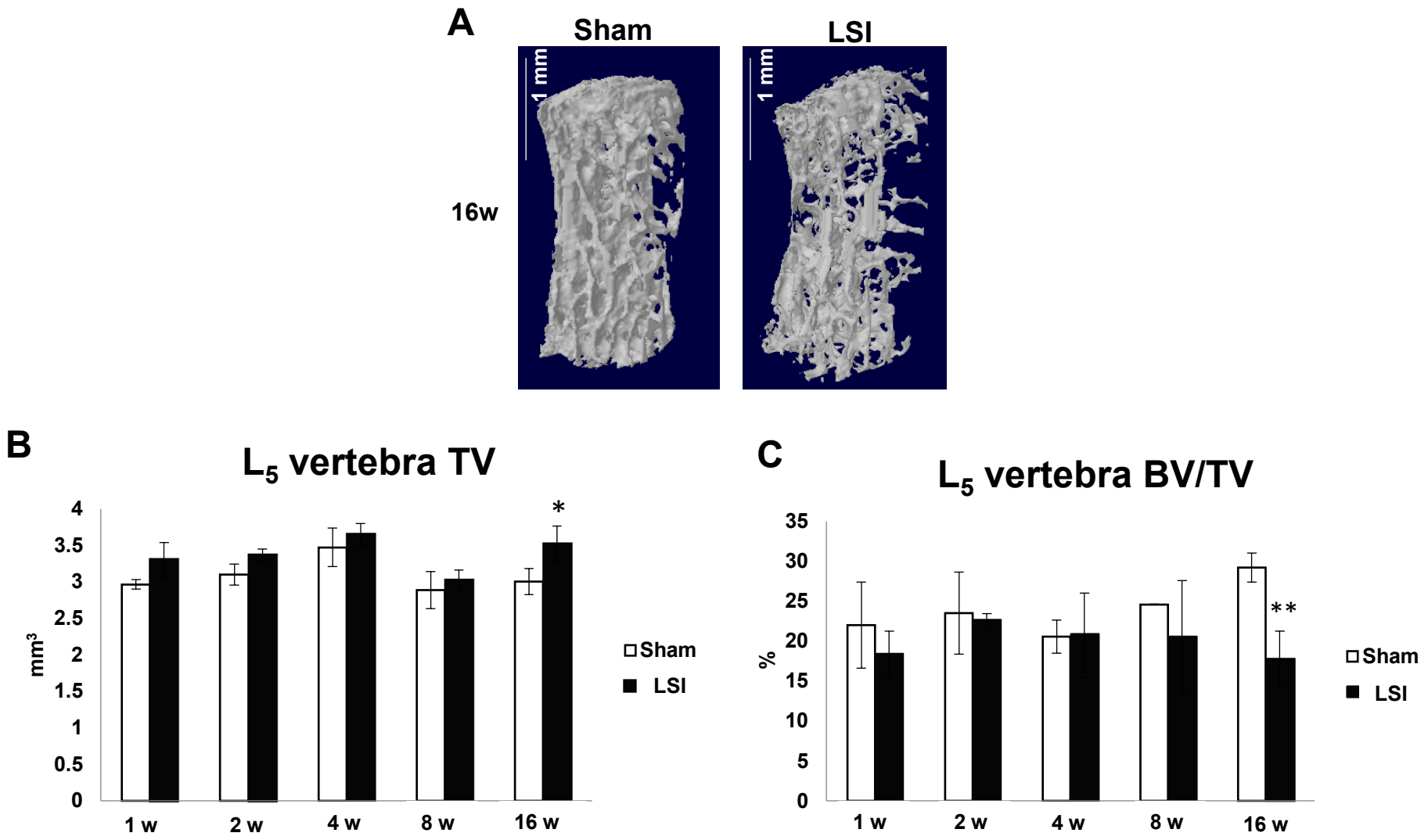
