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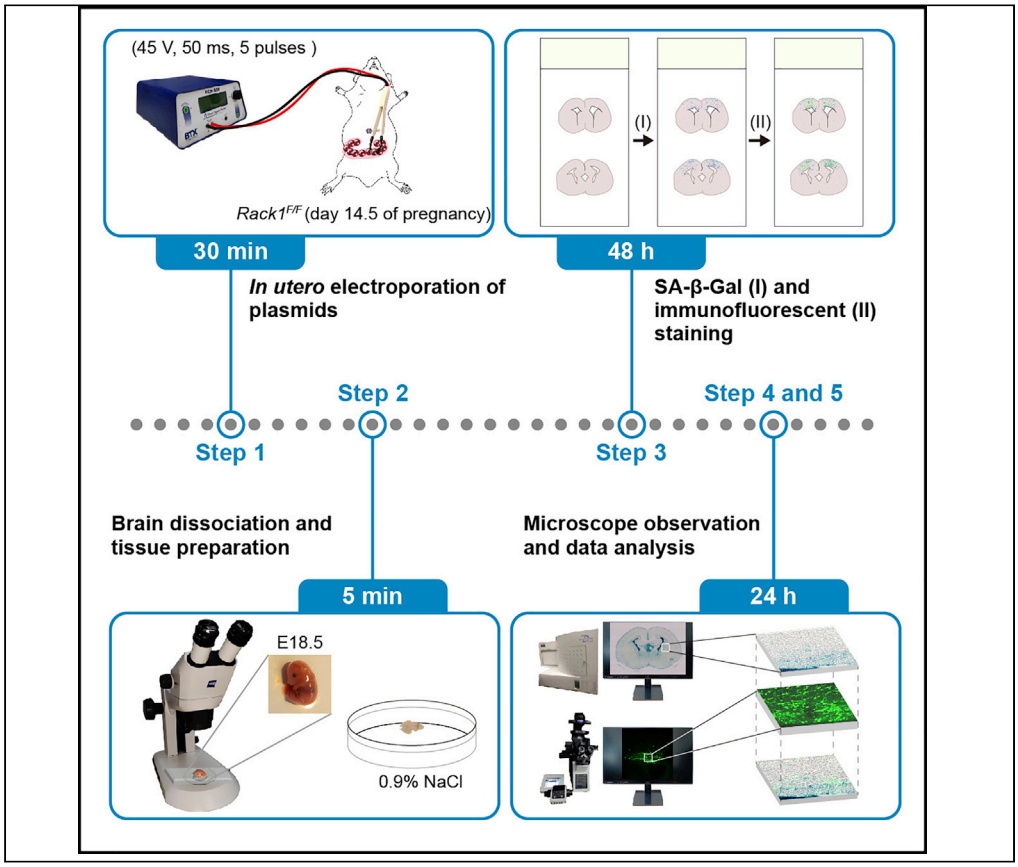
Protocol for analysis of senescent neuronal stem cells in genetic-modified embryonic mice using *in utero* electroporation technique



Here, we present a detailed protocol for identification and analysis of senescent neuronal stem cells (NSCs) in the developing mouse embryonic neocortex using *in utero* electroporation. We describe the procedures of *in utero* electroporation and brain slide preparation. We then detail the procedures of senescence-associated β-galactosidase (SA-β-Gal) staining and immunofluorescence staining, followed by microscope imaging analysis. This combined approach can be used to study the *in situ* senescence of *in utero* electroporated embryonic brains.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Identification of senescent cells in the developing brain by SA-β-Gal staining

In utero electroporation of pCAG-Cre-GFP plasmid into NSCs in loxP embryonic brains

SA-β-Gal and anti-GFP co-staining to identify the senescent NSCs in embryonic brain

A modified protocol for imaging and quantification of *in situ* labeled senescent NSCs

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Protocol for analysis of senescent neuronal stem cells in genetic-modified embryonic mice using *in utero* electroporation technique

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SUMMARY

Here, we present a detailed protocol for identification and analysis of senescent neuronal stem cells (NSCs) in the developing mouse embryonic neocortex using *in utero* electroporation. We describe the procedures of *in utero* electroporation and brain slide preparation. We then detail the procedures of senescence-associated β-galactosidase (SA-β-Gal) staining and immunofluorescence staining, followed by microscope imaging analysis. This combined approach can be used to study the *in situ* senescence of *in utero* electroporated embryonic brains.

For complete details on the use and execution of this protocol, please refer to Zhu et al. (2021).

BEFORE YOU BEGIN

In this protocol, we took Rack1 gene as an example to describe steps for labeling in situ senescent cells within the in utero electroporated embryonic brain. First, Rack1 deletion in neuronal stem cells (NSCs) was generated by pCAG-Cre-GFP plasmid (pCAG-dCre-GFP as control) in utero electroporation in the developing neocortex. Second, tissue dissection, cryosection, SA-β-Gal staining and immunofluorescence staining of slices were performed to acquire SA-β-Gal+, GFP+ and Sox2+ cells, respectively. Third, in situ labeled senescent cortical NSCs were analyzed to determine whether Rack1 can regulate the senescence of embryonic cortical NSCs. It should be noted that other genes that regulate the cellular senescence in developing cerebral cortex can also be studied using this method. The detailed materials, reagents, concentrations, and equipment related to this protocol can be found below. All procedures were carried out in accordance with animal welfare regulations and approved by the local Ethics Committee and local regulatory authorities of the institute.

Preparation of *Rack1$^{F/F}$* and related pregnant mice (about E14.5)

Timing: 15 days

1. Set up overnight (12 h–16 h) breeding in the afternoon by using one male and one *Rack1$^{F/F}$* or two *Rack1$^{F/F}$* females in each cage.
2. Check plugs of the females in the next morning.

