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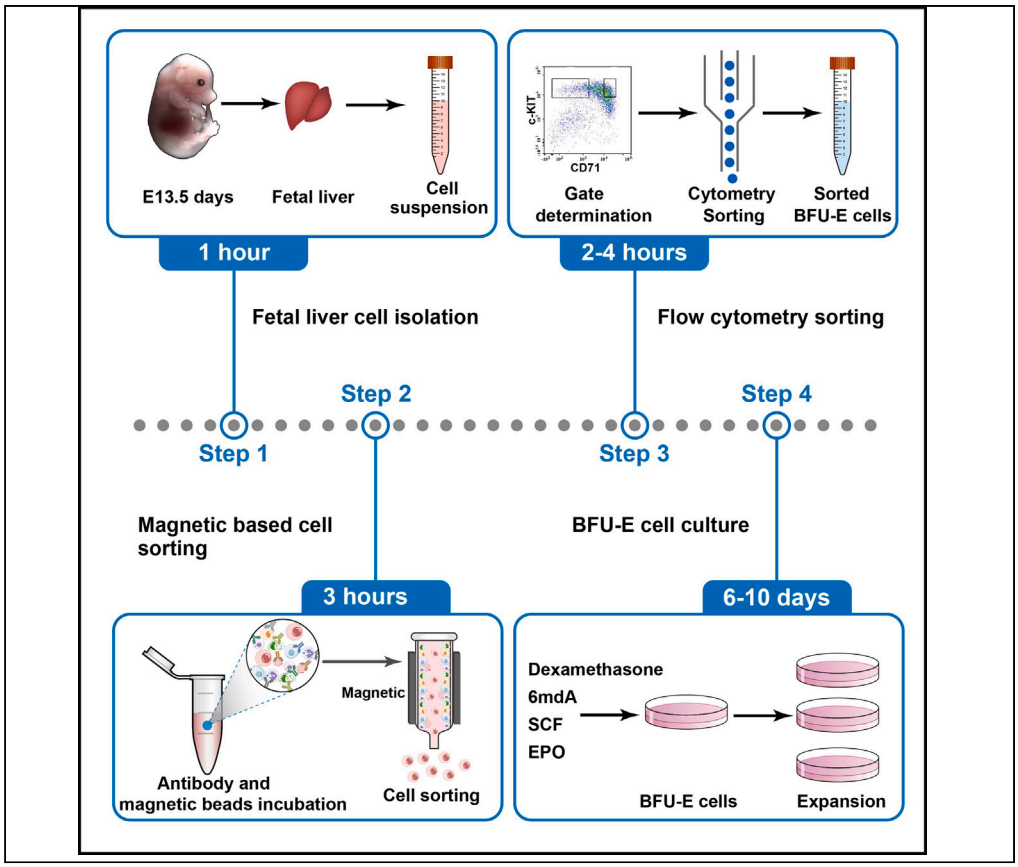
Protocol for sorting and culturing of mouse early erythroid progenitor BFU-E cells



Techniques allowing the long-term culture of the burst-forming unit of erythroid (BFU-E) progenitor cells are essential for understanding erythropoiesis. Here, we present a protocol for sorting mouse BFU-E cells and culturing them in a medium that promotes BFU-E cell expansion. We describe steps for isolating BFU-E cells from mouse fetal livers by combining magnetic microbeads with flow cytometry and culturing BFU-E cells with a specific expansion media. This approach can enhance the production of BFU-E cells.

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

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Highlights

Isolation of mouse BFU-E cells from fetal liver

Steps for magnetic bead-based sorting followed by flow cytometry

Expansion of BFU-E cells in specific expansion medium

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Protocol for sorting and culturing of mouse early erythroid progenitor BFU-E cells

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SUMMARY

Techniques allowing the long-term culture of the burst-forming unit of erythroid (BFU-E) progenitor cells are essential for understanding erythropoiesis. Here, we present a protocol for sorting mouse BFU-E cells and culturing them in a medium that promotes BFU-E cell expansion. We describe steps for isolating BFU-E cells from mouse fetal livers by combining magnetic microbeads with flow cytometry and culturing BFU-E cells with a specific expansion media. This approach can enhance the production of BFU-E cells.

