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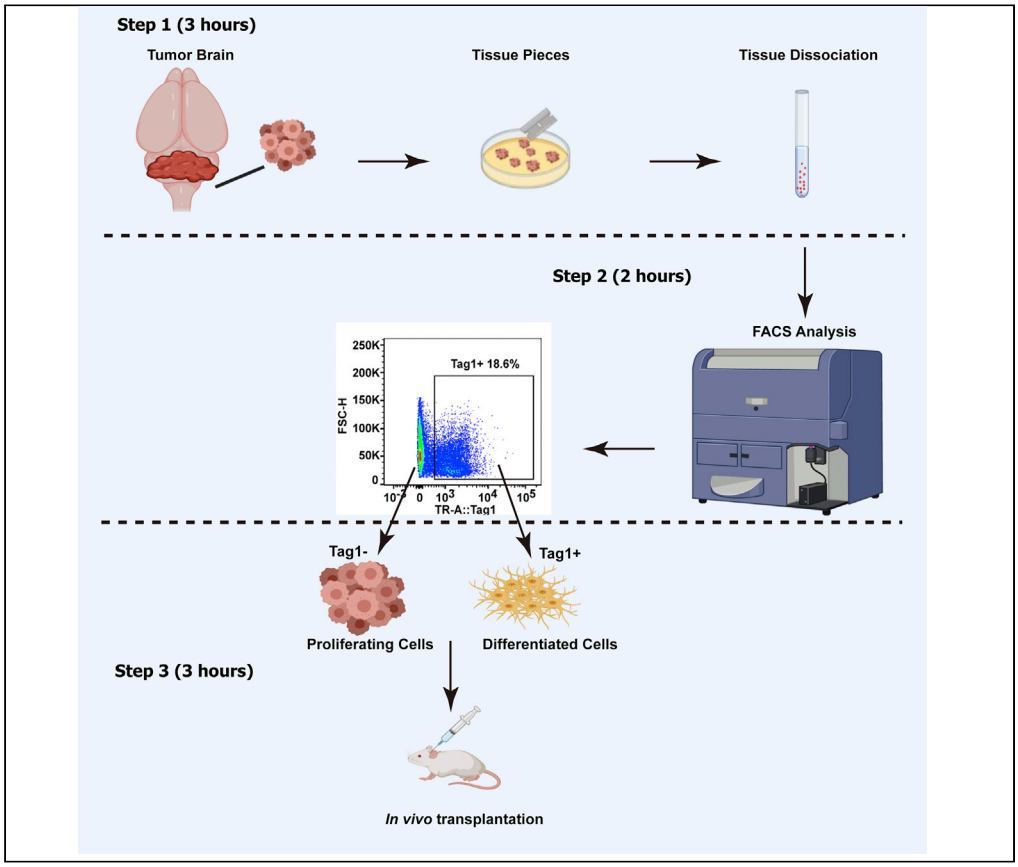
Protocol

Purification of differentiated tumor cells from medulloblastoma for transplantation into mouse cerebellum



This protocol provides the procedures for isolating differentiated tumor cells from medulloblastoma (MB) in mice. Procedures for transplantation into cerebella are also included to examine the tumorigenesis of differentiated MB cells. This protocol outlines the detailed steps required for (1) isolation of tumor cells from mouse MB, (2) purification of differentiated tumor cells by fluorescence-activated cell sorting, and (3) transplantation of tumor cells into cerebella. This protocol is useful to purify differentiated tumor cells for investigating mechanisms underlying MB progression.

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HIGHLIGHTS

Procedures for isolating tumor cells from mouse medulloblastoma

Purification of differentiated medulloblastoma cells by FACS

Technical details for injecting live tumor cells into mouse cerebella

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Purification of differentiated tumor cells from medulloblastoma for transplantation into mouse cerebellum

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SUMMARY

This protocol provides the procedures for isolating differentiated tumor cells from medulloblastoma (MB) in mice. Procedures for transplantation into cerebella are also included to examine the tumorigenesis of differentiated MB cells. This protocol outlines the detailed steps required for (1) isolation of tumor cells from mouse MB, (2) purification of differentiated tumor cells by fluorescence-activated cell sorting, and (3) transplantation of tumor cells into cerebella. This protocol is useful to purify differentiated tumor cells for investigating mechanisms underlying MB progression.

For complete details on the use and execution of this protocol, please refer to Cheng et. al. (2020).

BEFORE YOU BEGIN

Medulloblastoma (MB), the most common malignant brain tumor in children, predominately locate in the cerebellum. Approximately 30% of human MB cases have mutations in the hedgehog (Hh) pathway (Northcott et al., 2012). Conditional deletion of *Patched1* (*Ptch1+/-*, encoding an antagonizing receptor of hedgehog pathway) in cerebellar granule neuron precursors, causes MB formation in mice with 100% penetrance (Yang et al., 2008). We have recently identified a subset of tumor cells from mouse and human MB, undergoing terminal differentiation in tumor tissue (Cheng et al., 2020). Those differentiated MB cells express Tag1 (encoded by *Contactin 2*), a cell surface protein that is associated with neuronal migration and differentiation (Xenaki et al., 2011). Differentiated tumor cells have irreversibly lost their proliferative capacity as well as tumorigenic potential.

Here, we introduce procedures to purify differentiated tumor cells from mouse MB tissue, and evaluate the tumor-forming ability of differentiated cells by transplantation into cerebella.

Mouse breeding

Timing: [18 weeks]

1. Breeding *Math1-Cre/Ptch1*$^{fl/fl}$ mice
   a. *Math1-Cre* mice, and *Ptch1*$^{fl/fl}$ mice purchased from the Jackson Laboratory, are crossed to generate *Math1-Cre/Ptch1*$^{fl/fl}$ progeny (through intercrossing *Math1-Cre/Ptch1*$^{fl/wt}$ mice), which are genotyped by PCR as recommended by the Jackson Laboratory.