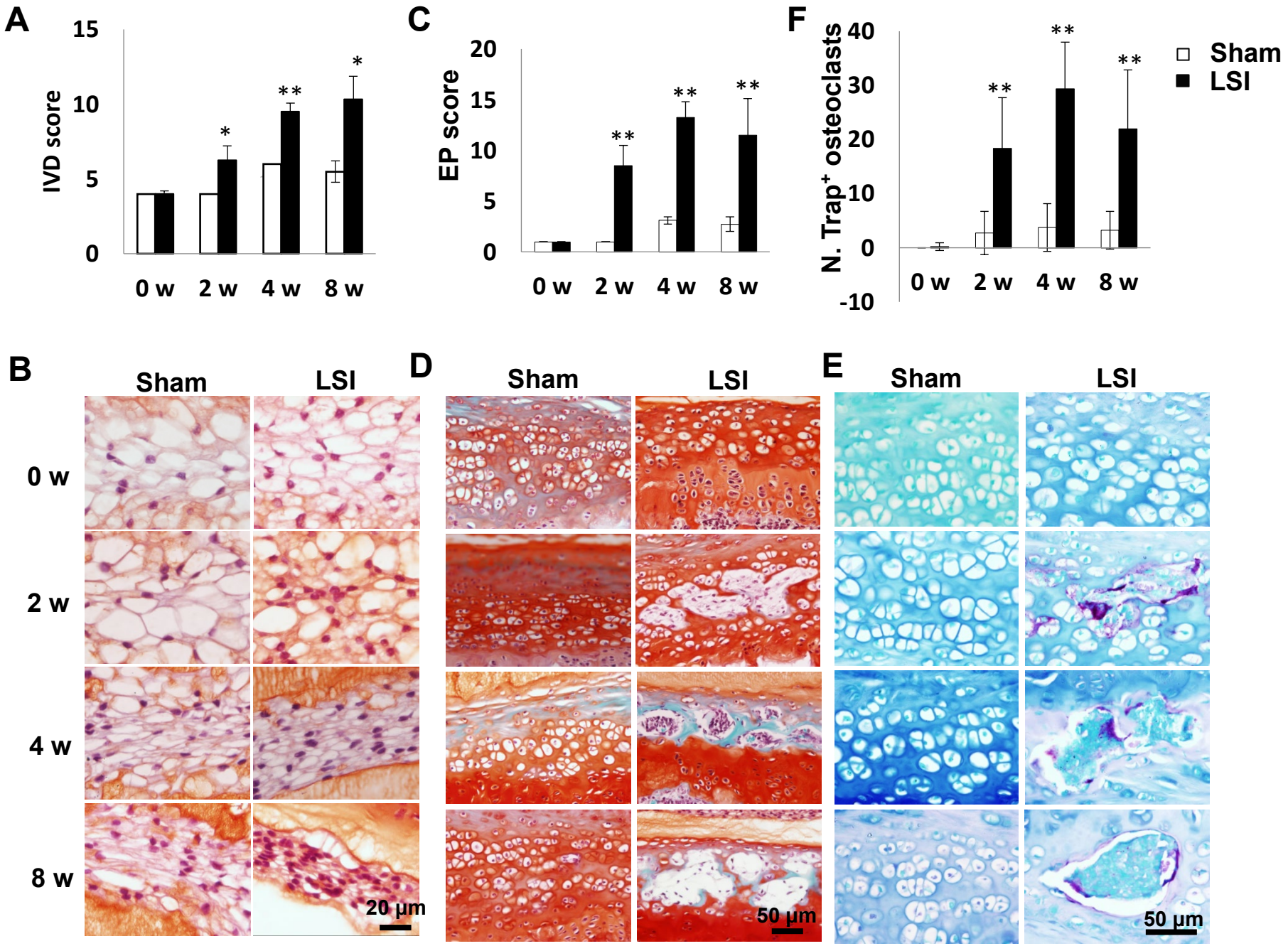