⚠ CRITICAL: Avoid setting up new crosses by using females when no plugs were observed the night before. Because the mouse brain develops quite quickly at early embryonic stages, it is imperative to calculate the embryonic days as accurately as possible.

⚠ CRITICAL: Prepare more than one pregnant female for each experiment to make sure you can harvest enough embryonic mice for your experiments. You may need 10–15 embryos for each plasmid to ensure enough embryonic mice can be collected to proceed further.

pCAG-dCre-GFP and pCAG-Cre-GFP plasmids extraction

Timing: 2 days

3. Transform the plasmids into *Escherichia coli* (*E. coli*) DH5α chemically competent cells and amplify at 37°C.
4. Extract and purify the plasmid DNA from *E. coli* using the EndoFree Maxi Plasmid Kit V2 (Tiangen). Adjust the final concentration of the plasmids as 1 μg/μL.

⚠ CRITICAL: Extract the plasmid immediately after the harvest of bacteria. The OD_{600} of the bacteria collected is more than 1.0. And prepare more than 100 mL 2×YT medium to amplify each plasmid.

⚠ CRITICAL: Adjust the final concentration of each plasmid to 1 μg/μl to facilitate further *in utero* electroporation. And aliquot the purified plasmids into tubes (20 μl for each tube) and store at −20°C.

Repackage of reagents in senescence-associated β-galactosidase staining kit

Timing: 1 h

5. Divide the β-galactosidase staining solution A and B into tubes to a final volume of 20 μL.
6. Divide Solution C and fixative into tubes to a final amount of 1 mL.
7. Divide X-gal solution into tubes to a final amount of 100 μL.
8. Store all of the reagents at −20°C.

⚠ CRITICAL: Keep all stocks on ice and aliquot them to avoid repeated freezing and thawing immediately upon receipt of the kit.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-GFP	Invitrogen	Cat# A11122; RRID: AB_221569
Mouse monoclonal anti-Sox2	Abcam	Cat# ab79351; RRID: AB_10710406
Goat anti-Rabbit IgY (H+L) CF®488	Biotium	Cat# 20012; RRID: AB_10559670
Goat anti-Mouse IgY (H+L) CF®568	Biotium	Cat# 20101; RRID: AB_10559187
Bacterial and virus strains		
Escherichia coli (E. coli) DH5α competent cells	TransGen Biotech	Cat# CD201-01
Chemicals, peptides, and recombinant proteins		
Sodium pentobarbital	Sigma-Aldrich	Cat# 52944-66-8
Sucrose	Sigma-Aldrich	Cat# 57-50-1

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mounting medium	Invitrogen	Cat# P36961
Proteinase K	cwbiotech	Cat# CW2584M
PCR 2× Mix buffer	cwbiotech	Cat# CW0690L
PBS	ZSGB-BIO	Cat# ZLI-9061
Depilatory cream	Veet	N/A
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat# 1933
Normal goat serum (NGS)	ZSGB-BIO	Cat# ZLI-9021
Paraformaldehyde	Solarbio	Cat# P1110
O.C.T. Compound	SAKURA	Cat# 4583
Saline	Solarbio	Cat# IN9000
2×YT medium	Sigma-Aldrich	Cat# Y2377
Fast Green	Sigma-Aldrich	Cat# F7252
Triton X-100	Sigma-Aldrich	Cat# X100
Critical commercial assays		
Senescence-associated β-galactosidase staining kit	Beyotime	Cat# C0602
EndoFree Maxi Plasmid Kit V2	TIANGEN	Cat# DP120
Experimental models: Organisms/strains		
Mouse: Rack1$^{F/F}$ (C57BL/6, E14.5 and E18.5)	(Zhao et al., 2015)	N/A
Oligonucleotides		
Primer used for genotyping: Rack1 loxP-F:	This paper	CGCTGCGCCTCTGGGATCTCA
Primer used for genotyping: Rack1 loxP-R:	This paper	TGGTGTGGCCGACAAATCGCC
Recombinant DNA		
pCAG-dCre-GFP	A gift from Dr. Weixiang Guo, Institute of Genetics and Developmental Biology, CAS	N/A
pCAG-Cre-GFP	A gift from Dr. Weixiang Guo, Institute of Genetics and Developmental Biology, CAS	N/A
Software and algorithms		
Image J	(Schneider et al., 2012)	https://imagej.nih.gov/ij/
NDP 2.0 view	Hamamatsu	https://www.hamamatsu.com/jp/en/product/type/U12388-01/index.html
GraphPad Prism8	Prism	https://www.graphpad.com/scientific-software/prism/
Olympus FV-1200	Olympus	https://www.olympus-lifescience.com.cn/es/support/downloads/
Olympus Fluoview Ver.4.2b View	Olympus	https://www.olympus-lifescience.com/es/support/downloads/#!dlOpen=%23detail847249651
Adobe Photoshop CC	Adobe	https://www.adobe.com/products/photoshop.html
Other		
Electro Square PoratorTM	BTX	ECM 830
Confocal Laser Scanning Microscope	Olympus	FV1200
NanoZoomer Digital Pathology	Hamamatsu	NanoZoomer2.0
Cryomold	Thermo Fisher Scientific	CRYOSTAR NX50
Incubator	Thermo Fisher Scientific	IMC18

MATERIALS AND EQUIPMENT

Mouse line: In this protocol, we choose Rack1 gene as a positive example to study the role of it in neuronal cell senescence. Before the experiment, the Rack1$^{F/F}$ mice are crossed with wild-type C57BL/6J mice to improve the stability and quality of the homozygous. The homozygous, heterozygous, and wild type mice were genotyped by PCR, and keep homozygous mice for pregnant female preparation.