For complete details on the use and execution of this protocol, please refer to Li et al..[1]

BEFORE YOU BEGIN

This protocol is developed for mouse BFU-E cell sorting and culturing *in vitro*. To avoid bacterial contamination, all materials and buffers require sterilization before use. The below describe these requirements in detail.

Institutional permissions

Prior to executing the protocol presented herein, investigators must acquire institutional permission and perform procedures following institutional guidelines.

All animal procedures in this protocol were approved by and performed according to the guidelines of the Animal Ethics and Welfare Committee of Research Center for Eco-Environment Sciences, Chinese Academy of Science, approval No. AEWC-RCEES-2021049.

Isolation and preparation of fetal liver cells

Timing: 20 min

1. Prepare 13.5-day pregnant for fetal liver cell isolation.
2. Set the dissecting station with the following materials:
   a. Clean benchtop.
   b. Autoclave sterilized scissors and tweezers.
   c. Sterilized 15 mL and 1.5 mL tubes.
   d. 30 μm pre-separation filters.
   e. 35 mm cell culture dish.
   f. 1X phosphate-buffered saline (PBS) and 0.5% BSA PBS solution.

Flow cytometer preparation

Timing: 2 h

3. Prepare 10 L sterilized 1X PBS as the sheath solution.
4. Prepare the flow cytometer by turning on the machine and confirming optimal settings.

Erythroid progenitor BFU-E cell culture preparation

Timing: 1 h

5. Prepare growth factor for basic expansion media:
   a. Dissolve recombinant murine stem cell factor (SCF) to 40 mg/mL with 1X PBS, 100 μL per tube, and store at −20°C.
   b. Dissolve recombinant human erythropoietin (EPO) in sterile water containing 0.1% BSA to 10, 000 U/mL, and store at −20°C.
   c. Dissolve 25 mg dexamethasone in 127 μL ethanol, dilute with 6.243 mL serum-free IMDM medium to a storage concentration of 10 mM, and store at −20°C.
6. All cells are cultured in a standard 5% CO_2 37°C humidified incubator and handled in a sterile cell culture biosafety cabinet.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Biotin anti-mouse lineage panel 0.2 μg of each antibody per million cells	BioLegend	Cat# 133307; RRID: AB_11124348
Biotin anti-mouse CD34 0.2 μg antibody per million cells	BioLegend	Cat# 128604; RRID: AB_1236371
Biotin anti-mouse CD41 0.2 μg antibody per million cells	BioLegend	Cat# 133930; RRID: AB_2572133
Biotin anti-mouse Sca-1 0.2 μg antibody per million cells	BioLegend	Cat# 108104; RRID: AB_313341
Biotin anti-mouse CD16/32 0.2 μg antibody per million cells	BioLegend	Cat# 101303; RRID: AB_312802
PE anti-streptavidin antibody 0.2 μg antibody per million cells	BioLegend	Cat# 410504; RRID: AB_2571915
APC anti-mouse CD117 (c-Kit) 0.2 μg antibody per million cells	BioLegend	Cat# 135108; RRID: AB_2028407
PE/Cy7 anti-mouse CD71 0.2 μg antibody per million cells	BioLegend	Cat# 113812; RRID: AB_2203382
Chemicals, peptides, and recombinant proteins		
N6-methyldeoxyadenosine (6mdA)	Sigma-Aldrich	Cat# M2389
Streptavidin microbeads	Miltenyi Biotec	Cat# 130-048-101
StemPro-34 SFM	Thermo Fisher Scientific	Cat# 10639011
Human EPO	PeproTech	Cat# 100-64-10
Murine SCF	PeproTech	Cat# 250-03-10
Dexamethasone	Sigma-Aldrich	Cat# D4902
IMDM (glutamine-free)	HyClone	Cat# SH30259
L-glutamine	Thermo Fisher Scientific	Cat# 25030081
Software and algorithms		
NovoExpress	ACEA	N/A
Prism9	GraphPad	https://www.graphpad.com/scientific- software/prism/

