

Generation and validation of *ROSA26^{mT-mG}* knock-in mouse with *CRISPR/Cas9* as a universal lineage tracing model

Behrouz Asgari Abibeiglou¹, Magid Fallahi¹, Mahdi Hesarak², Meghdad Yeganeh³, Mohammad Rezaei^{1,4}, Hossein Baharvand^{1,5}, Mohsen Basiri^{1,6*}, Yaser Tahamtani^{1,4*}

¹Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

²Department of Animal Sciences and Marine Biology, Faculty of Life Science and Biotechnology, Shahid Beheshti University, Tehran Iran

³School of Biotechnology, College of Science, University of Tehran, Tehran, Iran

⁴Department of Basic and Population Based Studies in NCD, Reproductive Epidemiology Research Center, Royan Institute, ACECR, Tehran, Iran

⁵Department of Developmental Biology, University of Science and Culture, Tehran, Iran

⁶Department of Hematology and Hematopoietic Cell Transplantation (T Cell Therapeutics Research Laboratories), City of Hope Beckman Research Institute and Medical Center, Duarte, CA, USA

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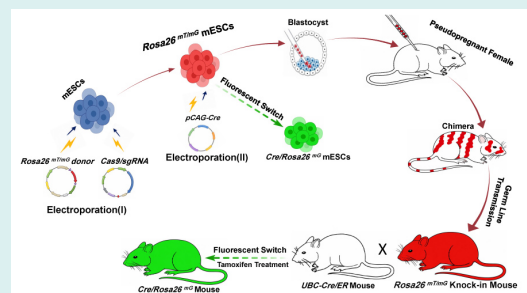
Abstract

Introduction: Transgenic *ROSA26^{mT-mG}* knock-in mice serve as an invaluable tool in developmental and differentiation studies, particularly for tracing and analyzing cell lineages throughout various stages of growth.

Methods: In this study, we employed the CRISPR/Cas9 system to precisely introduce desired genetic material ("*ROSA26^{mT-mG}*" vector) into the *Rosa26* locus of the mouse genome.

Results: Under fluorescent light, *ROSA26^{mT-mG}* knock-in mice exhibit a red phenotype in the absence of Cre recombinase. However, when crossed with the "*UBC-Cre/ER*" transgenic mouse, the membrane-targeted *tdTomato* (*mT*) gene is excised at the *LoxP* site, leading to the expression of the membrane-targeted green fluorescent protein (*mGFP*) gene, thereby changing the mouse's appearance from red to green under fluorescent light.

Conclusion: *ROSA26^{mT-mG}* knock-in mice can serve as universal lineage tracing models in a diverse array of developmental biology and regenerative medicine studies, enabling researchers to investigate differentiation pathways, and tissue regeneration mechanisms in a clear and visually interpretable manner.



Lineage tracing enables long-term tracking of progenitor cells and their descendants during development and differentiation.^{1,2} This information can be used for the isolation and transplantation of specific cells, while also enabling the observation of cell behavior in intact tissue or organism. There are several methods for lineage tracing, such as direct observation,³ transplantation of tissue and cells,⁴ labeling cells with dyes and radioactive tracers⁵ and introduction of genetic markers.⁶ However, one of the best methods for lineage tracing is labeling cells through genetic recombination, as this system allows for precise spatial and temporal control of gene expression.

Genetic recombination itself has two components; a conditional reporter gene and a recombinase enzyme,⁷ such as *Cre/loxP* recombination system.⁸ Here, we have generated a transgenic mouse with conditional reporter genes as a universal tool for lineage tracing. In this study, a double fluorescent reporter gene system is used, including a membrane-targeted *tdTomato* protein and a membrane-targeted green fluorescent protein (*mGFP*) with a ubiquitous promoter; *tdTomato* is expressed prior to *Cre* excision, and *mGFP* is expressed following *Cre* excision. In addition, a novel nuclear CRISPR/Cas9 system⁹ was used to direct the desired genetic material within "*tdTomato*/"



*Corresponding authors: Mohsen Basiri, Emails: mbasiri@coh.org, mohsenbasiri@gmail.com; Yaser Tahamtani, Emails: y.tahamtani@royan-rc.ac.ir, yasertahamtani@gmail.com



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mGFP" to *Rosa26* locus of mouse genome. Although generation of a similar model using conventional gene targeting methods was reported previously,^{10,11} here we successfully generated this knock-in *ROSA26^{mT-mG}* mouse using the CRISPR/Cas9 system during our study.