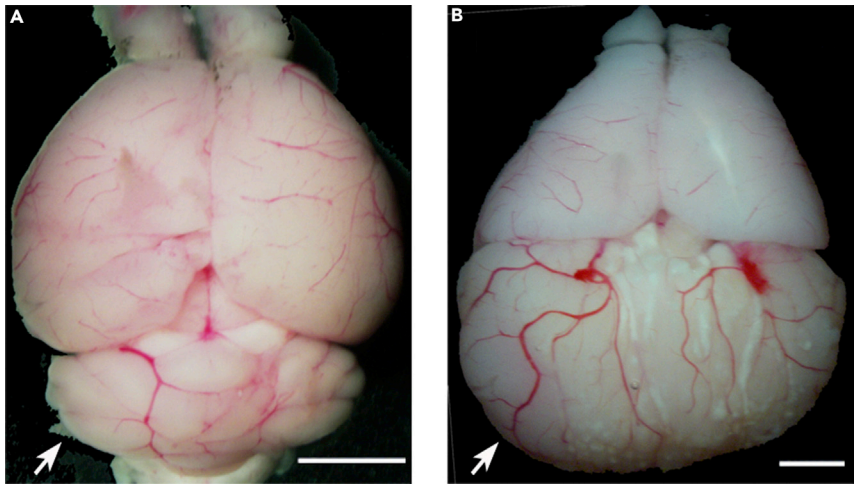

Figure 1. MB developed in a *Math1-Cre/Ptch1$^{fl/fl}$* mouse

Whole mount pictures of brains dissected from a wild type mouse (*C57BL/6J*) (A) and a *Math1-Cre/Ptch1$^{fl/fl}$* mouse (B), at 6 weeks of age. Arrows point to the cerebellum (A and B). Note that MB was formed in the cerebellum of the *Ptch1* mutant mouse (B). Scale bars, 4 mm.

b. Monitor *Math1-Cre/Ptch1$^{fl/fl}$* mice every two days for brain tumor signs including ataxia, hunched back and head tilt.
c. Tumor-bearing *Math1-Cre/Ptch1$^{fl/fl}$* mice are used for tumor cell isolation.

Note: *Math1-Cre/Ptch1$^{fl/fl}$* mice are expected to develop MB at 6–8 weeks of age with 100% penetrance (Yang et al., 2008) (Figure 1).

2. *CB17/SCID* mice obtained from Laboratory Animal Facility at Fox Chase Cancer Center, are used to maintain *Math1-Cre/Ptch1$^{fl/wt}$* colony.
3. Purchase *CB17/SCID* mice (6–8 weeks of age) from the Jackson laboratory, which are used for cerebellar transplantation.

Note: All animal work should be performed with the approval of the Institutional Animal Care and Use Committee.

Preparation of surgical materials for cerebellar transplantation

Autoclave surgical tools including scissors, forceps, needle holders, surgical gloves and a Hamilton syringe.

Note: It is recommended to use sterilization pouches to autoclave surgical tools.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-TAG-1	DSHB	Cat# 4D7/TAG1
Mouse IgM isotype control	Thermo Fisher	Cat# MA1-10438
Alexa Fluor 594 anti-IgM antibody	Invitrogen	Cat# 2084922
Chemicals, peptides, and recombinant proteins		
1× DPBS, calcium, magnesium	Thermo Fisher	Cat# 14040133

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bovine serum albumin (BSA)	Fisher Scientific	Cat# BP1600-100
Trypsin inhibitor	Roche	Cat# 17075029
EBSS (Ca++/Mg++ free)	Thermo Fisher	Cat# 14155063
DNase I (type II)	Worthington	Car# LS002139
Papain	Worthington	Cat# LS003127
L-Cysteine	Sigma-Aldrich	Cat# 30089
Neurobasal medium (NB)	Thermo Fisher	Cat# 21103049
B27 supplement	Thermo Fisher	Cat# 17504044
L-Glutamine	Gibco	Cat# 25030081
Sodium pyruvate	Thermo Fisher	Cat# 11360070
Penicillin and streptomycin	Gibco	Cat# 2029637
Trypan blue solution, 0.4%	Gibco	Cat# 15250061
Goat serum	Cell Signaling	Cat# 5425S
Saline solution	Thermo Fisher	Cat# R064432
NaOH (2N)	Millipore Sigma	Cat# 1091361000
Propidium iodide staining solution	Thermo Fisher	Cat# 00-6990-50
Ketamine	Vedco	Cat# AHO2WWH
Xylazine	Bimeda-MTC	Cat# 1XYL0038XYL006
Bupivacaine	AuroMedics Pharma	Cat# NDC55150-168-30
Ointment	Dechra	Cat# 17033-211-38
Experimental models: organisms/strains		
Mouse: Ptch1$^{loxp/loxp}$	Jackson Laboratory	https://www.jax.org/strain/030494
Mouse: Math1-Cre	Jackson Laboratory	https://www.jax.org/strain/011104
Mouse: CB17/SCID	Jackson Laboratory	https://www.jax.org/strain/001303
Mouse: C57BL/6J	Fox Chase Cancer Center	N/A
Software and algorithms		
FlowJo v10.4	Tree Star	http://www.flowjo.com/
Other		
Benchtop pH meter	Fisher Scientific	AB150
Centrifuge	Thermo Scientific	ST40R
Inverted microscope	Zeiss	Axiovert 40 CFL
BD FACS Aria II Cell Sorter	BD Biosciences	BD FACS AriaTM II
Small Animal Stereotaxic Frame	David Kopf Instruments	Model 900LS
Micromanipulator	David Kopf Instruments	Model 1760
Replacement blade	Excel	Cat# 84-327-1
Disposable transfer pipettes	Fisher Scientific	Cat# 13-711-20
Disposable glass Pasteur pipettes	Fisher Scientific	Cat# NC0541803
Pipettes	Gilson	Pipetman F123601/2
Pipette tips	Fisher Scientific	Cat# 10-320-703
Eppendorf tubes	Fisher Scientific	Cat# 05-402-18
15 mL Conical centrifuge tube	Fisher Scientific	Cat# 14-959-53A
50 mL Conical centrifuge tube	Fisher Scientific	Cat# 05-539-13
Membrane filters (0.22 μm)	Millipore Sigma	Cat# GSWP01300
Membrane filters (0.45 μm)	Millipore Sigma	Cat# HAWP04700
6 cm Plastic dishes	CytoOne	Cat# cc7682-3359
60 mL Syringe	BD Biosciences	Cat# 309653
10 mL Syringe	BD Biosciences	Cat# 309604
40 μm Cell strainer	Falcon	Cat# 352340
Hemocytometer	Hausser Scientific	Cat# 486560
Surgical scissors	Fine Science Tools	Cat# 14090-09
Dissection scissors	Fine Science Tools	Cat# 14060-09
Scalpel handle	Fine Science Tools	Cat# 10004-13
Scalpel blades	Fine Science Tools	Cat# 10023-00
Forceps	Fine Science Tools	Cat# 11252-00
Cotton swabs	Fisher Scientific	Cat# 22-029-488