Senescence-associated β-galactosidase staining buffer		
Reagent	Final concentration	Amount
Solution A (potassium ferrocyanide)	10 μL/mL	500 μL
Solution B (potassium ferricyanide)	10 μL/mL	500 μL
Solution C (citric acid-sodium phosphate)	880 μL/mL	44 mL
X-gal solution	100 μL/mL	5 mL
Total	**n/a**	**50 mL**
Storage temperature: −20°C; maximum time for storage: 12 months.		

Note: Use fresh-made senescence-associated β-galactosidase staining buffer in each staining process. Other commercial senescence-associated β-galactosidase staining kits are optional, such as the kit from the Cell Signaling Technology (#9860). In particular, the β-galactosidase activity in senescent cells can also be detected by X-Gal staining at pH 6.0.

Proteinase K solution		
Reagent	Final concentration	Amount
Proteinase K	**10 mg/mL**	100 mg
ddH_2O	n/a	10 mL
Total		**10 mL**
Storage temperature: −20°C; maximum time for storage: 3 months.		

Note: Working concentration is 0.2 mg/mL.

Bovine serum albumin solution		
Reagent	Final concentration	Amount
bovine serum albumin	**30 mg/mL**	300 mg
TritonX-100	0.3% (V/V)	30 μL
PBS	0.01 M	9.97 mL
Total		**10 mL**
Storage temperature: −20°C; maximum time for storage: 3 months.		

Normal goat serum solution		
Reagent	Final concentration	Amount
normal goat serum	**10% (V/V)**	1 mL
TritonX-100	0.3% (V/V)	30 μL
PBS	0.01 M	8.97 mL
Total		**10 mL**
Storage temperature: −20°C; maximum time for storage: 3 months.		

Sodium pentobarbital solution		
Reagent	Final concentration	Amount
Sodium pentobarbital	**1 mg/mL**	10 mg
0.9% NaCl	n/a	10 mL
Total		**10 mL**
Storage temperature: 4°C; maximum time for storage: 7 days.		

Note: Use fresh-made Sodium pentobarbital solution. We also encourage the use of other legal anesthetics, such as inhaled anesthetics isoflurane or urethane.

Alternatives: inhaled anesthetics isoflurane or urethane.

Plasmid solutions		
Plasmids	Final concentration	Amount
pCAG-dCre-GFP or pCAG-Cre-GFP	1 μg/μL	99 μL
Fast Green	1 μg/mL	1 μL
Total		**100 μL**
Storage temperature: −20°C; maximum time for storage: 6 months.		

Electroporation settings	
Items	Amount
Voltage	40 V
Pulse time	50 ms
Interval time	950 ms
Frequency	5

Note: The electroporation starts after the electrode is pressing the cortex and ends before the electrode leaves the mouse.

STEP-BY-STEP METHOD DETAILS

In utero electroporation of plasmids in the mouse embryonic cortex

Timing: 30 min each pregnant female mouse

This section describes the procedures for the *in utero* electroporation of plasmids in the mouse embryonic cortex according to previous reports (Meyer-Dilhet and Courchet, 2020; Pacary and Guillemot, 2020; Saito, 2006). Sterile procedures are required during the operation (Figure 1). Due to electroporation is accomplished with cesarean section at gestational age embryonic day (E14.5), all surgical instruments necessary for cesarean section surgery are heat sterilized to reduce contamination risk.

Note: Pre-heat the normal saline at 38°C, and prepare the cesarean section operating table. Adjust and ensure the voltage, time and frequency of electroporation. Prepare the plasmids used in this procedure and mix the plasmid with Fast Green (0.1 mg/mL, Sigma-Aldrich) at the ratio of 99:1 (v/v). Try to finish the electroporation and section as fast as possible to keep high viability of embryonic and pregnant mice.

1. Sterilize the surgery tools, operation area and electrode with 75% ethanol.
2. Anesthetize pregnant Rack1$^{F/F}$ mouse by injecting 1% sodium pentobarbital (6 g/kg).

⚠ CRITICAL: Prepare the anesthetic when it is used. And try to choose other legal anesthetics that are less reactive to mouse.

⚠ CRITICAL: The pregnant mice are anesthetized one by one. Next pregnant mouse is anesthetized when the cesarean section of above one has finished.

3. Slightly put the anesthetized pregnant mouse on the operation table and fix the limbs with adhesive tape. Hair is removed from the abdomen of the mouse using a commercially available depilatory cream.
4. Cover the body of the mouse with sterile gauze, except for the abdomen and head. And the abdomen of the mouse is disinfected with betadine.

