WITHDRAWN: The Optimal Condition of Poly(I: C)-Induced Knockout of Cre Recombinase for Mx1-Cre/ Cre-*lox*P Mice

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Abstract

Background

The Cre-*lox*P system is widely applied for conditional knockout mice, commonly used to study the function of specific genes. Although some different promoters drive Cre expression, the poly(I: C)-inducible Mx1-Cre is the most commonly used to delete the target gene in experimental hematology. However, the optimal induction knockout condition for *Mx1-Cre/ Cre-loxP* mice using the Poly(I:C)-inducible Cre-*lox*P conditional system remains unclear. Here, we present two different components and three injection protocols of poly(I: C) to find the optimized condition.

Results

The results showed that the better knockout efficiency of Cre-*lox*P in mice injected with pure poly(I: C) has than those injected with poly(I: C) with some components. From the perspective of lethal genes (*Brg1*), data showed that mice injected with a single high dose (500 μg) of pure Poly (I:C) had a lower knockout rate. For mice injected media-dose (10μg/g) poly(I: C) triple, which induced a high knockout rate, but the mortality rate was still high. Importantly, the mice injected low-dose (6μg/g) poly(I: C) triple, both the knockout rate and survival rate of mice was high. Similarly, the knockout rate of non-lethal mice injected with media-dose (10μg/g) or low-dose (6μg/g) poly(I: C) triple was very high, but injected with a single high dose (500 μg) of pure poly(I: C) had a low knockout rate.

Conclusion

Our studies provided the optimized condition for using poly(I: C)-inducible effective knockout and maintaining the survival rate for the Cre-*lox*P mice, which might be applied in other knockout mice for this system to ensure both the gene knockout and the mice survival.

Background

Due to genetic and pathophysiological similarities between mice and humans, conditional knockout mice are frequently used to study normal and malignant diseases, including hematopoiesis[1]. The so-called conditional knockout approach has been used to enable gene knockout in a tissue/cell (spatial control) and/or time (temporal control) specific manner[2]. At present, the conditional knockout mice models are generated by Cre-loxP system, FLP-FRT system and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) system[3–6]. Although there are numerous methods to implement conditional knockout, the commonly used conditional knockout mice models are generated by flanking the *lox*P site of the target gene fragment, which can be removed by Cre recombinase.

The Cre-loxP system contains two major components: the enzyme Cre recombinase and the loxP sites[7]. The Cre recombinase, which is controlled by a cell-specific promoter to allow selective deletion of genes of interest, specifically recognizes the two *lox*P sites[8]. Depending on the orientation of the two *lox*P

sites, Cre recombinase either excises or inverts the transgene sequences inserted between the two *lox*P sites[9–11]. The Mx1-Cre model is the most commonly transgenic mice, specifically expressed in hematopoietic stem cells (HSC), in which Cre recombinase is under the control of a type I interferon-inducible promoter (Mx1)[12]. This promoter is silent in healthy mice, while it can be induced to high levels of transcription by administration of interferon-alpha (IFN-α), interferon-beta (IFN-β), or synthetic double-stranded RNA (for instance, poly(I: C), an IFN inducer)[12–14]. The times, doses and methods of poly(I: C) injection wrere complex and chaotic[15–18]. To investigate the influencing factors for poly(I: C) inducible on the knockout rate of target genes, we performed the experiments of components, dosage, injection time for poly(I: C), based on the Mx1-Cre and specific knockout *Brg1* or *Alkbh5* gene, to construct $Brg1^{fl/fl}$*Mx1-Cre* or $Alkbh5^{fl/fl}$*Mx1-Cre* mice.

Brg1 gene, also known as *smarca4*, was the core component of the SWI/SNF ATP-dependent chromatin remodeling complex, and it was early embryonic lethality in mice [4, 19, 20]. *Alkbh5* was identified as RNA m6A demethylase, which is a non-lethal gene according to the International Mouse Phenotype Association (http://www.mousephenotype.org/) [21]. Here we used *Brg1* and *Alkbh5* as a model gene for establishing gene knockout of the Cre-*lox*P system. We compared two poly(I: C) with different components and three injection protocols, and found that the mice injected low-dose (6μg/g) poly(I: C) triple could keep the high knockout efficiency and high survival rate for $Brg1^{fl/fl}$*Mx1-Cre* mice, and $Alkbh5^{fl/fl}$*Mx1-Cre* mice injected media-dose (10μg/g) or low-dose (6μg/g) poly(I: C) triple could be guaranteed *Alkbh5* complete knockout, indicating that appropriate experimentally-induced knockout protocols should be explored when conducting target gene conditional knockout studies.