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Pre-separation filters (30 μm)	Miltenyi Biotec	Cat# 130-041-407
LS columns	Miltenyi Biotec	Cat# 130-042-401
LS column adapter	Miltenyi Biotec	Cat# 130-090-544
1.5 mL tube	Axygen	Cat# YC-HC01008
15 mL tube	Corning	Cat# 430791
35 mm cell culture dish	Corning	Cat# CLS430165
Clean benchtop	Thermo Fisher Scientific	N/A
FACSAria II	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

1 X PBS		
Reagent	Final concentration	Amount
NaCl	140 mM	80 *g*
Na_2HPO_4-$12H_2O$	4.3 mM	35.8 *g*
KCl	2.7 mM	2 *g*
KH_2PO_4	1.4 mM	2.4 *g*
H_2O	N/A	10 L
Total	**N/A**	**10 L**
Store at 25°C after sterilized for up to 6 months.		

0.5% BSA PBS solution		
Reagent	Final concentration	Amount
BSA	0.5% (W/V)	0.25 *g*
1 X PBS	N/A	50 mL
Total	**N/A**	**50 mL**
Store at 25°C for up to 1 week.		

Expansion media:		
Reagent	Final concentration	Amount
StemPro-34 SFM	N/A	39.5 mL
L-Glutamine	2 mM	400 μL
SCF (40 μg/mL)	100 ng/mL	100 μL
EPO (10,000 U/mL)	0.5 U/mL	2 μL
Dexamethasone (10 mM)	10 μM	4 μL
6mdA (20 mM)	100 μM	80 μL
Total	**N/A**	**40 mL**
Store at 4°C for up to 1 month. Warm at 37°C before use.		

Note: The addition of 6mdA was found to significantly enhance the proliferation of early erythroid progenitor BFU-E cells in the concentration of 10–100 μM, to maximize the proliferation potential of BFU-E cells; the concentration of 6mdA used is 100 μM.

Note: 6mdA needs to be added at an early stage during BFU-E culture. Especially, adding 6mdA after just BFU-E sorting can achieve the best effect of promoting proliferation.

STEP-BY-STEP METHOD DETAILS

Fetal liver cells isolation

Timing: 1 h

This protocol has been developed to isolate and culture early erythroid progenitor BFU-E cells. Sacrifice a pregnant mouse at 13.5 days gestation (approximately 6–9 fetuses) and place these fetuses in one tube for further isolation.

1. Dissect a pregnant mouse at 13.5 days gestation, cut the skin, and take out the uterus. Troubleshooting 1.
2. Use fine tipped forceps to tear the uterus in the gap between the fetuses and squeeze out the fetuses.
3. Place fetuses into PBS in a culture dish, wash once, and place in a new culture dish.
4. Squeeze the fetal liver from a fetus, remove connective tissues surrounding the liver, and put it into a 15 mL tube.

Note: If using non-wild type or treated mice, place the fetus separately for further isolation.

5. Dissociate fetal liver cells by pipetting with 4 mL PBS-BSA, generating a single cell suspension.
6. Pass the cell suspension through a 30 μm pre-strainer into a new 15 mL tube. Count cell and centrifuge for 5 min at 300 × *g* at 4°C.

Magnetic based cell sorting

Timing: 2 h

The magnetic based erythroid cell sorting is adapted from McIver et al.[2] and Flygare et al.[3] All magnetic cell sorting procedures are performed on a clean benchtop.

7. Lineage negative (Lin^-) cells isolation.
   a. Adjust the cell concentration of fetal liver cells to 100 × 10^6 cells/mL with PBS-BSA in a 1.5 mL tube.

   Note: Because subsequent antibody addition requires a certain volume, each 1.5 mL tube has 500 μL PBS-BSA containing 50 × 10^6 fetal liver cells.

   b. Add 0.2 μg of each antibody of the biotin anti-mouse lineage panel per million cells, and incubate at 4°C for 15 min.

   Note: Before antibody incubation, it is recommended a blocking step, e.g., 10% serum.

   c. Wash off unbound antibody with 1X PBS, and centrifuge for 5 min at 300 × *g* at 4°C.
   d. Resuspend cells with 500 μL PBS-BSA, add 2 μL streptavidin microbeads per million cells and incubate at 4°C for 30 min.
   e. Pass the labeled fetal liver cells through the LS column according to manufacturer's instructions: https://www.miltenyibiotec.com/CN-en/products/ls-columns.html#130-042-401.
      i. Collect Lin^- cells and centrifuge.
      ii. Adjust cell concentration to 100 × 10^6 cells/mL for the second round of magnetic cell sorting.