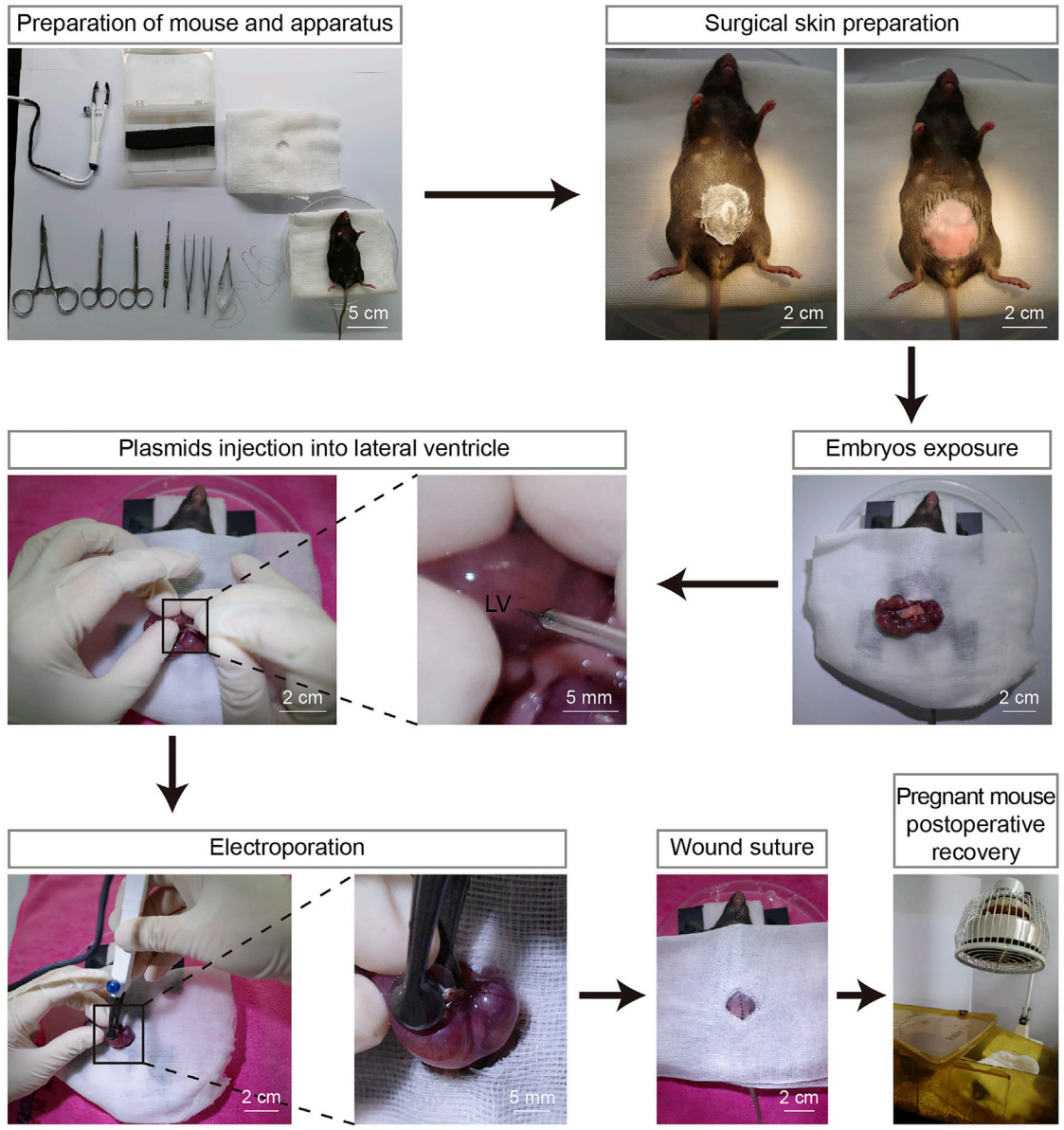

Figure 1. Schematic representation of *in utero* electroporation of plasmids into E14.5 mouse embryos

LV: lateral ventricle.

5. At the middle of lower abdomen, lift the skin with tweezers and make an incision about 1 cm with ophthalmic scissors.

   Note: The incision of the mouse is kept moist with warm (about 37°C) sterile saline.

6. Gently pull out the uterine horn with scissors to expose the embryos.
7. For each embryo, inject the lateral ventricle with 1 μg of plasmids DNA (pCAG-dCre-GFP or pCAG-Cre-GFP) mixed with 1% Fast Green (v/v) for a total volume of 1 μL using a glass pulled micropipette, respectively. The plasmids are injected into the mouse according to the list in the table. Troubleshooting 1.

8. The indicated expression constructs were then electroporated using an ECM 830 electroporator (BTX) with five 50 ms pulses separated by 950 ms intervals at 40 V.
9. Each group of plasmids are injected into five different embryos in at least three independent operations.
10. After electroporation, the uterine horns are carefully repositioned into the abdominal cavity.
11. The cavity is bathed with pre-warmed sterile saline, and the wound is sutured closed.
12. The mice are left under observation on a heating pad until recovery, then returned to home cage and monitored for pain carefully.

Brain dissociation and tissue preparation and frozen section

Timing: 4 days

This section describes the procedures for brain dissociation from embryos at E18.5, brain fixation, dehydration and frozen section (Figure 2).

13. Prepare the electroporated pregnant mice at E18.5 and euthanize them by intraperitoneal injection of sodium pentobarbital (6 g/kg), which is approved by the animal welfare committee.

Note: Try to choose the legal anesthetics that are less reactive to mouse.

14. Slightly put the anesthetized pregnant mouse on the operation table and dissect the abdomen of anesthetized pregnant mouse with fine scissors.
15. Take out all the embryos from the pregnant mouse under binocular microscope and put them in 37°C normal saline.
16. Dissect the brains under binocular microscope by using fine microsurgical tweezers and collect the brain into 4% paraformaldehyde in a 10 mL centrifuge tube one by one. And make a mark on the tube (Figure 2). Troubleshooting 2.

⚠ CRITICAL: There is a meninges on the surface of the brain. Carefully remove the meninges by using fine microsurgical tweezers.

17. Fix the brains in paraformaldehyde for 24 h, dehydrate with gradient 15% sucrose (Sigma, 57-50-1) for 24 h and 30% sucrose for 48 h, respectively.
18. Place the tissues in 1:1 30% sucrose/OCT (Tissue-Tek) solution for 1–2 h at 4°C, in 100% OCT compound for 1 h at 4°C, embed them in 100% OCT compound and froze in Cryomold at −20°C (Thermo Fisher, CRYOSTAR NX50).
19. Store the sections in −20°C refrigerator for subsequent experiments.

Senescence-associated β-galactosidase (SA-β-gal) staining

Timing: 26 h

This section describes the procedures for the senescence-associated β-galactosidase (SA-β-Gal) activity detection of sections from mice brains. The staining procedures are carried out according to protocol supplied by the manufacturer (C0602, Beyotime).

Note: For all technique replicates, sections from both groups are stained simultaneously, using the same batch of staining buffer to avoid inter-assay variation.

20. Take the slices out from freezer and warm them up to room temperature (20°C–25°C) for 10 min.
21. Fix brain sections in fixative for 15 min at room temperature (20°C–25°C).