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Needle holders (1.5mm)	Fine Science Tools	Cat# 12003-15
Sterilization pouches	Fisher Scientific	Cat# 01-812-50/54
Gloves	Denville	Cat# 1159G05
Pasteur pipet	Fisher Scientific	Cat# 13-678-20A
Unbeveled Hamilton syringe	Hamilton	Cat# 76350-01
Beveled 18-gauge needle	BD Biosciences	Cat# 5302622
Nylon skin suture (with needle)	Fisher Scientific	Cat# NC1646047
Deltaphase Isothermal Pad	Braintree Scientific	Cat# 39DP
Nutrition gel	ClearH2O	Cat# 72-06-5022

MATERIALS AND EQUIPMENT

4% Bovine serum albumin (BSA):

Reagent	Final concentration (mM or μM)	Amount
BSA (1 g)	4%	
1× DPBS	n/a	25 mL
Total	**n/a**	**25 mL**

Store aliquots (400 μL) at −20°C for up to 6 months

DPBS+BSA:

Reagent	Final concentration (mM or μM)	Amount
BSA (4%)	0.02%	1 mL
1× DPBS	n/a	199 mL
Total	**n/a**	**200 mL**

DPBS+BSA Store at 4°C

Ovomucoid (Ovo):

Reagent	Final concentration (mM or μM)	Amount
BSA (1 g)	5%	
Trypsin inhibitor (1 g)	5%	
1× DPBS	n/a	20 mL
Total	**n/a**	**20 mL**

Store aliquots (1 mL) at −20°C for up to 6 months

DNase I:

Reagent	Final concentration (mM or μM)	Amount
DNase I (40,000 U)	12500 U/mL	
EBSS	n/a	3.2 mL
Total	**n/a**	**3.2 mL**

Store aliquots (200 μL) at −20°C for up to 6 months.

Note: DNase I is sensitive to physical denaturing. Therefore, do not vortex the DNase during preparation. Mix it by gently flicking the tube or pipetting. Minimize the freeze-thaw cycles of DNase I stock (3 cycles maximum).

Fluorescence-activated cell sorting (FACS) buffer:		
Reagent	Final concentration (mM or μM)	Amount
Goat serum	10%	0.5 mL
Neural basal medium	n/a	4.5 mL
Total	**n/a**	**5 mL**
Store aliquots (1 mL) at 4°C.		

Note: Prepare DPBS+BSA solution and FACS buffer weekly. No detergents (Triton-X100 or Tween-20) should be included in the FACS buffer.

Anesthetic solution for cerebellar transplantation:		
Reagent	Final concentration (mM or μM)	Amount
Ketamine (100 mg/mL)	10 mg/mL	1 mL
Xylazine (20 mg/mL)	0.5 mg/mL	0.25 mL
Saline solution	n/a	8.75 mL
Total	**n/a**	**10 mL**
Store the solution at 20°C–25°C (up to 6 months).		

Note: Ketamine needs to be prepared under sterile conditions. Ketamine is a controlled reagent and the records for its usage must be kept securely.

Analgesics solution:		
Reagent	Final concentration (mM or μM)	Amount
Bupivacaine (5 mg/mL)	0.25%	1 mL
Saline solution	n/a	1 mL
Total	**n/a**	**2 mL**
store at 20°C–25°C (up to 6 months)		

Note: Bupivacaine needs to be prepared under sterile conditions.

STEP-BY-STEP METHOD DETAILS

Tissue dissociation

Timing: 3 hrs

This section describes the steps for MB tissues dissociation.

1. On the day of tumor tissue dissociation, prepare the following solutions:
   a. Papain solution:
      i. Dissolve 100 U papain in 10 mL DPBS.
      ii. Add 200 μL DNase I, and mix by inversion. The final concentration of DNase I is 250 U/mL.
      iii. Add 2 mg L-cysteine into the solution.
      iv. Adjust pH value until the solution becomes orange (same shade as DPBS, pH, 7.0–7.3) using 4–6 μL 2N NaOH.
      v. Filter the solution with a 0.45 μm membrane.
   b. Ovo solution:
      i. Add 200 μL DNase I in 5 mL DPBS, and mix by inversion.

ii. Add 1 mL Ovo stock solution into the above solution.

c. NB-B27 culture medium:

i. Mix 38 mL neurobasal medium, 800 μL B27 supplement, 400 μL penicillin/streptomycin, 400 μL L-glutamine and 400 μL sodium pyruvate.

ii. Adjust pH value of the medium to 7.2–7.4 with 2N NaOH (4–6 μL), using a bench-top pH meter.

Note: The above volume of prepared solutions including papain and Ovo solutions are enough for dissociation of one tumor from *Math1-Cre/Ptch1*$^{fl/fl}$ mouse. Prepare another set(s) of solutions for dissociation of more than 2 tumors. The pH value of NB-B27 medium is critical for the *in vitro* survival of MB cells.

2. Euthanize a *Math1-Cre/Ptch1*$^{fl/fl}$ mouse (at 6–8 weeks of age, Figure 1) with CO_2 following the routine procedure approved by the Institutional Animal Care and Use Committee at Fox Chase Cancer Center.
3. Remove the mouse head with a cut posterior from the ears using surgical scissors. Using the scissors, make a midline incision in the skin. Flip the skin over the eyes.
4. Hold the head with forceps, access the brain by inserting microdissection scissors into the foramen magnum and cutting straight towards the eyes.
5. Using forceps, peel away the skull and pinch off the cerebellum. Remove the brain stem (anterior to the cerebellum) as much as possible using forceps.
6. Cut cerebellar tissue into small (2–3 mm) pieces on a 6 cm plastic dish (with 1–2 mL ice cold PBS) using a replacement blade, and bring to tissue culture hood for further dissociation.