Note: The use of LS column can also reduce the residue of magnetic bead-bound cells.

8. $Lin^-CD34^-CD41^-CD16/32^-Sca1^-$ cells isolation.
   a. Incubate Lin^- cells with 0.2 μg of each antibody of the biotin anti-mouse CD34, biotin anti-mouse CD41, biotin anti-mouse Sca-1, and biotin anti-mouse CD16/32 per million cells for 15 min at 4°C.
   b. Wash off unbound antibody with 1X PBS.
   c. Add 2 μL streptavidin microbeads per million cells, and incubate at 4°C for 30 min.
   d. Pass the labeled fetal liver cells through the LS column according to manufacturer's instructions https://www.miltenyibiotec.com/CN-en/products/ls-columns.html#130-042-401, collect $Lin^-CD34^-CD41^-CD16/32^-Sca1^-$ cells.

Flow cytometry sorting for BFU-E cells

Timing: 3–4 h

This step is adapted from Flygare et al.[3] to get higher purity BFU-E cells.

9. Count cells, centrifuge 5 min at 300 × *g* at 4°C and resuspend cells with 400 μL PBS-BSA.

   Note: After magnetic based cell sorting, about 10×10^6 cells will be obtained from 9 embryos at E13.5 days.

10. Assemble 0.2 μg of each antibody per million cells: APC anti-mouse c-Kit (CD117), PE anti-Streptavidin, and PE/Cy7 anti-mouse CD71.

    Note: Because most live/dead dyes are harmful to cells, cell debris and dead cells are simply excluded according to SSC and FSC.

11. Single stain control construction.
    a. Harvest 2×10^5 cells in 100 μL PBS-BSA.
    b. Add each antibody individually to separate tubes.
    c. One tube with 2×10^5 cells unstained for use as a no-stain control.
12. Incubate antibodies and cells in the dark at 4°C for 15–20 min.
13. Centrifuge 5 min at 300 × *g* at 4°C, and wash off unbound antibody with 1 mL PBS, collect cells by centrifuge 5 min at 300 × *g* at 4°C.
14. Resuspend cells with 2 mL PBS for cell sorting with a flow cytometer.

    Note: When the sorter being prepared, the cells should be stored at 0.5% BSA-PBS solution at 4°C.

15. Set the voltage and regulate the compensation of PE, PE/Cy7, and APC fluorescence using single stain control (Figures 1A–1C).
16. For flow cell sorting, exclude cell debris and dead cells according to SSC and FSC (Figure 1D), take PE-streptavidin negative cells as gate 1 (Figure 1E), BFU-E cells are sorted from Kit^+ fraction according to CD71 expression (10% lowest CD71 expression part ($CD71^{10\%low}$))[3] (Figure 1F). Troubleshooting 2.

Expansion culture

Timing: 6–10 days

The expansion medium promotes the rapid expansion of early erythroid progenitor cells as referred to McIver's describe.[2] SCF (Kit receptor agonist) and dexamethasone (glucocorticoid receptor agonist) stimulate the expansion of early erythroid progenitor cells. Especially, we add