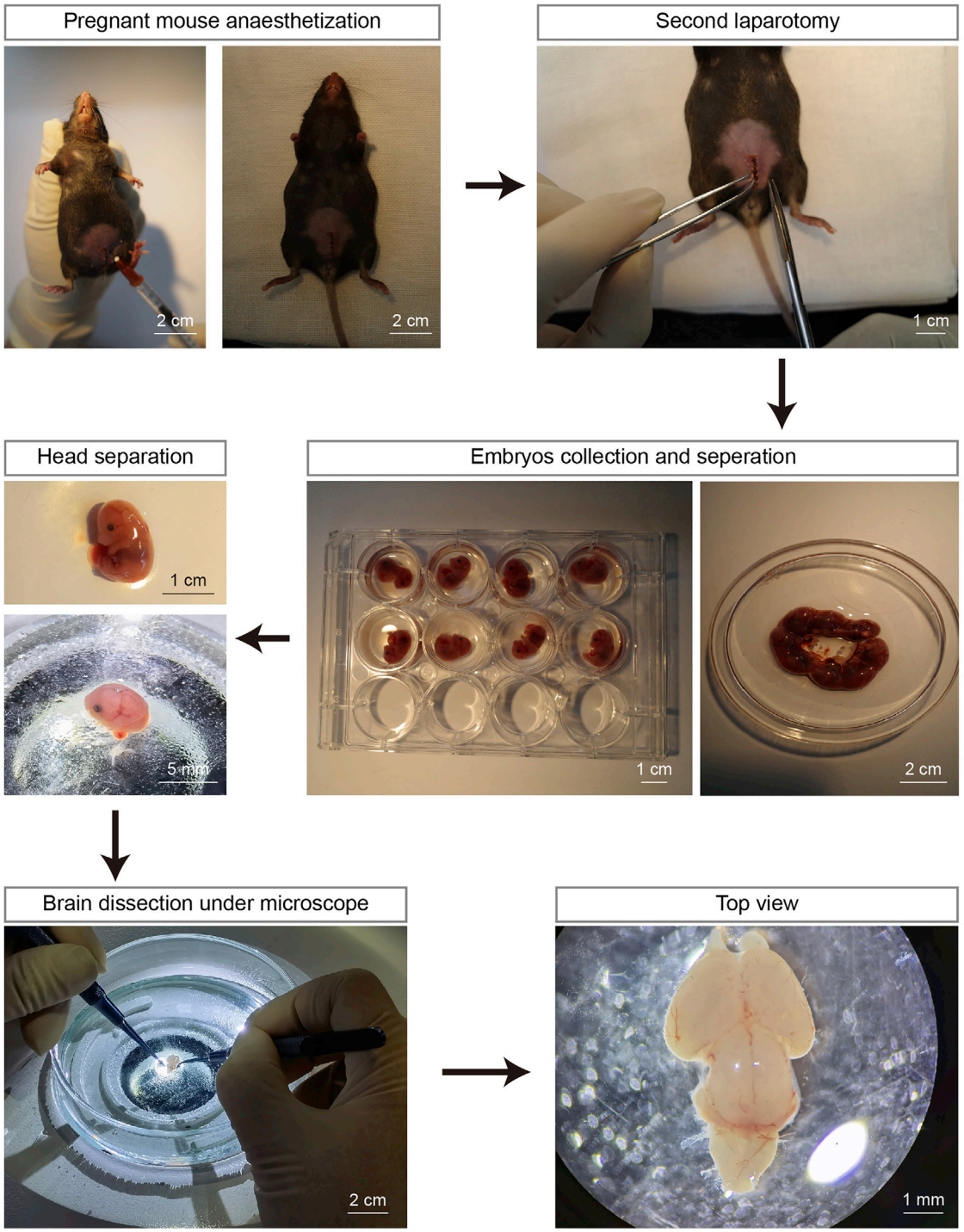

Figure 2. Schematic representation of mouse brain collection steps from E18.5 mouse embryos

Note: This is an optimized step. The fixative is from the senescence-associated β-galactosidase (SA-β-Gal) staining kit and its main composition is 4% PFA. This step can be ignored if the fixed time in Step17 is longer enough. Otherwise, this step is necessary (such as large or fresh tissue used in the experiment). In addition, in our experiment, we found two-step

fixation did not affect the staining quality and experimental results compared to one-step fixation.

22. Wash the slices three times with PBS for 5 min each and prepare the SA-β-Gal solution. Each 1 mL SA-β-Gal staining solution is mixed with 10 μL solution A (potassium ferrocyanide), 10 μL solution B (potassium ferricyanide), 100 μL X-Gal and 880 μL solution C (citric acid-sodium phosphate buffer).
23. Stain the slices immediately with SA-β-Gal staining solution (pH 6.0) for 24 h in the incubator at 37°C protected from light. Troubleshooting 3.
24. Rinse the slices with PBS for three times after the blue color is developed.

Immunofluorescent staining

Timing: 24 h

These procedures are performed after the SA-β-Gal staining procedures.

25. After washing the slices with PBS, the slices are permeabilized with 1% PBST (1% Triton X-100 in PBS) for 20 min at room temperature (20°C–25°C).
26. Block the slices with 3% bovine serum albumin (BSA) or 10% normal goat serum (NGS) in 0.3% PBST for 1 h at room temperature (20°C–25°C).
27. Incubate the brain samples on the slide with the primary antibody overnight (12 h–16 h) at 4°C. Primary antibodies and dilutions (diluted in 3% BSA in 0.3% PBST) are as following: anti-GFP (1:500) and anti-Sox2 (1:400).

Note: The plasmids coding for green fluorescent protein (GFP) tagged Cre or dCre are used for *in utero* electroporation in this experiment. In particular, the SA-β-Gal staining is prior to immunofluorescent staining, and this step reduces the intensity of GFP autofluorescence causing the GFP signal much weaker. To better illustrate the GFP signals, we used anti-GFP labeling to amplify the GFP signals. It should be noted that the immunofluorescent staining of other proteins can also be performed according to experimental requirement.