Note: To reduce the risk of contamination, always wipe the working surface in the hood with 70% ethanol and turn on UV light in the hood for 30 min each time before and after using the hood.

7. Using a disposable plastic pipette, transfer tissue pieces to a 15 mL conical tube containing 10 mL Papain solution with DNase I and L-cysteine, and incubate at 37°C in a water bath for 30 min.

Note: Try to minimize the amount of PBS (less than 500 μL) transferred with the tissue.

8. Aspirate off the papain solution, and add 2 mL Ovo solution to tissue pieces.
9. Gently triturate the tissue pieces using a Pasteur glass pipette (fire polished) with a bulb.

Note: Bubbles should be avoided at this stage for an effective dissociation. It is recommended to pipette up and down 5 times using a Pasteur pipet.

10. Let the tissue pieces settle for 1 min at 20°C–25°C, and transfer the top 1 mL suspension to a cell collection tube (a 15 mL conical tube).
11. Add another 1 mL Ovo solution, and triturate again using a Pasteur pipet with a bulb.
12. Let the tissue pieces settle for 1 min, and transfer the top 1 mL suspension to the cell collection tube.
13. Add another 1 mL Ovo solution, and triturate using a P1000 pipette (set to 1 mL) with a 1000 μL sterilized pipette tip. Pipette up and down 5 times.
14. Let the tissue pieces settle for 1 min, and transfer the top 1 mL suspension to the cell collection tube.
15. Repeat steps 13 and 14 for another two times, and remove top 1.5 mL suspension to the cell collection tube.
16. Transfer all remaining solution/cells to a 1.5 mL Eppendorf tube.
17. Triturate with a P200 pipette (set to 200 μL) with a 200 μL pipette tip. Pipette up and down 5 times.

18. Let the tissue pieces settle for 1 min, and transfer top solution (approximate 300 μL) into the cell collection tube. (Problem 1)

Note: Discard the remaining solution (~200 μL) at the bottom of the tube, which contains tissue debris.

19. Centrifuge cells (220 × *g*) in the cell collection tube for 10 min at 4°C.
20. Aspirate the supernatant and resuspend the cell pellet in 6 mL DPBS-BSA.
21. Strain cells through a cell strainer (40 μm) into a 50 mL tube.

Note: This step will remove the tissue chunks that are not thoroughly dissociated by the trituration. Some liquid is often underneath the strainer after straining dissociated cells. Make sure to suck off liquid from underside of the strainer, to minimize cell loss.

22. Transfer the cell suspension from the 50 mL tube to a 15 mL conical tube.
23. Centrifuge for 10 min, 220 × *g* at 4°C. Remove the supernatant.
24. Wash the cells with 2–3 mL PBS.
25. Repeat steps 23 and 24 for another 2 times.
26. After the last centrifugation, resuspend the cell pellet in 5 mL NB-B27.
27. Take 10 μL of above cell suspension into an Eppendorf tube and add 190 μL 0.4% Trypan Blue. Mix gently to count the number of harvested cells and examine cell viability.
28. Using a P200 pipette, take 100 μL of Trypan Blue-treated cell suspension and apply to a glass hemocytometer. Gently fill both chambers underneath the coverslip, allowing the cell suspension to be drawn out by capillary action.
29. Under an inverted microscope, focus on the grid lines of the hemocytometer with a 10× objective.
30. Count live (unstained) cells in all 4 sets of 16 corner squares. Dead cells stained with Trypan Blue in 4 sets of 16 corner squares are counted separately to measure cell viability.

Note: When counting, cells are only counted when they are set within a square or on the right-hand or bottom boundary line.

31. Take the average count of live cells from each of the sets of 16 corner squares, multiply by 10^4 to get the concentration of Trypan Blue-treated cell suspension, and further multiply by 20 to correct for the 1:20 dilution from the addition of Trypan Blue solution.

Note: The final value is the number of viable cells/mL in the original cell suspension (5 mL NB-B-27). 6–9 × 10^7 tumor cells (1.2–1.8 × 10^7/mL in 5 mL NB-B27) can be obtained from a *Math1-Cre/Ptch1*$^{fl/fl}$ mouse at 6–8 weeks of age.

32. To calculate the cell viability, add together the live and dead cell count from each set of 16 corner squares to obtain a total cell count. Divide the live cell count by the total cell count to calculate the percentage viability. (Problem 2)

Note: More than 95% of cells should be alive. If over 5% of cells are dead, the cell suspension should be washed with NB-B27 medium by centrifugation (220 × *g* for 5 min).

Collection of differentiated tumor cells by FACS

Timing: 2 hrs

This section describes the steps for immunolabelling tumor cells and purifying differentiated cells by FACS.

Note: All steps for cell immunolabelling should be performed on ice, and all centrifugations should be operated at 4°C.

33. Centrifuge the cell suspension at 220 × *g* for 5 min and resuspend the cell pellet in FACS buffer.

Note: The ideal density of cells for immunolabelling should be approximately 2 × 10^6 cells/ mL.

34. Incubate the cells with the primary antibody against Tag1 (1:50, mouse IgM) or mouse IgM isotype control (1:500) in FACS buffer for 30 min.
35. Wash the cells with 2 mL PBS, centrifuge at 220 × *g* for 5 min and remove the supernatant. Repeat this step for 3 times.
36. Incubate the cells with Alexa Fluor 594 goat anti-mouse IgM antibody in FACS buffer (1:200, goat IgG) for 30 min in the dark.
37. Wash the cells with 1 mL of FACS buffer and centrifuge at 220 × *g* for 5 min. Repeat this step for 3 times.
38. Add 5 μL of Propidium Iodide (PI) staining solution per 100 μL of cell suspension.
39. Incubate for 15 min on ice in the dark. Do not wash the cells.
40. Exclude dead cells from the cell suspension on a BD FACS ARIA-II cytometer. (Problem 3)

Note: Typically, the percentage of dead cells (PI+) in total cells should be less than 5%.

41. Harvest Tag1+ cells and Tag1- cells by FACS (gating strategies are described in the section of expected outcomes). Purified cells are collected into 2–3 mL cold NB-B27 medium.