Methods

Mice

All animals were bred in specific pathogen-free facilities at Third Military Medical University. $Brg1^{fl/fl}$ mice (C57BL/6) were a kind gift from Professor Shi[22]. *Mx1-Cre* mice (C57BL/6) were purchased from Shanghai Model Organisms. To generate $Brg1^{fl/fl}$*Mx1-Cre* or $Alkbh5^{fl/fl}$*Mx1-Cre* mice, $Brg1^{fl/fl}$ or $Alkbh5^{fl/fl}$ mice were crossed with *Mx1-Cre* transgenic mice. Mice were genotyped by PCR performed on DNA extracted from tail biopsies. All animal experiments were conducted in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experimental protocol was approved by Laboratory Animal Welfare and Ethics Committee of Third Military Medical University. The study was carried out in compliance with the ARRIVE guidelines.

Poly(I:C) treatment

Polyinosinic-polycytidylic acid sodium salt was purchased from Sigma-Aldrich, and polyI-polyC was purchased from GE Healthcare. For conditional deletion of *Brg1 or Alkbh5 in vivo*, Cre expression was induced by Polyinosinic-polycytidylic acid sodium salt or polyI-polyC (all called poly(I: C)), and the former contains about 1% free nucleotides, while the latter no other components. poly(I: C) is soluble in 0.9%

saline, and its concentration is 2mg/mL. The comparison of the two poly(I: C) is shown in Supplementary Table 1.

Isolation of bone marrow cells

Age-matched C57BL/6 mice (from 7 to 9 weeks) were sacrificed by cervical dislocation and bones from hind legs. Bone marrow (BM) was extracted by flushing the hind legs with a 5ml syringe in RPMI1640, and Red Blood Cell Lysis Buffer (Solarbio, China) was used to lyse red blood cells. The suspension was filtered through a 40-μm mesh and centrifuged for 5 min at 1,500 rpm.

Real-time PCR (q-PCR)

For total RNA isolation, bone marrows cells from Poly(I:C)-induced mice were sorted into Trizol (RNAiso Reagen) and RNA isolation was performed according to the manufacturer's instructions (Takara, Japan). RNA samples were transcribed into complementary DNA (cDNA) using the PrimeScript RT reagent Kit according to the manufacturer's instructions (Takara, Japan). RT-qPCR measurement of individual cDNAs was performed using SYBR Green Master Mix (Takara, Japan). RT-qPCR reactions were carried out on BioRad cyclers with the following primers: *Brg1* forward, 5'-TCGCGCTCATCACATACCTC-3'; *Brg1* reverse, 5'-GCGAAGCTGTGGGACAAAAG-3'; *Alkbh5* forward, 5'-CGCGGTCATCAACGACTACC-3'; *Alkbh5* reverse, 5'-ATGGGCTTGAACTGGAACTTG-3'; *Actin* forward, 5'-ACCTTCTACAATGAGCTGCG-3'; *Actin* reverse, 5'-CTGGATGGCTACGTACATGG-3'.

Western blot (WB)

Western blot analysis was performed using standard protocols. Mouse antibody against Brg1 (Santa Cruz, USA) and Alkbh5 (Sigma, USA) was all used at a 1:1000 dilution, and mouse anti-GAPDH antibody (Proteintech, USA) was used at a 1:1000 dilution. GAPDH were as the inner control and each experiment was repeated triple.

Hematoxylin-eosin (H&E) stain

The tissues were fixed in 4% paraformaldehyde for 48 hours, and then embedded in paraffin. 4-μm sections were obtained from paraffin block using a microtome (Leica RM2255, Germany) and stained with H&E. Samples were immersed in xylene and alcohol for deparaffinization and rehydration, stained with hematoxylin for 5 min, stained with eosin for 3 seconds and followed by dehydration with graded alcohol and clearing in xylene. Slides were mounted using a synthetic resin (Entellan; Merck, Germany).