28. Wash the sections three times with PBS (5 min each).
29. Incubate the sections with secondary antibodies for 3 h at room temperature (20°C–25°C). Alexa Fluor 568- or Alexa Fluor 488-conjugated fluorescent secondary antibodies and dilutions (diluted in 3% BSA in 0.3% PBST) are as the ratio of 1:500.
30. Wash the sections five times with PBS (5 min each).
31. Mount the slices with medium containing DAPI (Invitrogen, P36961).

Microscope observation

Timing: 12 h

These procedures follow the staining and start after the slides are air dried completely.

32. Use confocal microscope (Olympus FV-1200; Olympus, Japan) to obtain the fluorescence imaging picture.

Note: It is pivotal to acquire the fluorescence imaging pictures firstly, because the strong light will attenuate the fluorescence signal. And makes a special symbol on the slide in each brain sample to distinguish each sample.

33. Use a digital slide scanner (NanoZoomer Digital Pathology; Hamamatsu Photonics, Shizuoka, Japan) with a 20× objective to acquire brightfield photomicrographs of SA-β-Gal staining. Troubleshooting 4.

EXPECTED OUTCOMES

In the present, the observation of the target cells and senescent cells at the same tissue sample is rare simultaneously due to the different staining and scanning model. Most of the SA-β-Gal staining and immunofluorescent staining results are independent. In this study, our co-labeling of the senescent NSCs in the manipulation of plasmids by in utero electroporation provides a direct method of studying the molecules regulating the senescence of cells in the developing neocortex (Zhu et al., 2021). Senescence of NSCs has emerged as a novel approach to control stem cell fate across species in embryonic development (Munoz-Espin et al., 2013; Storer et al., 2013). And cellular senescence is associated with NSC deregulation in neurodegeneration and may contribute to pathological phenotypes in neurodevelopmental disorders (Bussian et al., 2018; Nicaise et al., 2019; Riessland et al., 2019). However, the role of cellular senescence in NSCs during normal neurodevelopment and associated mechanisms remains largely unknown. Therefore, the purpose of this protocol is to provide an essential and reliable method to identify and label senescent cells in the developing brain in situ.

Senescence-associated β-galactosidase (SA-β-Gal) activity is the most widely used assay to detect senescence (Collado and Serrano, 2006; Dimri et al., 1995), which is based on the increased lysosomal content of senescent cells (Kurz et al., 2000). Here, we provide a feasible protocol to identify and investigate the potential molecular mechanisms underlying the cellular senescence in NSCs. In this protocol, by in utero electroporation of the CAG promoter drove GFP expression with Cre or dCre (destabilized Cre) control plasmids in the developing embryonic brain within the pregnant Rack1$^{F/F}$ mice, the GFP/Sox2 double positive (GFP+Sox2+) and SA-β-Gal/GFP/Sox2 triple positive (SA-β-Gal+GFP+Sox2+) NSCs within the VZ/SVZ represent plasmid electroporated NSCs and senescent plasmid electroporated NSCs, respectively. In the pCAG-dCre-GFP electroporated (control) embryonic brain, our data showed the ratio (percentage) of SA-β-Gal+GFP+Sox2+ and GFP+Sox2+ is 2.19 $\pm$ 0.76% in control condition (Figure 4B). This means that the number of senescent cells (SA-β-Gal+Sox2+) significantly increased in the neocortex electroporated with pCAG-Cre-GFP, compared to that of the pCAG-dCre-GFP electroporated neocortex. This result indicates that ablation of Rack1 can induce the senescence of NSCs in the developmental neocortex. Therefore, this approach could be feasibly applied to validate the potential role of candidate genes in the regulation of the cellular senescence in the developing embryonic cortex.

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: 24 h

This step offers the picture reconstruction of two different microscopes (Figure 3). Because the files need enlargement to zoom overlapping area, a super powerful computer should be provided.

1. Use FV10-ASW 2.1 software (Olympus, Waltham, MA, USA) to export the fluorescent images.
2. According to the corresponding fluorescent image, use the NDP 2.0 view software (Hamamatsu, Japan) to adjust the appropriate magnification and angle, and export the SA-β-Gal staining images.
3. Then use Adobe Photoshop CC software (Adobe photoshop, San Jose, CA, USA) to open the fluorescent image and SA-β-Gal staining image and adjust the brain sample to the same direction. Duplicate the background layer and use the move tool to drag and drop the layer of SA-β-Gal image to the front of fluorescent image.