The *ROSA26^{mT-mG}* donor plasmid and *gRNA^{ROSA26}/Cas9* (guide RNA) vector were obtained from Vector Bank of Royan Institute. The *ROSA26^{mT-mG}* donor plasmid has two reporter genes i.e., *tdTomato* and *mGFP* with ubiquitous promoter and antibiotic resistance cassette for

Neomycin. Reporter genes of *ROSA26^{mT-mG}* are conditional because *tdTomato* is flanked by the *loxP* sequences. We used CRISPR/Cas9 system in order to target the donor construct within the intron 1 of *Rosa26* locus. The *gRNA^{ROSA26}/Cas9* vector encodes gRNA and Cas9 enzyme (Fig. 1A; Supplementary file 1). The gRNA guides Cas9 enzyme to intron 1 of *ROSA26* locus causing a double strand break. Consequently, the desired *tdTomato/mGFP* genetic material of *ROSA26^{mT-mG}* vector aligns with intron 1 of *Rosa26* locus through its upstream and downstream

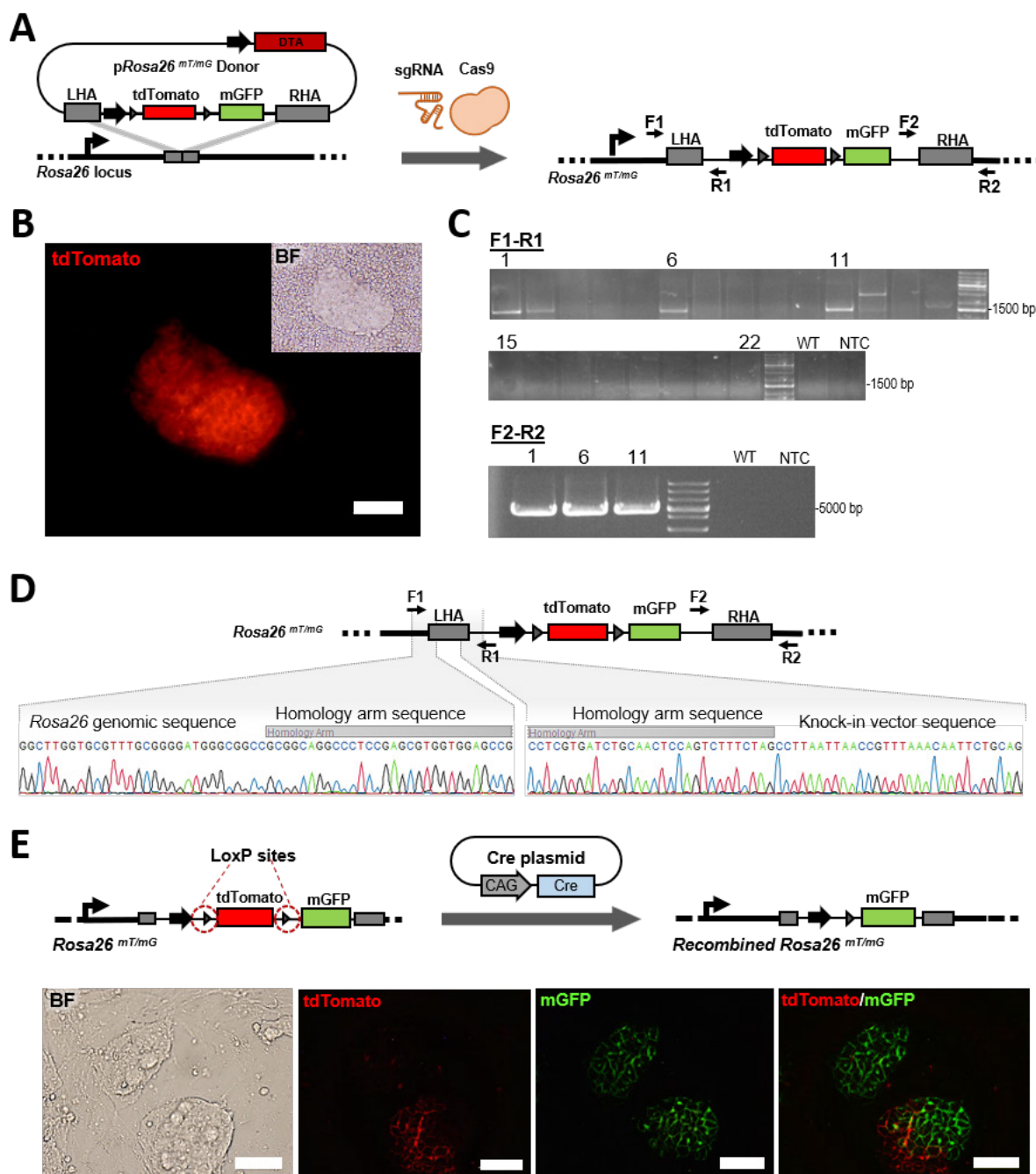


Fig. 1. Generation and validation of the *ROSA26^{mT-mG}* knock-in mESC line. **A.** Schematic of CRISPR/Cas9 knock-in strategy for inserting the *ROSA26^{mT-mG}* reporter cassette into the *Rosa26* locus and positions of specific primers for recombination confirmation. **B.** Representative images of targeted mESC colony showing *tdTomato* expression. **C.** PCR validation of successful knock-in into intron 1 of the *Rosa26* locus. Expected band sizes: 1450 bp (LHA) and 5000 bp (RHA). **D.** Sequencing of the F1/R1 PCR amplicon confirmed correct targeted insertion of the knock-in vector. The junction between the homology arm and its flanking sequences is shown, with endogenous genomic sequence on one side and vector-derived sequence on the other. **E.** Schematic of Cre-mediated recombination leading to expression from the CAG promoter and representative images of knock-in mESCs after Cre electroporation, showing bright-field (BF), *tdTomato*, and *mGFP* fluorescence. Scale bar: 50.0 μ m. Abbreviations: RHA: right homology arm; LHA: left homology arm; DTA: diphtheria toxin A; NTC: no template control; WT: Wild Type; CAG: Chicken beta-actin promoter.