Note: Tag1+ cells typically account for 15%–20% of tumor cells harvested from *Math1-Cre/ Ptch1*$^{fl/fl}$ mice at 6–8 weeks of age. (Problem 4)

42. Keep purified cells on ice (up to 4 h) for transplantation into mouse cerebella.

Cerebellar transplantation of differentiated tumor cells

Timing: 3 hrs

This section provides steps for injection of differentiated tumor cells into the cerebella of immunocompromised mice (*CB17/SCID* mice), to test the tumorigenicity of differentiated cells.

43. Centrifuge the cell suspension at 220 × *g* for 5 min and resuspend the cell pellet in NB-B27 medium. Quantify the number of live cells using a hemocytometer as described in steps 27–32.
44. Dilute the cell suspension to a density of 4–6 × 10^4 cells/ μL in NB-B27 medium, and keep on ice until use.

Note: We routinely inject 5–8 μl of cell suspension (2–5 × 10^5 tumor cells) into the cerebellum of each *CB17/SCID* mouse. However, if more cells need to be transplanted into each mouse, the cell density may be scaled up accordingly.

45. Anesthetize *CB17/SCID* mouse for brain surgery:
    a. Weigh mice using an animal weighing scale.
    b. Administer Ketamine/Xylazine solution via intraperitoneal injection (at a dosage of 10 μL per *g* of mouse weight).
    c. Inject 0.1 mL bupivacaine subcutaneously into the loose skin over the neck (where the incision will be).

d. The proper level of anesthesia in mouse should be confirmed by verifying a lack of pain response after tail pinching.

Note: The mice should be ready for the surgery within 10 min following the injection of ketamine/xylazine.

46. Place anesthetized mouse on a Kopf stereotaxic frame. Gently tighten the ear bars on the stereotaxic frame, so that they support mouse head and the head is centered. Adjust the height of the bite plate to make sure that mouse head is level on the stereotaxic frame. Press gently on the top of mouse head to ensure that the head does not move (Figures 2A and 2B). (Problem 5)

Note: After placing mouse on the stereotaxic frame, watch the mouse carefully as too much pressure through the ear bars may cause respiratory distress.

47. Apply non-antibiotic ointment on mouse eyes to ensure that they remain moist while the mouse is anesthetized and during recovery.
48. Using a sharp scalpel or scissors, make an incision (approximately 1/2″) in the midline of the scalp over the position of the cerebellum (Figure 2C).
49. Using a cotton swab with PBS, gently scrape away muscles and tendons covering the skull.
50. Drill a small hole (2 mm in diameter) in the skull over the cerebellum using a hand-held beveled 18-gauge needle (Figures 2D and 2E). Remove skull pieces from the drill point by forceps. (Problem 6)

⚠ CRITICAL: The hole on the surface of the skull should be positioned at 5–6 mm posterior to the intersection point of sagittal suture line and coronal suture line (Figures 2E and 2F). The depth of the hole should be just through the skull, but not to the beneath cerebellar tissue. Place a beveled 18-gauge needle on the surface of the skull, and gently rotate the needle to make a hole. Must not strongly press the needle over the skull to avoid piercing into beneath cerebellum.

Note: The small hole in the skull made in this step does not affect mouse survival or cause brain herniation.

51. Fill a Hamilton syringe (with an unbeveled 24-gauge needle) with cell suspension (5–8 μL) to be implanted and mount the syringe on a micromanipulator.

Note: The total volume of cell suspension should not be more than 8 μL for each injection.

52. The syringe loaded with purified cells (Tag1+ or Tag1- tumor cells) is introduced through the skull and into the cerebellum at a depth of 2–3 mm through the small hole made at step 50, by manually operating the micromanipulator though the lateral (Y-axis), probe (X-axis) and vertical (Z-axis) directions.

Note: Because the cerebellum is located beneath the forebrain, introduce the syringe needle at an angle of 40°–60° (relative to the mouse head) to target the cerebellum (as shown in Figure 2G). The depth of the needle injection is controlled by a micromanipulator. Fine positioning on Z-axis gives 1 mm movement per revolution.

⚠ CRITICAL: To prevent efflux of the injected cell suspension, penetrate the syringe (loaded with cell suspension) into the cerebellum to a depth of 4 mm and withdraw the needle slowly by 1–2 mm. This step will create some space in the cerebellum for injected cells.