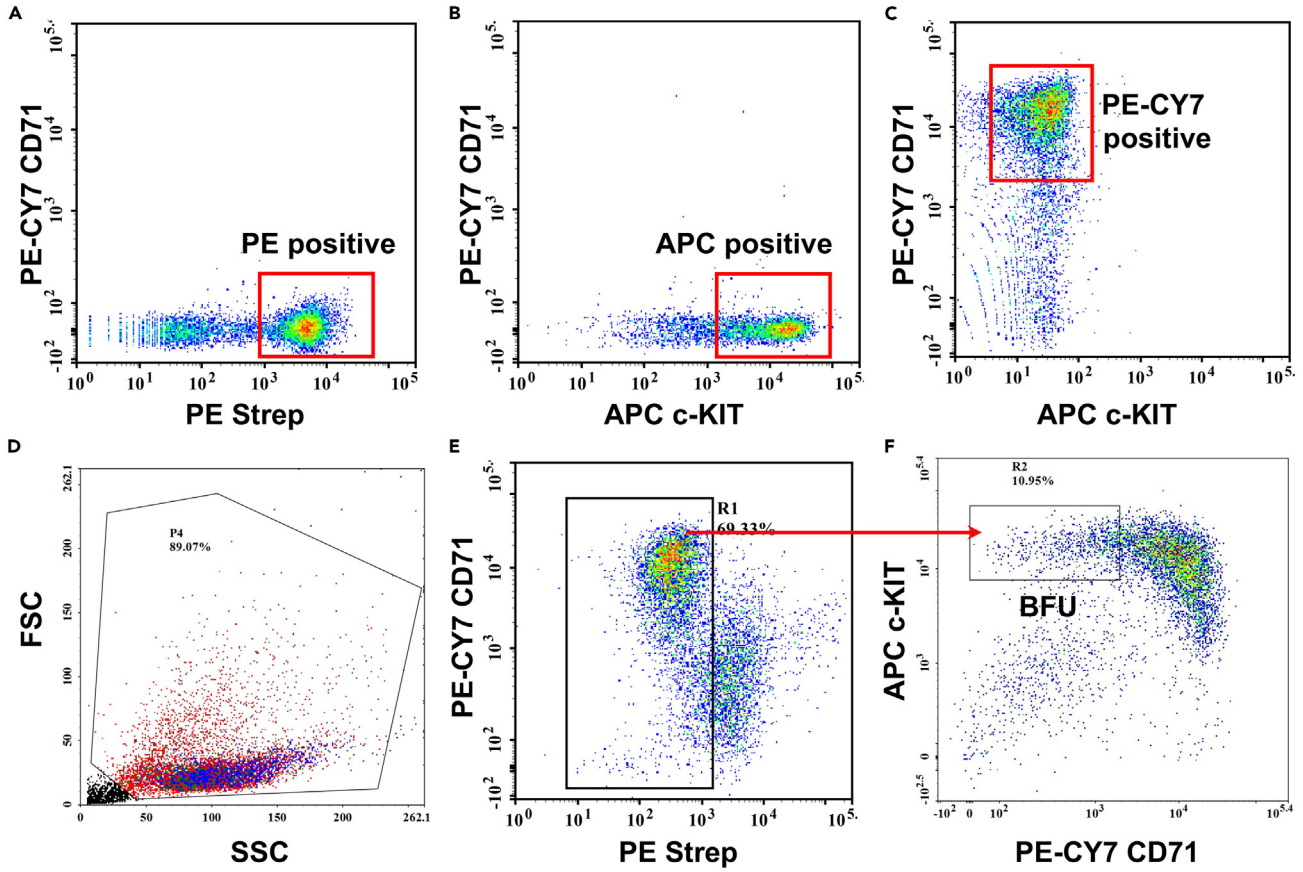

Figure 1. BFU-E cell sorting with flow cytometry

(A) Compensation and gating of PE, (B) APC, and (C) PE/Cy7 fluorescence using single strain control.

(D)FSC and SSC gating.

(E) Streptavidin negative population for BFU-E sorting.

(F) BFU-E sorting by flow cytometry according to the expression of CD71 and c-Kit, c-Kit^{+}CD71^{-} represents BFU-E.

N6-methyladenosine (6mdA) during the expansion culture of BFU-E cells, which can promote the proliferation of early erythroid progenitor cells in the range of 10–100 μM (Figure 2).

Note: In this protocol, we test the concentration of 6mdA in the range of 10–100 μM; we speculate that 6mdA has the same effect when the concentration is greater than 100 μM.

17. Plate the sorted BFU-E cells in expansion media at a concentration of 0.25–1 × 10^6 cells/mL in 6-well plate. Troubleshooting 3.

Note: The concentration of cells is important for BFU-E cell expansion, it is necessary to keep the cells in the optimal concentration range (0.25–1 × 10^6 cells/mL) with fresh expansion media.