homology arms, while Cas9 enzyme of *gRNA^{ROSA26}/Cas9* creates a specific DNA cut allowing recombination to occur through the homologous recombination (Fig. 1A). The V6.5 mouse embryonic stem cell (mESCs) line (derived from C57BL/6 $\times$ 129/sv) was obtained from Royan Stem Cells Bank. These cells (5×10^6 cells) were electroporated (Vol:250v, Capacitance:500 μ F, Cuvette:4mm, TC (Time Constant):17.2 ms) with *ROSA26^{mT-mG}* donor plasmid (25 μ g) and the *gRNA^{ROSA26}/Cas9* (50 μ g) vectors. Twenty-two single-cell-derived tdTomato-positive mESC colonies (Fig. 1B) were screened by locus-specific junction PCR using primer pairs that bridge the endogenous target locus and inserted vector sequence across each homology arm (F1/R1 and F2/R2). Three colonies (1, 6, and 11) positive at the left junction by F1/R1 primer pair were also confirmed at the right junction using F2/R2 primers, with no amplification from wild-type genomic DNA (Fig. 1C). Sequencing of the F1/R1 product further verified the expected linkage of genomic flanking sequence, homology arm, and inserted vector sequence (Fig. 1D). The three correctly targeted mESC clones, 1, 6, and 11, were also subjected to karyotype analysis and clone 11 was selected for microinjection after confirmation of the expected sequence and male karyotype.

To evaluate functionality of conditional reporter genes, *Rosa26^{mT-mG}* knock-in mESCs were electroporated (250 V, 500 μ F, 4 mm cuvette, TC of 12.7 ms) with a Cre recombinase-expressing plasmid (28 μ g) to induce transient

Cre expression (Fig. 1E). Two days after electroporation, tdTomato reporter gene expression was abolished in the majority of the cells ($\leq 70\%$), as Cre recombinase excised the tdTomato sequence at the loxP sites, thereby activating mGFP expression (Fig. 1E). The expression of both transgenes along with positive PCR results on both ends of the transgene.