Statistical Analysis

Data were processed using GraphPad Prism v.7. Difference analyses were performed using unpaired Student's t-tests. Kaplan-Meier plots were analyzed by the log-rank test. A value of $P < 0.05$ was considered statistically significant and was denoted as *< 0.05; **< 0.01; ***< 0.001 compared with control group.

Results

Given that the Cre-*lox*P system is the wide-usage and powerful strategy for conditional gene knockout mice, we applied it for *Brg1*gene as the model, with the Mx1-Cre for HSC to construct the conditional *Brg1*(*Brg1*$^{fl/fl}$*Mx1-Cre*) (*Brg1-CKO*) and *Alkbh5* knockout (*Alkbh5*$^{fl/fl}$*Mx1-Cre*) (*Alkbh5-CKO*) mice. To optimize the knockout condition, based on both knockout rate and survival of CKO mice, we explored the different strategies, different components of poly(I: C), various injection doses and injection schedules.

The poly(I: C) components influences the knockout of *Brg1-CKO* mice.

To investigate the influence of poly(I: C) components, we firstly constructed *Brg1*$^{fl/fl}$*Mx1-Cre* mice by injecting two kinds of poly(I: C), and injected 10μg/g poly(I: C) containing free nucleotides every second day for a total of 3 intraperitoneal injections (Fig. 1A), and analyzed the mice 15 days post-injection. The data showed that there was an increasing trend of the body weight in mice, though there was no significant difference in *Brg1-CKO* mice before and after poly(I: C) injection, as well as for the *Brg1*$^{fl/fl}$ mice. The body weight of the two groups was increased significantly when compared with that before the injection after poly(I: C) injection for 15 days (Fig. 1B). However, only the *Brg1-CKO* mice were died after poly(I: C) injection, without significant difference in the survival rate of the two groups (Fig. 1C). The results of q-PCR and WB showed that the expression of *Brg1* was significantly lower in samples from *Brg1-CKO* mice than in those from *Brg1*$^{fl/fl}$ mice, and a small portion of *Brg1-CKO* mouse samples slightly decreased *Brg1* expression (Fig. 1D, 1E). Consistently, histological analysis showed that naive cells were increased slightly in the BM of the *Brg1-CKO* mice (Fig. 1F), implying that poly(I: C) with other components could induce the incomplete loss of *Brg1* expression.

The poly(I: C) without other components affects the mortality of *Brg1-CKO* mice.

To reduce the expression of *Brg1* more efficiently, we used poly(I: C) without other components, and the injection schedule and dosage had not been changed (Fig. 2A). Although the body weight of control mice was no significant difference before and after poly(I: C) injection, the *Brg1-CKO* mice showed a prominent reduction of body weight (Fig. 2B). At the time of mice sacrificed, we found an increased body weight of *Brg1*$^{fl/fl}$ mice, whereas *Brg1-CKO* mice were severely reduced (Fig. 2B). Mice derived from *Brg1-CKO* treated with poly(I: C) showed a significant survival disadvantage (Fig. 2C). The expression of *Brg1* was found to be decreased in the *Brg1-CKO* mice, which was compared with *Brg1*$^{fl/fl}$ mice (Fig. 2D). Western blot analysis showed complete loss of *Brg1* in poly(I: C)-treated *Brg1-CKO* mice (Fig. 2E). Histological analysis of BM in *Brg1-CKO* mice revealed the enhanced naive cells (Fig. 2F). Collectively, these results suggest that the expression of *Brg1*was effectively knock out, but the mortality rate of mice was high.

Multiple low-dose poly(I: C) injections affect the rate of knockout and survival in *Brg1-CKO* mice.