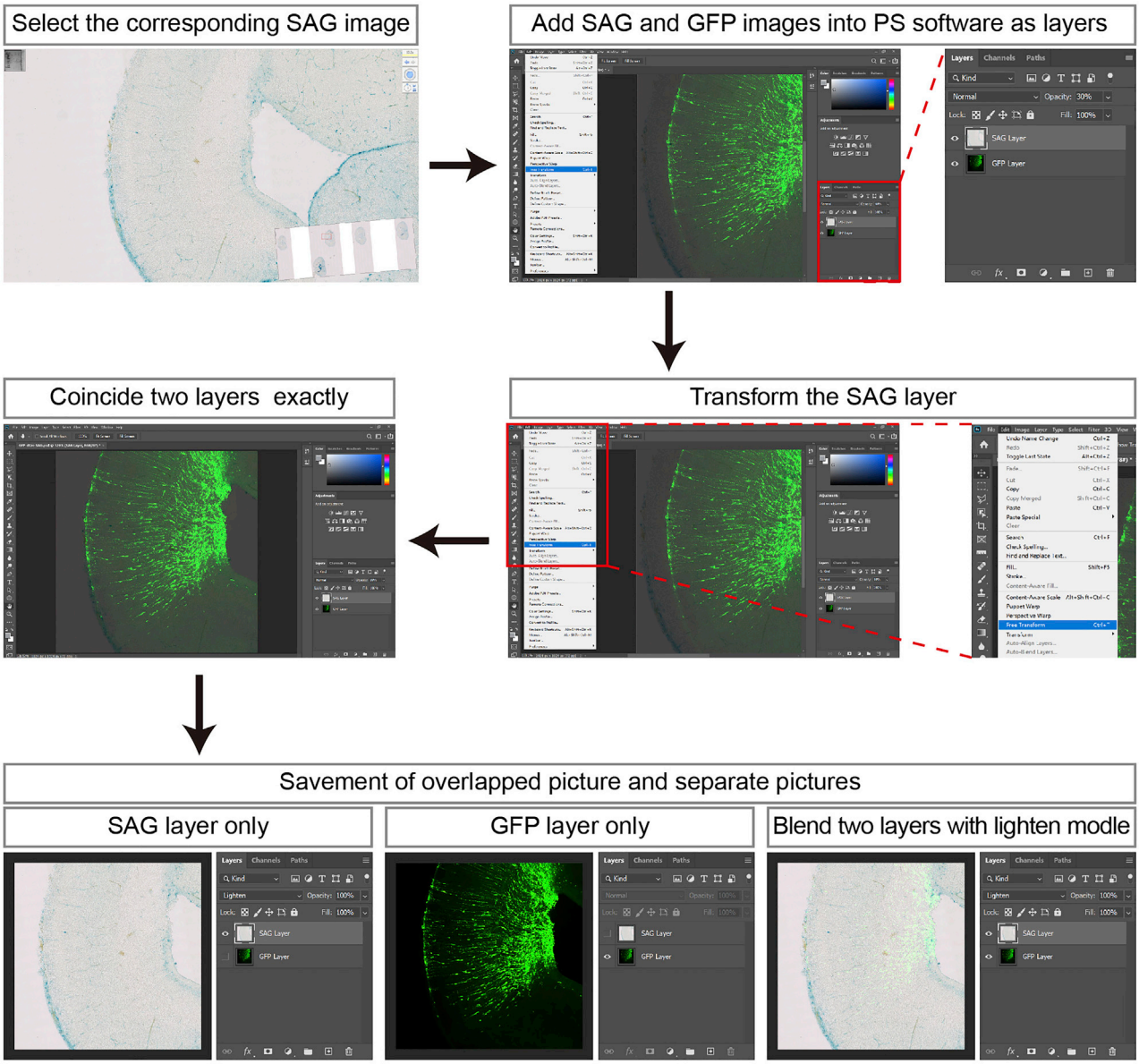

Figure 3. Schematic representation of the overlapping of fluorescent images and bright images

SAG: SA-β-Gal staining.

Note: The fluorescent images and wide-field images from the same brain slide were taken from different imaging instruments, respectively, which make the view field of those images do not perfectly fit well. Therefore, it is necessary to select a suitable reference frame and finely adjust the orientation of the images before merging them. It should be noted that this step is very convenient to operate in Photoshop. If the images were taken from the same instrument, the merge of fluorescence and wide-field images can also be done in Fiji-ImageJ.

4. Set up the opacity of SA-β-Gal image layer as 30%–60%, adjust the brightness and contrast of copped layer of fluorescent image to change the location, direction and size of SA-β-Gal layer conveniently.

5. Choose the translucent SA-β-Gal image layer and the "Edit-Free Transform" in menu bar to adjust the location, direction and size of SA-β-Gal layer and make it finely overlap with the fluorescent image whose brightness and contrast have been adjusted.
6. Adjust the opacity of SA-β-Gal image layer as 100%, delete the fluorescent image whose brightness and contrast have been adjusted, and set the blending mode for SA-β-Gal image layer as "Hard Light". Troubleshooting 5.
7. Hide the SA-β-Gal image layer, display the fluorescent image layer and serve the image to acquire the changed fluorescent image. Hide the fluorescent image layer, display the SA-β-Gal image layer to acquire the changed SA-β-Gal image.
8. Simultaneously display the SA-β-Gal image layer and fluorescent image layer, and serve the image as the overlapped result.
9. Use Image J to open these cropped and overlapped images. The SA-β-Gal+ cells are senescent cells, the GFP+ and Sox2+ cells are cells that have been successfully electroporated with plasmids and NSCs, respectively.
10. Illustrate and merge the GFP/Sox2 double positive (GFP+Sox2+) and SA-β-Gal/GFP/Sox2 triple positive (SA-β-Gal+GFP+Sox2+) NSCs within the VZ/SVZ, respectively (Figure 4A).
11. Calculate and compare the ratio of SA-β-Gal+GFP+Sox2+ cells and GFP+Sox2+ NSCs between pCAG-dCre-GFP and pCAG-Cre-GFP electroporated neocortex, respectively.
12. The result shows that the ratio of SA-β-Gal+GFP+Sox2+ cells and GFP+Sox2+ cells in the pCAG-Cre-GFP electroporated brain (Rack1 knockdown) is much higher than that in pCAG-dCre-GFP electroporated brain (negative control) (Figure 4B). This indicates that loss of Rack1 in developing NSCs induces the cell-autonomous senescence.

LIMITATIONS

Limited experiment time

The protocol described here is used for investigating the role of candidate genes in the regulation of cellular senescence in developing neocortex. The time points for experimental operation are very important. According to our study, E13.5 and E14.5 are the best stages for this protocol (Zhu et al., 2021). *In utero* Electroporation is a technology suitable for analyzing roles of candidate genes not only in embryonic development but also in higher order function of the nervous system (Saito, 2006). The operation time ranges from E11.5 to E15.5. Therefore, the method is adaptable for the studies of roles of the genes involved in embryonic development and disease, rather than the cellular senescence at adulthood and the following age.