After characterization of *Rosa26^{mT-mG}* mESCs, line #11 was injected into the host blastocyst of NMRI mouse strain for generation of chimera mice. The blastocysts were harvested from the super-ovulated female mice. Subsequently, 10–15 *Rosa26^{mT-mG}* mESCs were transferred into each blastocyst (Fig. 2A–2B). Approximately 10 injected embryos were transplanted into the uterine horns of pseudo-pregnant recipient female mice by surgery (Fig. 2C). Microinjection of V6.5 *Rosa26^{mT-mG}* ES cells into 129/BL6 blastocysts resulted in 131 injected embryos, of which 47 developed to term. Among the live-born pups, 19 chimeras were obtained (40%). Thirteen chimeric males were test-mated, and germline transmission was confirmed in 1 case (7%). The coat color and expression of *tdTomato* suggested successful generation of chimeric mice. Further, germline transmission of the adult chimeras was confirmed when they were crossed with wild-type mouse (Fig. 2D–2E). The resulting *Rosa26^{mT-mG}* mouse strains generated through backcrossing are summarized in Table 1.

To evaluate functionality of conditional reporter gene in

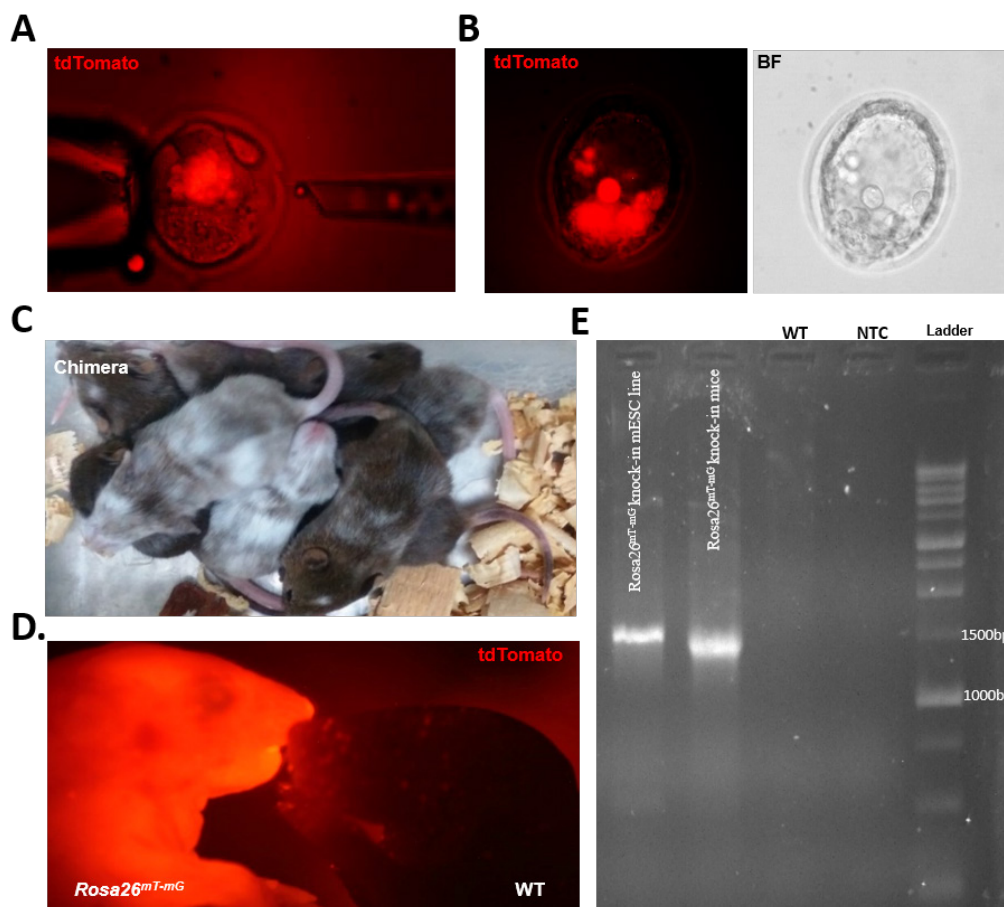


Fig. 2. Generation of *Rosa26^{mT-mG}* knock-in mice. **A.** Microinjection of *Rosa26^{mT-mG}* targeted mESCs into a blastocyst. **B.** mice after transfer of chimeric blastocysts into pseudopregnant females. **D.** Breeding of chimeric mice with wild-type to produce germline-transmitted *Rosa26^{mT-mG}* knock-in mice. Abbreviations: BF: bright field; WT: wild type, NTC: no template control.