Figure 7



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Chlortetracycline Hydrochloride Eye Ointment	Shanghai General Pharmaceutical Co., Ltd.	H31021931	Prevent eye dry, Prevent wound infection
C57BL/6J male mice	Tian-jiang Pharmaceuticals Company (Jiangsu, CN)	SCXK2018-0004	Animal model
Disposable medical towel	Henan Huayu Medical Devices Co., Ltd.	20160090	Platform for surgical operation
Inhalant anesthesia equipment	MIDMARK	Matrx 3000	Anesthesia
Isoflurane	Shenzhen RWD Life Technology Co., Ltd.	1903715	Anesthesia
Lidocaine hydrochloride	Shandong Hualu Pharmaceutical Co., Ltd.	H37022839	Pain relief
Medical suture needle	Shanghai Pudong Jinhuan Medical Products Co., Ltd.	20S0401J	Suture skin
Ophthalmic forceps	Shanghai Medical Devices (Group) Co., Ltd. Surgical Instruments Factory	JD1050	Clip the skin
Ophthalmic scissors(10cm)	Shanghai Medical Devices (Group) Co., Ltd. Surgical Instruments Factory	Y00030	Skin incision
silk braided	Shanghai Pudong Jinhuan Medical Products Co., Ltd.	11V0820	Suture skin

Small animal trimmer	Shanghai Feike Electric Co., Ltd.	FC5910	Hair removal
Sterile surgical blades(12#)	Shanghai Pudong Jinhuan Medical Products Co., Ltd.	35T0707	Muscle incision
Veet hair removal cream	RECKITT BENCKISER (India) Ltd	NA	Hair removal
Venus shears	Mingren medical equipment	Length:12.5cm	Clip the muscle and spinous process

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• **Protocol Highlight:**
1) Unhighlight anesthesia steps as it will not be filmed.

Completed.

• **Results:**
1) Remove the numbered list and avoid subheadings.
2) Mention statistical test used.
Completed.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
Combined with Reviewer's comments and made changes marked in blue.

• **Figures:**
1) Remove text "Figure #" from all figures.
2) Figure 2,3,4: Scale bars will be useful.
3) Avoid crashing units e.g. 1mm should be changed to 1 mm and 16w should be 16 w.
4) Please fix the units on the axes. i.e., "3" in "mm3" should be superscripted.

Completed.

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Completed.

• **Table of Materials:**
1) Please sort in alphabetical order.

Completed.

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Added.

Comments from Peer-Reviewers:

_____

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Liu et al. investigate a lumbar spine instability for IVDD in a mouse model using radiographic, uCT, and histologic assessments. However, the study has several major flaws that should be remedied.

Major Concerns:
First, the details of design for the study need to be described, which is important for the reader to repeat the experiment, such as the number of animals in each group and the protocol for uCT and histology. Second, the standard for IVDD should be defined by positive control and negative control. Third, the discussion part is the weak link of the paper and makes it difficult to be considered it for publication. Authors should focus on the protocol and not only the surgery process, but also the measure of the experiment and how to make sure the model is successful.

Thank you for Reviewer's valuable comments. The number of animals in each group was indicated in figure legends. We did not add the protocol for uCT and histology since 1) the manuscript focuses on animal model only 2) the protocol for uCT and histology can be customized in different lab that will not affect the results. We agree positive control is a supportive evidence. However, so far, mouse IVDD model is still controversial and there is no ideal mouse IVDD model that can be chosen as a positive control. That is also why we developed this mouse IDD model. Although we did not have a positive control, we did apply golden standard for IDD assessment such as volume of IVD (Fig 4C) and IVD score (Fig 7A). In the discussion part, we added the measure of the experiment as suggested.

Minor Concerns:
Abstract
The results only tested L4-5 IVD, this should be specified.

Added.

Introduction
The mechanism of spinal instability (SI) cause IDD should be explained and the references need to be added.

Methods
The total numbers of animals should be described.

It is indicated in figure legends.

The details for sham groups should be described?

It is described in PROTOCOL 3.7.

The uCT and histology used within the protocol should be described in details or cited, like the parameters of uCT and the thickness of slices.
Added cited reference.

Results
Healthy control should be added, not only sham groups.

We thank for the suggestion. However, we do not have healthy control data. We added this suggestion in discussion part.

The whole discs' structures need to be shown by histology, including NP and AF.

The zoom out images are not accessible at this time. We appreciate reviewer's understanding.

What about the other levels of IVD, L3-4 and L 2-3?
The other levels of IVD exhibit similar pathological changes in LSI groups as compared with same levels of IVD in Sham groups. However, since different levels of IVD exhibit different normal phenotype. For example, the upper level of IVD has more vacuoles in NP and less porosities in EP compared to lower IVD. To avoid being redundant, we only showed results of $L_{4\text{-}5}$.