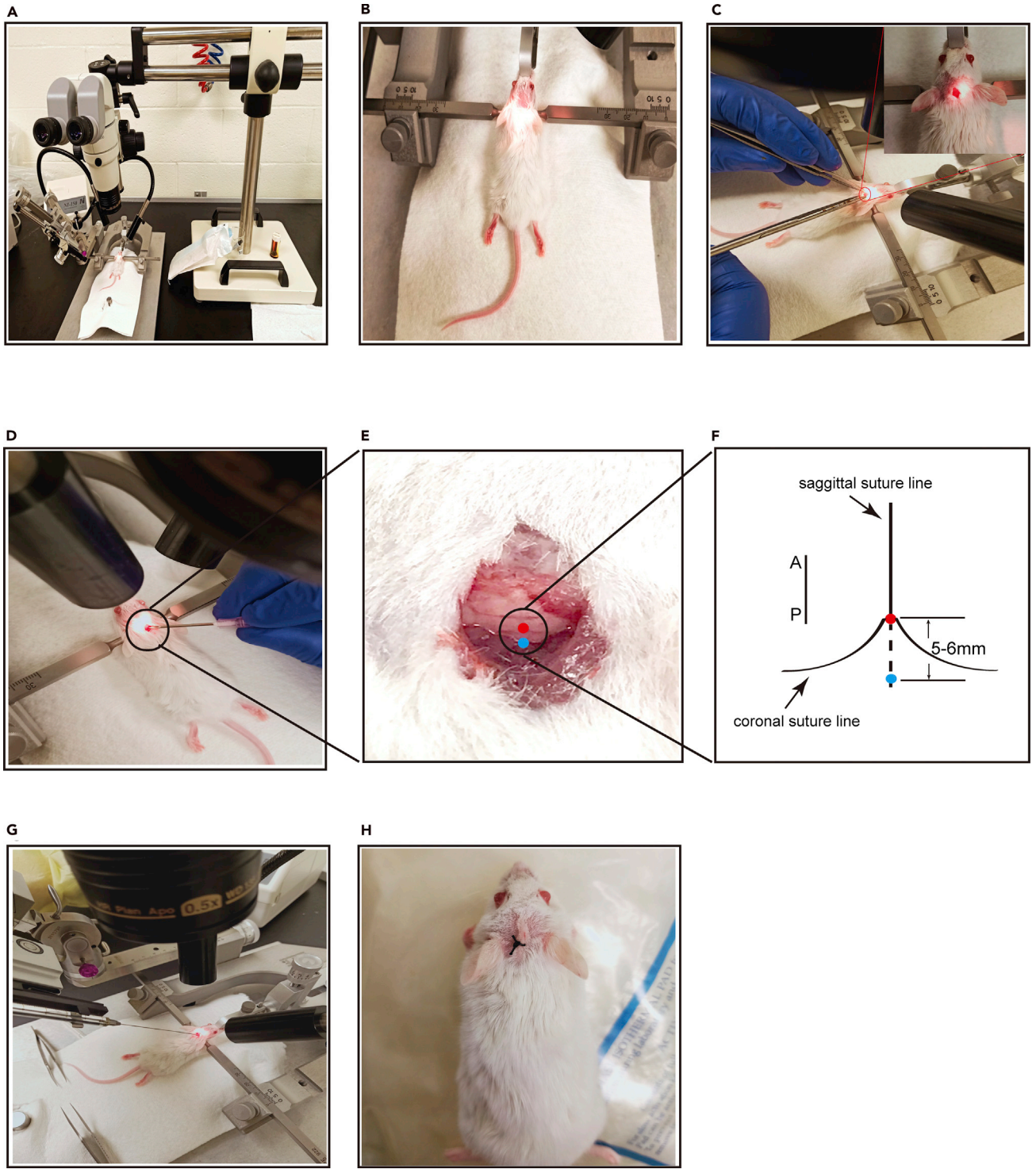

Figure 2. Procedures for cerebellar transplantation of MB cells

After being anesthetized, a *CB17/SCID* mouse was mounted on a stereotaxic frame (A), with ear bars positioned just below the mouse ear (B). A 10–14 mm incision was made through the midline of the scalp (C). An inset in C shows a close-up image of the incision. A small hole (2 mm in diameter) was drilled on the skull over the cerebellum using a hand-held needle (D and E). To locate the cerebellum beneath the skull, find the intersection of sagittal suture line and lambdoid suture lines (red solid circle). The cerebellum is located at 5–6 mm posterior to the intersection (blue solid circle), where the small hole should be made over the skull (F). A, anterior; P, posterior. Following the injection, the syringe should be left in place for 2 min to prevent efflux of injected cell suspension (G). After that, the mouse was removed from the stereotaxic frame, and the incision was sutured (H).

53. Inject the cells slowly, after which the needle should be left in place for 2 min to avoid flow-back of the injected cells (Figure 2G).

Note: It takes approximately 2 min to inject 8 μL of cell suspension into the cerebellum.

54. Remove the mouse from the stereotaxic frame. Close the incision by pulling the edges of the skin together and suturing by a 18″ PC-1 needle with a needle holder and Nylon 6-0 suture (Figure 2H). A simple interrupted pattern is recommended to close the incision, as it minimizes the risk of skin dehiscence. Tie sutures using square knots with 4 throws. The knots should be loose enough to allow for blood flow at the skin edge to minimize tissue ischemia.

Note: The Nylon sutures should be removed within 7 days after the surgery.

55. Before being put back into cages, mice should be placed on a warming pad until they start moving (for approximate 20–30 min).
56. Put back the mice into cages, and observe the mice until they regain a normal level of activity (including movement, feeding and drinking). (Problem 7)
57. Monitor the mice for signs of tumor development in the cerebellum (ataxia, head tilt and hunched back etc.). (Problem 8)

Note: It typically takes 2–3 months for mice to develop tumors after cerebellar injection of 2–5 × 10^5 MB cells. However, the tumor latency varies depending on the number of injected cells, as well as age of recipient mice. Tumor formation in recipient mice can be further confirmed by brain dissection combined with histopathological analyses.

EXPECTED OUTCOMES

This protocol describes procedures for isolation of tumor cells from MB tissue, purification of differentiated tumor cells by FACS and cerebellar transplantation of tumor cells.

Following the above protocol, we isolated tumor cells from a *Math1-Cre/Ptch1*$^{fl/fl}$ mouse at 6 weeks of age. Finally, we obtained 8.5 × 10^7 tumor cells after tissue dissociation. Less than 3% of dead cells were found in the final cell suspension, detected by trypan blue exclusion test. We then immunostained the cell suspension with an antibody against Tag1 or an isotype control (mouse IgM) for 30 min, and a secondary antibody (Alexa Fluor 594 goat anti-mouse IgM antibody) for an additional 30 min. After staining with Propidium Iodide (PI) for 15 min, the cell suspension was then analyzed for fluorescence by flow cytometry.

For cell sorting, all samples were collected on a BD FACSAria II running BD FACSDiVa software v. 7.0. Dead cells were excluded from the sample on the basis of PI fluorescence. After that, doublet discrimination was performed through the gating of coincident datapoints on a plot of the FSC-A vs. FSC-H pulse processing parameters. Discrete populations of this subset displayed in an SSC vs FSC plot permitted identification of viable cells while aiding debris exclusion. Alexa Fluor 594 labeled Tag1+ cells present in upstream populations were excited at 594 nm and detected at a bandpass of 616/23 nm. Fluorescence background was established using the isotype control. Tag1+ gating was then set to minimize inclusion of false positives in the collected fraction.

As shown in Figure 3A and 3B, majority of tumor cells were alive based on the PI fluorescence. Cell debris and clumps were sequentially excluded based on profiles of FSC-H vs SSC-A as well as profiles of FSC-H vs FSC-A. Approximate 18.6% of single tumor cells were found positive for Tag1, and almost no Alexa Fluor 594 signal was detected in control cells after labelling with the isotype IgM. We then harvested Tag1+ tumor cells (Tag1+, 18.6%) as differentiated MB cells. Tumor cells negative for Tag1 (Tag1-, 78.9%) were also collected as a control.