Table 1. Mouse strains generated by backcrossing

Strain	Background	Provisional designation*	Generation
BL6-congenic <i>Rosa26^{mT-mG}</i>	C57BL/6 (inbred)	C57BL/6-Gt(<i>ROSA</i>)26Sor tm (<i>ACTB</i> -tdTomato,-EGFP) <i>Rgtl</i> /Rgtl	N13
NMRI-backcrossed <i>Rosa26^{mT-mG}</i>	NMRI (outbred)	NMRI-Gt(<i>ROSA</i>)26Sor tm (<i>ACTB</i> -tdTomato,-EGFP) <i>Rgtl</i> /Rgtl	N15

*Rgtl is the registered laboratory abbreviation of Royan Gene Targeting Lab through the Institute for Laboratory Animal Research (ILAR) International Laboratory Code Registry.

Rosa26^{mT-mG} knock-in mouse, we crossed it with Tg(*UBC-Cre/ER*) transgenic mouse that allows tamoxifen-inducible, ubiquitous Cre recombination (Fig. 3A). When fibroblasts derived from F1 of this cross (*UBC-Cre-ER/ Rosa26^{mT-mG}*) were treated with tamoxifen (1 mM/ml for 4 days), *tdTomato* reporter gene was removed and *mGFP* was expressed in cells ($\leq 60\%$) due to Cre recombinase activity (Fig. 3B). Also, oral

administration of tamoxifen (2 mg/mouse for 5 days) in F1 transgenic Tg(*UBC-Cre/ER*)/ *Rosa26^{mT-mG}* mice resulted in *mGFP* expression instead of *tdTomato* (Fig. 3C).

In conclusion, in this study we reported successful generation of *Rosa26^{mT-mG}* knock-in mouse as an efficient tool for lineage tracing, as it can be crossed with any transgenic mice expressing *Cre* under the control of a