To improve the survival, we adopted the original injection schedule and changed the injection dose from 10μg to 6μg poly(I: C) per gram of body weight (Fig. 3A). In terms of body weight, we found that the two groups of mice also remained unaffected before and after poly(I: C) injection. The weight of control mice

was increased significantly on day 15 more than that of mice before poly(I: C) injection, while that of *Brg1-CKO* mice was lost severely (Fig. 3B). Although *Brg1-CKO* mice treated with poly(I: C) showed a shorter survival time than that from *Brg1*$^{fl/fl}$ mice, the survival rate of the former can reach 50% (Fig. 3C). Notably, both the results of qPCR and WB showed a complete loss of *Brg1* expression in poly(I: C)-treated *Brg1-CKO* mice (Fig. 3D, 3E). We also observed more naive cells in the BM of the *Brg1-CKO* mice (Fig. 3F). Taken together, these results suggest that knocking out *Brg1* expression and improving the survival of mice could be effective when reducing the poly(I: C) injection dose with multiple injection times.

A single high-dose poly(I: C) injection will reduce knockout efficiency in *Brg1-CKO* mice.

Considering that a single high-dose injection of poly(I: C) can also knock down target genes efficiently [17], we implemented it as the scheme (Fig. 4A). On the 4th day of poly(I: C)-induced knockout, two groups of mice had a normal weight, whereas all mice had a significant increase on the 15th day (Fig. 4B). In addition, we found that *Brg1-CKO* mice had died partially, and significant differences were observed (Fig. 4C). By contrast, both the results of qPCR and WB analysis indicated that *Brg1* expression was incompletely lost in *Brg1-CKO* mice (Fig. 4D-4E). The naive cells in the BM of *Brg1-CKO* mice did not change significantly (Fig. 4F), indicating that a single high-dose of poly(I: C) is not suitable for *Brg1-CKO* mice.

Multiple media or low-dose poly(I: C) injections can ensure complete depletion of *Alkbh5* mice with non-lethal phenotypes.

To rule out that this drug injection protocol is only applicable to brg1 mice, we use *Alkbh5*$^{fl/fl}$*-Mx1-Cre* mice to verify these three injection protocols. We found that both the results of qPCR and WB showed *Alkbh5* can be knocked out completely with 10μg poly(I: C) per gram of body weight injection protocol (Fig. 5A-5B), and *Alkbh5* can also be completely knocked out with 6μg poly(I: C) per gram of body weight injection protocol (Fig. 5C-5D). Consistent with the results of previous studies, *Alkbh5* can only be partially knocked out with a single high-dose of poly(I: C) (Fig. 5E-5F). Taken together, these results suggest that to ensure that the expression of the target gene is completely exhausted, 10μg or 6μg poly(I: C) per gram of body weight injection protocol can be used.

Before the mice were sacrificed, observe the weight gain of the mice of all injection protocols (Supplementary Fig. 1), but no weight change of the mice was observed before and after 10μg or 6μg poly(I: C) per gram of body weight injection protocol (Supplementary Fig. 1A, 1C), and the weight gain of the mice was observed before and after poly(I: C) per gram of body weight injection protocol (Supplementary Fig. 1B).

Discussion

The Cre-*lox*P conditional system is a wide-usage and powerful strategy for gene knockout mice, but the knockout efficiency is varied in different strategies. To optimize the knockout condition and keep the survival rate of lethality mice, here, we investigated the different components, different injection doses

and injection schedules of poly(I: C). We found that low-dose (6μg/g) poly(I: C) injection triple could knockout the gene expression of *Brg1* efficiently and maintain the high survival rate of mice, Meanwhile, *Alkbh5* can be completely knocked out, which is a non-lethal knockout mouse.

The phenotype was positively correlated with the degree of target gene knockout, that is, the higher knockout rate of target genes, the more obvious the phenotype of mice[23–25]. We observed that when the poly(I: C)-induced loss of *Brg1* expression was incomplete, the naive cells in the bone marrow of *Brg1-CKO* mice were slightly changed or unchanged, whereas when the *Brg1* expression was completely deleted, the naive cells in the bone marrow of *Brg1-CKO* mice were increased significantly. This indicates that the degree of *Brg1* expression censorship is positively correlated with the phenotype of *Brg1* mice. Furthermore, our experiments showed that *Brg1* was essential for hematopoietic development and provides important evidence for the next research.