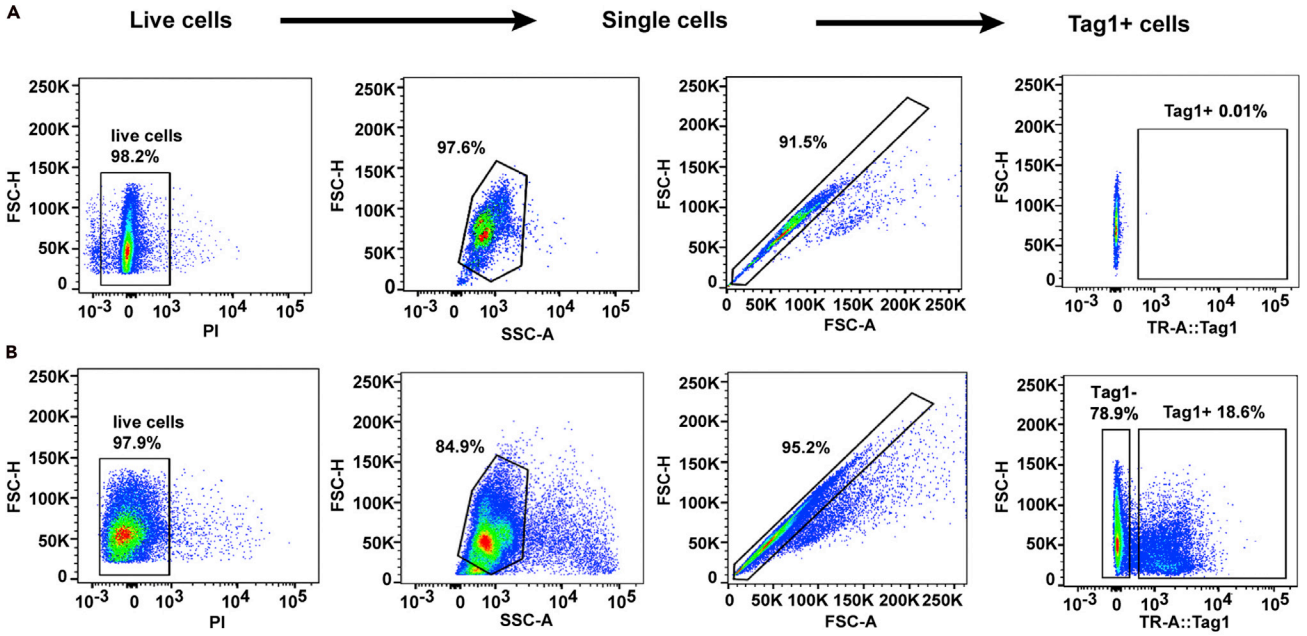

Figure 3. A gating strategy for collecting Tag1+ as well as Tag1- MB cells

MB cells were immunostained with an isotype antibody control (A) or an antibody against Tag1 (B), and then with an Alex fluor 594-conjugated secondary antibody. Dead cells were firstly excluded based on PI staining vs FSC-H. After that, single and intact cells were obtained after sequentially gating based on profiles of FSC-H vs SSC-A, and profiles of FSC-H vs FSC-A. Selected single cells were then fractionated based on Alexa Fluo 594 signal fluorescence. Approximately 18.6% of tumor cells were found positive for Tag1, whereas almost no fluorescent cells were detected in tumor cells after labelling with the isotype antibody control (A). Besides differentiated MB cells, Tag1- tumor cells, accounting for 78.9% of tumor cells, were also harvested for following transplantation.

Tag1+ or Tag1- MB cells were transplanted into the cerebella of *CB17/SCID* mice (6–8 weeks). 2 × 10^5 cells were injected into each recipient mouse following the procedures described above. 6 *CB17/SCID* mice were transplanted in each group (with Tag1+ cells or Tag1- cells). It took approximately 3 h for injecting 12 *CB17/SCID* mice (starting from anesthetizing mice till returning all mice to cages). All mice regained normal movement, feeding and drinking within 1 h after being put back into cages. Finally, all *CB17/SCID* mice injected with Tag1- cells developed MB in their cerebella (the median survival days: 53 days, Figure 4), showing tumor bearing signs including gait ataxia and domed head (Methods video S1). However, no tumors were formed in *CB17/SCID* mice after transplantation with Tag1+ cells, exhibiting normal gaits and head shape (Methods video S2).

LIMITATIONS

This protocol introduces procedures to purify differentiated tumor cells from MB tissue by FACS and examine their tumorigenicity by cerebellar transplantation. The overall strategy could be utilized to collect/study differentiated cells from many other types of brain tumors. However, this protocol uses Tag1 as a cell surface marker to define differentiated tumor cells in MB tissue. The expression of Tag1 protein is relatively restricted to differentiated granule neurons in developing cerebellum as well as differentiated MB cells. Further studies are necessary to identify cell markers expressed by differentiated tumor cells in other types of brain tumors such as glioma and meningioma.

TROUBLESHOOTING

Problem 1

Many tissue chunks remain at the bottom of the tube after the trituration (step 18).

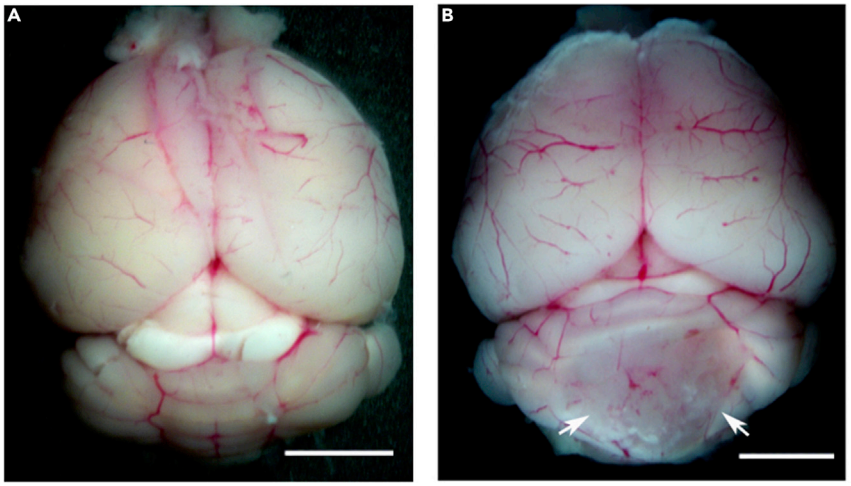

Figure 4. A MB derived from Tag1- tumor cells upon cerebellar transplantation

Purified Tag1- cells and Tag1+ cells were transplanted into the cerebella of *CB17/SCID* mice. By 8 weeks following the transplantation, no tumors were detected in the cerebella of *CB17/SCID* mice transplanted with Tag1+ MB cells (A). However, Tag1- MB cells developed into tumors (arrows, B) in the cerebella of *CB17/SCID* mice with 100% penetrance. Scale bars, 4 mm.