Limited areas of the brain to study

In this protocol, the area chose to study is the developing mouse cortex. In addition, *in utero* electroporation is also applied in the gene delivery in mouse auditory brainstem and hindbrain, such as rhombic lip (David et al., 2014; Holland et al., 2012). Using a triple-electrode probe, the special high-performance and site-directed *in utero* electroporation are achievable (dal Maschio et al., 2012). The studied brain areas of investigating the cellular senescence will expand greatly, when combining with other *in utero* electroporation technique. Besides the *in utero* electroporation, stereotactic virus injection combined with the SA-β-Gal and immunofluorescent staining is also applied to study the cellular senescence mechanism of various cells in different brain regions. Hence, this limitation can be overcome by the combination with other gene delivery method.

High resolution original picture images are need

To ensure the same cells are completely overlapped within the field of brightfield microscope vision and confocal microscope vision, it is better to image the entire sample and zoomed local area at the highest possible resolution, respectively, because final selected pictures are enlarged many times. Meanwhile, each image should make a special maker to distinguish them clearly. This process is time-consuming, but necessary, which will generate a huge benefit for data treatment and analysis. Due to the size of the data, for data analysis and image reconstruction, a powerful computer is needed.

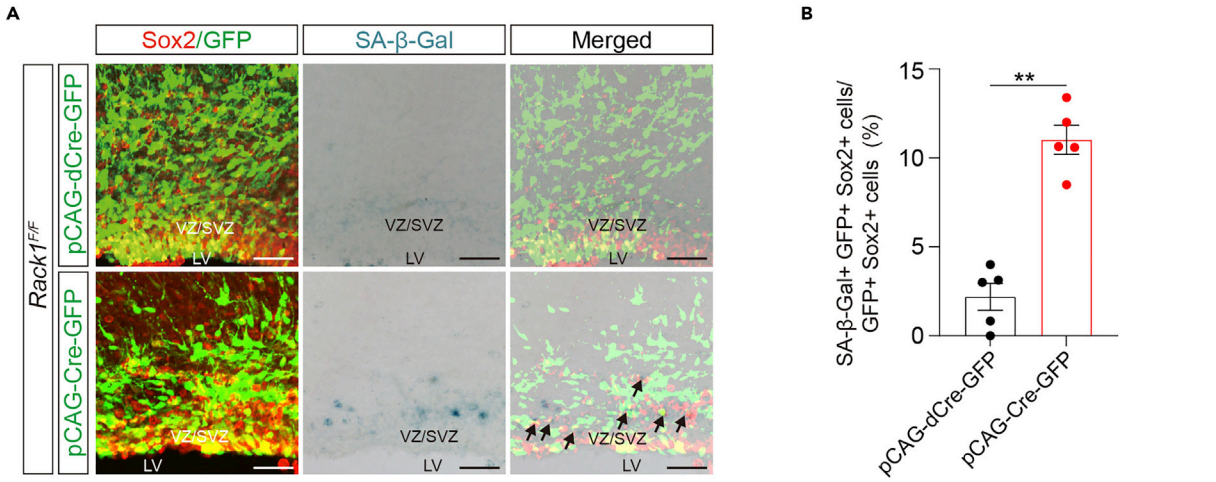

Figure 4. The deletion of Rack1 in NSCs induces cell-autonomous senescence

(A) Co-localization assay of SA-β-Gal+ senescent cells with dCre-GFP+ control or Cre-GFP+ electroporated NSCs within the VZ/SVZ by *in utero* electroporation. Scale bars, 50 μm.

(B) Quantification of the SA-β-Gal+, GFP+, and Sox2+ (triple positive) cells in electroporated cortical sections within the VZ/SVZ. VZ: ventricular zone; SVZ: subventricular zone; LV: lateral ventricle. Data represent mean ± SEM. p** < 0.0001, n = 5 mice. Unpaired *t*-test.

TROUBLESHOOTING

Problem 1

Low viability of pregnant mice and embryonic mice after *in utero* electroporation (step 7).

Potential solution

Before the formal experiment, all the surgical procedures must be very skilled. Try to shorten the operation time and keep all embryos in wet all the time with the hot-prepared saline to reduce the embryos mortality. Try your best to touch the embryos slightly. The operation time of each pregnant must be shorten than 30 min. After the operation, all pregnant mice are maintained under a warm and quiet environment until they wake up. Notably, the anesthesia of pregnant mice is very important. It is pivotal to prepare the anesthetic drug before the operation and use the drug according to the weight of the pregnant mice strictly.

Problem 2

Keep original brain tissue forms from embryonic mice (step 16).

Potential solution

The entire process of brain tissue dissection must be completed under a stereomicroscope with sterile forceps and the undamaged anatomical structure of brains are retained. Do not pull the tissue during the dissection. And the fixture is followed the dissection.

Problem 3

Low signal of SA-β-Gal staining (step 23).

Potential solution

Upon receipt, the reagents in senescence-associated β-galactosidase staining kit should be aliquoted and stored at −20°C. Use the fresh staining solution each time, and the volume of the staining solution is sufficient to prevent drying. Extending the staining time is also suggested, if the staining of tissue samples derived from the different mouse groups is simultaneous.

Problem 4

Low resolution of brightfield photomicrographs of X-gal staining (step 33).

Potential solution

When scanning, focus of samples is maintained during image acquisition by manual adjustment. Do the best to choose more focus points to improve the picture clarity.

Problem 5

The distorted overlapping effect of brightfield scanning image and laser confocal image (step 6).

Potential solution

Adjust the overlay mode.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Haitao Wu (wuht@bmi.ac.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

H.W. designed the experiments. L.C., Y.L., and Y.W. performed experiments. S.L. and X.C. performed the mice breeding and genotyping experiments. L.C. performed the data analysis and prepared the manuscript. H.W. revised the protocols.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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