18. Cells are cultured at 37°C and 5% CO_2 in a sterile cell culture incubator.

EXPECTED OUTCOMES

The main goal of this protocol is to generate more BFU-E cells *in vitro*. SCF, dexamethasone, and EPO are commonly used to enhance the proliferation of erythroid progenitor cells. These growth

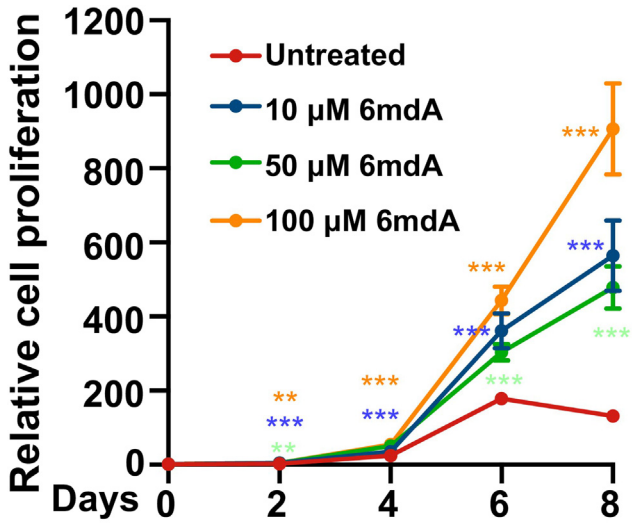

Figure 2. 6mdA promotes the proliferation of BFU-E cells

(A) Relative cell proliferation rate of BFU-E cells 6mdA treatment was analyzed by MTS. Each treatment contained six replicates. Data are presented as mean+/-SD. P values were calculated by one-way ANOVA for the 6mdA treated group compared with the no-6mdA (untreated) group of each day (no significance were not shown, *P < 0.05, **P < 0.01, ***P < 0.001).

factors, together with 6mdA can expand BFU-E cells more than 5,000 times (from 0.02 million to 105 million BFU-E cells after 8 days expansion).[1]

Most BFU-E cells could express Ter119 protein and produce hemoglobin after differentiation induction.[1] Therefore, the BFU-E cells which isolated and cultured using this protocol can be used for erythropoiesis exploration.

LIMITATIONS

This method is more suitable for sorting BFU-E cells from mouse fetal liver. Because of fewer BFU-E cells proportion and much antibody consumption for negative cell depletion, it is difficult to use this pipeline to sort BFU-E cells from adult mouse bone marrow cells.

Adding 6mdA during BFU-E cell culture significantly improves the proliferative capability, but after about 10 days of culture, the expansion of BFU-E cells would be sharply decreased. Subsequent exploration of erythropoiesis *in vitro* should be conducted within 10 days.

TROUBLESHOOTING

Problem 1

Mice are not 13.5 days pregnant (related to Step 1).

Potential solution

During E12.5 days to E17.5 days, the fetal liver is the main erythropoiesis tissue of mice.[4,5] Thus, the fetal liver from E12.5–E15.5 days fetus can be used as the material for sorting BFU-E cells. However, we found E13.5 days fetal liver is most suitable.

Problem 2

The obtained BFU-E cells are fewer than expected (related to Step 16).

Potential solution

If we get fewer BFU-E cells than we expected, the problem is most likely to arise at magnetic based cell sorting using the LS column. When passing the fetal liver cells through the LS column, the LS column needs to be moistened with 2 mL PBS, then pass the cell suspension through the column. Until cell suspension completely passes the column, wash with 3 mL PBS or PBS-BSA solution three times. This way can greatly increase the number of collected cells following the manufacturer's instructions.

Problem 3

BFU-E cells are growing slowly (related to Step 17).

Potential solution

Long *in vitro* cell sorting procedures (at least 6 h) will affect cell activity, BFU-E cells proliferate slowly in the first two days and proliferate rapidly from day 3 expansion. In addition, increasing the initial cell culture concentration is helpful for the proliferation of cultured BFU-E cells. To count cells and adjusting cell concentration in the optimal range (0.25×10^6–1×10^6 cells/mL in expansion media) is also important.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hailin Wang (hlwang@rcees.ac.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper does not report original code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

H.L.W. conceptualized and supervised the project. Y.L. and Z.Y.L. performed the experiments. H.L.W. and Y.L. wrote the article.

DECLARATION OF INTERESTS

We have a Chinese patent pending review (2023105116380).

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