This study found that poly(I: C) components, injection doses and protocols were related to the knockout efficiency of the target gene and mouse mortality. Research reports showed that triple injections of 10μg/g pure poly(I: C) can completely delete the target gene[15, 26], and the mortality of mice was related to the lethal phenotype of the target gene[4]. In this study, *Brg1* can be completely deleted when pure poly(I: C) used this scheme, but the mortality of *Brg1-CKO* mice was too high, which was not conducive to the implementation of the next research. The targeted mutation of *Brg1* is reported to be conferred early embryonic lethality in mice[4], so the death of *Brg1-CKO* mice was due to its lethal phenotype. Though few studies on poly(I: C) with other components using this protocol, we showed that the *Brg1* deletion was incomplete when poly(I: C) with other components used this injection protocol. Studies had shown that the poly(I: C) with other components contained high endotoxin and high molecular weight, which led to its poor effect[27]. This may be caused by the free nucleic acid, which may affect the absorption or performance of poly(I: C). This may be one of the reasons for its low knockdown rate. Therefore, when the researchers used the poly(I: C) with other components, mice received 5 or more doses of poly(I: C) (25μg/g of body weight every other day) injection intraperitoneally to ensure the complete deletion of the target gene[28, 29]. To improve survival, we reduced pure poly(I: C) from 10μg/g to 6μg/g according to the literature[15], thereby ensuring that *Brg1* was completely knocked out and improving survival. Although a single injection of 500μg poly(I: C) was reported to induce complete deletion of the target gene without affecting the death of mice[18], we used this experimental protocol to find that the *Brg1* knockout was incomplete. This proved that a single injection of high-dose (500μg) poly(I: C) does not necessarily guarantee the knockout efficiency. In addition, we use poly(I: C) induced knockout *Alkbh5*, the findings with the previous agreement, 10μg/g or 6μg/g poly(I: C) can ensure *Alkbh5* full knockout, while a single high doses (500μg) poly(I: C) does not guarantee *Alkbh5* knockout completely. For lethal phenotype mice to ensure the knockout rate and survival simultaneously, we can reduce the dose of poly(I: C) and increase the number of poly(I: C) injections, while non-lethal phenotype mice to ensure complete knockout, we can choose media (10μg/g) or low-dose (6μg/g) poly(I: C) multiple injections.

Conclusion

Altogether, our results suggest that poly(I: C) without other components induces *Brg1* knockout efficiency higher. Moreover, a single high-dose (500μg) poly(I: C) injection can not guarantee complete knockout of *Brg1* and *Alkbh5*, and triple media-dose (10μg/g) poly(I: C) injections can cause higher mortality in *Brg1-CKO* mice with a lethal phenotype as well. However, low-dose (6μg/g) poly(I: C) injections in a triple can ensure that *Brg1* expression is completely depleted and improve the survival of *Brg1-CKO* mice, and can also induce complete knockout of *Alkbh5*. This indicates that reducing the dose of poly(I: C) and increasing the number of poly(I: C) injections can ensure gene knockout and survival rates of mice.

Abbreviations

HSC: Hematopoietic stem cells

BM: Bone marrow

WB: Western blot

H&E: Hematoxylin-eosin stain

Declarations

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics declarations

All animal experiments were conducted in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experimental protocol was approved by Laboratory Animal Welfare and Ethics Committee of Third Military Medical University. The study was carried out in compliance with the ARRIVE guidelines.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Authors' Contributions

BJY contributed to study conception and design, performing the research and drafted the manuscript as the first author. HBW, YYL, FFX and MK contributed to data acquisition, analysis and interpretation, ST, ZD and TTJ contributed to formal analysis, validation and investigation. YS, SL and PHZ contributed to administrative support. LZ contributed to the study conception and design, data analysis, drafted, critical revision of the manuscript and funding. All authors have read and approved the manuscript.