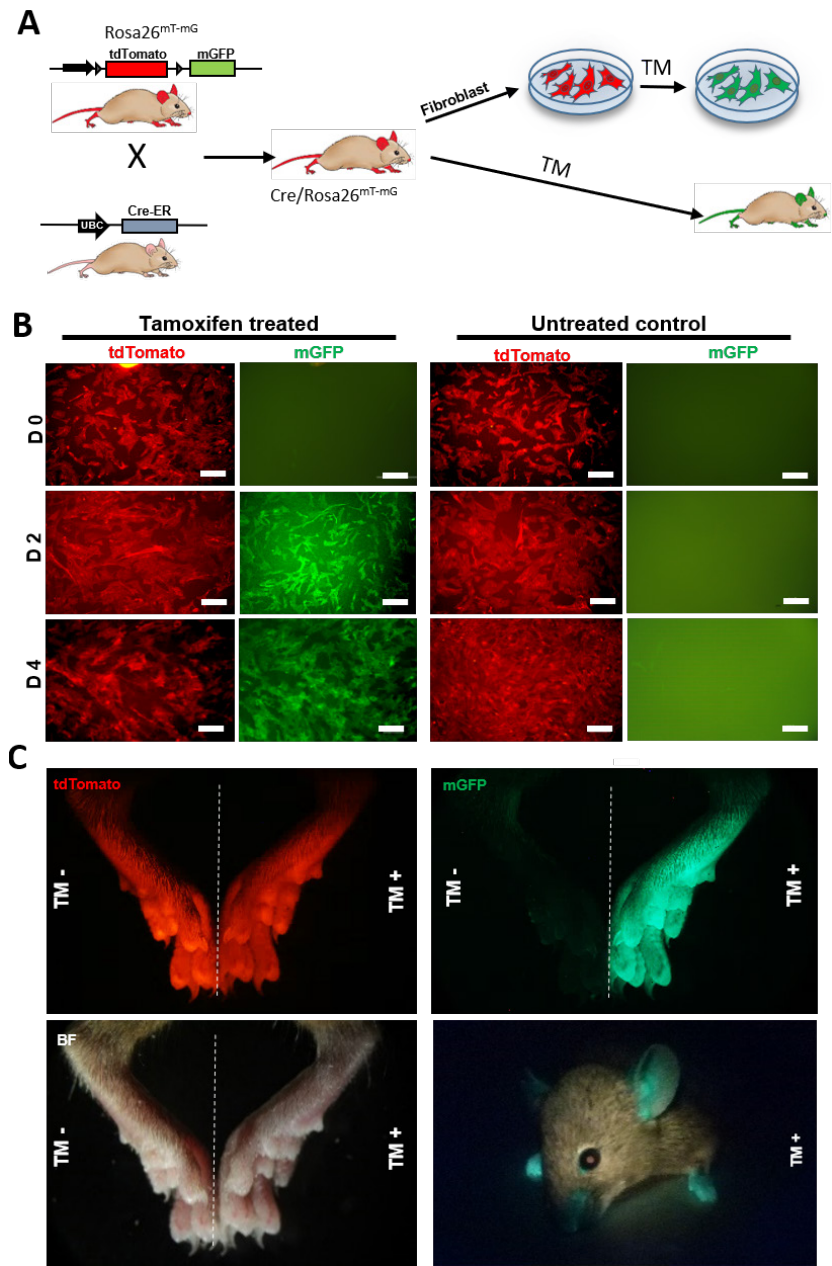


Fig. 3. Generation of *Cre/Rosa26^{mT-mG}* mice. **A.** A schematic representation of *Cre/Rosa26^{mT-mG}* mice generation and validation of recombination in these mice and tail-tip fibroblasts isolated from them after tamoxifen treatment. **B.** Tail tip fibroblasts isolated from *Cre/Rosa26^{mT-mG}* mice treated or not with tamoxifen. The cells ($\leq 60\%$) represent *mGFP* expression suggesting tamoxifen-induced *Cre* activity. **C.** *Cre/Rosa26^{mT-mG}* mice treated or not with tamoxifen. Tamoxifen treatment leads to *mGFP* expression in some cells through *Cre* expression, although cells not affected by tamoxifen remain red. Abbreviations: TM: tamoxifen, D: day, UBC: ubiquitous. Scale bar: 50.0µm

cell type-specific promoter to permanently label defined cell populations, trace their lineages, and follow their fate decisions during development, even after the promoter driving Cre expression is turned off during differentiation. Therefore, the knock-in mouse established in this study can be used to track different cell types, providing a better understanding of the function of various cells in vivo.

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Authors' Contribution

Conceptualization: Behrouz Asgari Abibeiglou, Mohsen Basiri.

Formal analysis: Magid Fallahi, Mahdi Hesarakhi, Meghdad Yeganeh, Mohammad Rezaei, Mohsen Basiri.

Funding acquisition: Hossein Baharvand, Yaser Tahamtani.

Supervision: Mohsen Basiri, Hossein Baharvand, Yaser Tahamtani.

Writing-original draft: Behrouz Asgari Abibeiglou.

Writing-review & editing: Magid Fallahi, Mahdi Hesarakhi, Meghdad Yeganeh, Mohammad Rezaei, Mohsen Basiri, Yaser Tahamtani.

Competing Interests

There is no conflict of interest to disclose.

Declaration of AI-Assisted Tools in the Writing Procedure

ChatGPT (OpenAI) was used to correct spelling and grammar errors and improve readability of the text. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Ethical Approval

All animal experiments were approved by the Medical Research Ethics Committee of Royan Institute (Approval No. IR.ACECR.ROYAN.REC.1396.120) and were conducted in accordance with the approved protocols of the Animal Laboratory Sciences Department at Royan Institute.

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Supplementary files

Supplementary file 1. Sequences of CRISPR/Cas9 components and donor template Used for ROSA26^{mT-mG} knock-in mouse generation.

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