Discussion
The authors should not just repeat the steps, instead modify and troubleshoot the critical steps in the protocol.

Added.

Figures
Figure 2 and figure 3 should be combined.

Combined.

Figure 5 and figure 6 need to be simplified and rearranged.
Completed.

Reviewer #2:
This manuscript describes a mouse model of lumbar disc degeneration by removing spinal processes and

associated ligaments. This method and results have been reported in 2016. Although the spinal instability model is interesting, the writing can be improved significantly. Please correct typos and grammar through the text.

We thank for Reviewer's comments. We carefully went through the full text and had typos and grammar corrected.

Specific points:
1. Introduction: it would be better to mention the difference between rodents and humans. Yet, animal models still serve as valuable tools to understand pathological processes of disc degeneration.

Added as Reviewer suggested.

2. Protocol: as this is supposed to be a detailed method, please describe clearly the details of animals, procedures, and postoperative care. For example, micro-dissection tools, suture size, animal age, gender, etc. if animals were fully anesthetized, what is the purpose of lidocaine injection?

We appreciate Reviewer's reminder. Local lidocaine injection is not necessary here and we have it removed.

We added animal information to 1.3.1, postoperative care was mentioned in 3.5, 3.6. Other details were added and marked in blue.

3. Results: how the x-rays and micro-CT were taken and analyzed? What methods were used to score IVD degeneration? The purpose of showing trap staining? Figure 8B showed degenerative changes in sham animals? It is hard to distinguish the sham from LSI groups.

We added citation for detailed method of micro-CT, IVD score. Trap staining indicates occurrence of bone remodeling in endplate. It might be helpful for investigators who study on IDD related to osteoclast induced cytokines. Figure 7B left panel showed NP changes by IVD maturation (0 w represents 8-week-old, 2 w represents 10-week-old, 4 w represents 12-week-old, 8 w represents 16-week-old), and right panel showed accelerated NP shrinkage induced by LSI. The relatively significant difference is a reduction in intracellular vacuoles of NP cells.

4. Most of these figures were published in 2016. It may not be necessary to shown all these images here again.

This protocol focuses on animal model, so we added 16 weeks post LSI data that has not been published to show the long-term effect of LSI surgery (Figure 4,5,6). We added radiographic images that show the differences between Sham and LSI (Figure 3). Figure 1 was modified to better depict the model at anatomic level. Only Figure 7 is the same as previous publication. We kept it here to show the histological changes in addition to radiographic changes.

Reviewer #3:
Manuscript Summary:
The model described is interesting and it will helpful for the research community. As it is hard to imaging how the video will look like there is one major concern regarding this manuscript.
Most of the figures describe the μCT analysis of the IVD degeneration, whereas the protocol described in details the surgical procedures. It seems like the manuscript was reviewed before the the resubmitted version is quite detailed.

This protocol focuses on animal model. We applied μCT analysis to evaluate the success of the model.

Major Concerns:

Spe-wise protocol to perform the disc volume analysis is needed, preferably with screen shots and explanation how it was done. This should also be part of the video in eventually.

We appreciate Reviewer's suggestion. The protocol focuses on LSI surgery. We believe IVD volume measured by μCT or MRI will be customized in different labs and will not significantly affect the results. Details about μCT measurement have been described in our previous publication (Bian, Q., Ma, L., et al. Mechanosignaling activation of TGFβ maintains intervertebral disc homeostasis. Bone research 5, 17008, doi:10.1038/boneres.2017.8 (2017). Bian, Q., Jain, A., et al. Excessive activation of tgfβ by spinal instability causes vertebral endplate sclerosis. Scientific Reports 6, 27093, doi:10.1038/srep27093 (2016)).

Minor Concerns:
The μCT image in Fig 4C is very noisy, meaning the threshold was not chosen correctly.

We thank for Reviewer's comments. We will adjust the threshold to make the 3D construction with less noisy in future. At this time, we removed background of the images.

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I am going to introduce generation of animal models on JoVE journal, and will re-use partial Figure1,2,3. The new preparing manuscript focuses on how to generate animal model.

I look forward to your reply,

Best,
Qin Bian