Potential solution

Papain solution needs to be prepared freshly at the day of tumor tissue dissociation. Make sure pH value of the papain solution is around 7.0–7.3 (based on the orange shade of the solution or measurement by a pH meter), which is important for papain activation.

It is important to cut the tumor tissue into 2–3 mm pieces for papain digestion (step 6). Larger tissue pieces may cause incomplete papain digestion, which could affect the efficiency of trituration.

Problem 2

Too few live cells/too many dead cells are obtained from MB tissue after the dissociation (step 32).

Potential solution

70–90 million of tumor cells can be harvested from one cerebellum in *Math1-Cre/Ptch1*$^{fl/fl}$ mouse at 6–8 weeks of age. Incomplete trituration of tumor tissue often results in low cell yield from the dissociation. Try to triturate the tissue pieces gently and slowly. During the trituration, ensure to draw up the solution but not the tissue pieces (steps 9–18).

Always centrifuge cells at 4°C during tumor cells isolation. The centrifugal speed should not be over 250 g. Excessive centrifugation may cause damage of MB cells.

Problem 3

The percentage of dead cells (PI+) in cell suspension is higher than 2% after PI staining (step 40).

Potential solution

Always perform cell immunolabelling procedures on ice. For staining cells for surface antigens (including Tag1), tumor cells can not be fixed. In addition, cells should be analyzed within 3 h after PI staining, due to the potential adverse effects on the viability of cells left in the presence of PI for prolonged periods.

Problem 4

Almost no positive cells or too many positive cells are detected in tumor cells after immunostaining with the anti-Tag1 antibody (step 41).

Potential solution

No detergents should be included in FACS buffer, which may destroy most of cell surface antigens, resulting in no positive cells after the immunostaining. In addition, all the immunostaining procedures should be performed on ice. Many cells may undergo apoptosis after being kept at 20°C–25°C for over 10 min, which could result in too much unspecific fluorescent signal (autofluorescence) from dying cells.

Problem 5

The mouse head is tilted after being placed on the stereotaxic frame (step 46).

Potential solution

The mouse head is tilted on the stereotaxic frame, indicating that the ear bars or the bite plate are in the wrong place. This may cause mouse respiratory distress or death. In addition, the tilted head may move, interfering with the following surgery.

If the mouse head is not level on the stereotaxic frame, loose the ear bars on both sides of the mouse. Lock the left ear bar in place, to make sure the mouse head is centered. Adjust the right ear bar to make sure the mouse head is level in the left/right direction. The final step is to bring in the bite plate and adjust the height to ensure the mouse head is level in the up/down direction.

Problem 6

Bleeding at the drill point by a 18-gauge needle used for making a hole on mouse skull (step 50).

Potential solution

In the case of bleeding at step 50, immediately pull out the needle, and press the drill point (needle entrance) using a cotton swab for 20–30 s. The bleeding is easily stopped and will not interfere with the following injection of tumor cells.

Problem 7

Mice die during the surgery or within 3 days following cerebellar transplantation (step 56).

Potential solution

The mouse mortality during the surgery is often caused by overdosage of ketamine during the anesthetization. In this protocol, we inject mice with Ketamine (100 mg/kg)/xylazine (30 mg/kg) by intraperitoneal injection, which normally provides a surgical level of anesthesia for 20–30 min. More than 100 mg/kg ketamine likely results in mouse death. If the mouse is still responsive to tail pinch after 20–30 min following the above anesthetic regimen, start to anesthetize another mouse, instead of giving additional ketamine/xylazine.

Mice may die within 3 days after the surgery because of infection. Mice should be monitored daily within 5 days after the surgical procedure. Examine the surgical incision to ensure that it is intact, dry and clean. Excessive redness, a thick discharge or swelling are common symptoms of surgical site infection. If this is the case, contact veterinarians for further evaluation or treatment. To avoid infections, always autoclave the surgical tools as well as gloves for transplantation, and all personnel performing the surgery should strictly follow aseptic techniques for survival rodent surgery. Silk suture must not be used for skin closure after the surgery, due to an increased potential for infection as well as a local tissue reaction.

After the surgery, mice may have difficulty reaching food and/or drink in the cage. Provide nutritionally fortified water gel to mice that cannot access food and/or drink.

Problem 8

No tumors are developed in mice transplanted with Tag1- MB cells (step 57).

Potential solution

Tag1- tumor cells should develop into tumors in *CB17/SCID* mice with 100% penetrance. Decreased incidence of tumor formation may be due to the injection of unhealthy cells. Tumor cells should be kept on ice all the time before the injection. In addition, tumor cells may flow back after being injected into the cerebellum, which could compromise the tumor formation in *CB17/SCID* mice. Always inject the cells slowly into the cerebellum (step 52), and leave the syringe in place for additional 2 min after the injection, to prevent the efflux of injected cells (step 53).

It is necessary to practice the transplantation procedure by injection of unsorted tumor cells (2–5 × 10^5 cells) into cerebellum of immunocompromised mice, until the tumor incidence rate reaches over 90% (indicating that the protocol is well-established).

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact, Zeng-jie Yang (zengjie.yang@fccc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets and codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100409.

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AUTHOR CONTRIBUTIONS

Z.Y. and Y.Y. designed the experiments, analyzed and interpreted the data, and wrote the manuscript. Y.Y. and Y.Q. performed the experiments and interpreted the data and designed the figures. Y.C., Q.F., and D.G. participated in the interpretation of the data. All the authors critically reviewed, edited, and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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