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Figures

Figure 1

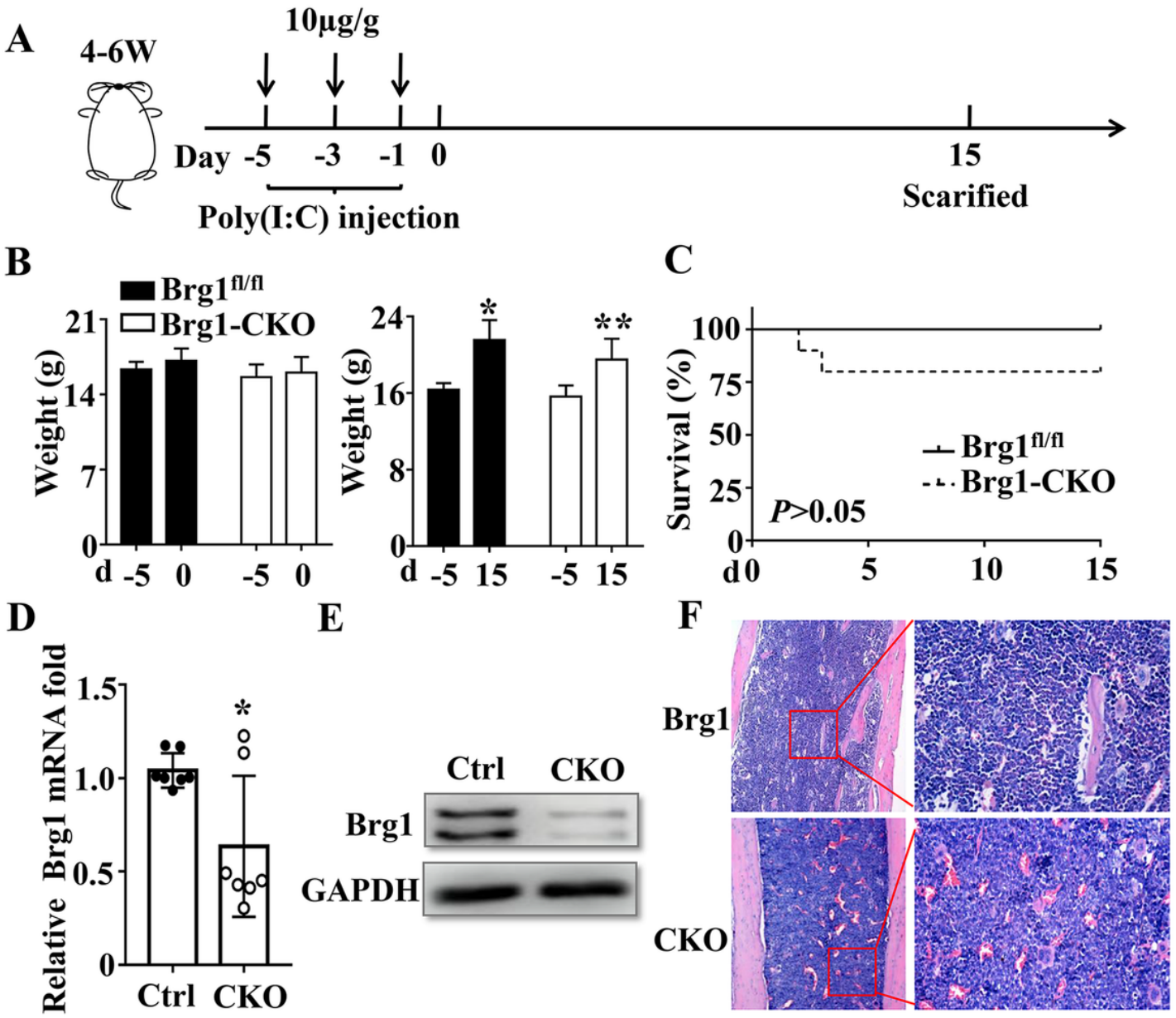

Figure 1

The poly(I: C) components influence the knockout of Brg1-CKO mice. A The schematic diagram of the deletion of Brg1 by using poly(I: C). B Total body weight of Brg1fl/fl (n=6) and Brg1-CKO (n=6) mice. C Kaplan-Meier curves of Brg1fl/fl (n=10) and Brg1-CKO (n=10) mice injected with Poly(I:C) containing other components. D The mRNA expression data of Brg1detected by Real-time PCR (q-PCR) exhibits expression levels of Brg1. (Brg1fl/fl=1.04 vs Brg1-CKO=0.64). E The protein expression of Brg1 measured by Western blot (WB) in Brg1fl/fl and Brg1-CKO mice after poly(I: C) injection for 15 days. F The representative images of hematoxylin-eosin (H&E) from the bone marrow of Brg1fl/fl and Brg1-CKO mice15 days after induction. * < 0.05, **< 0.01, ***< 0.001 vs. Brg1fl/fl mice.

Figure 2

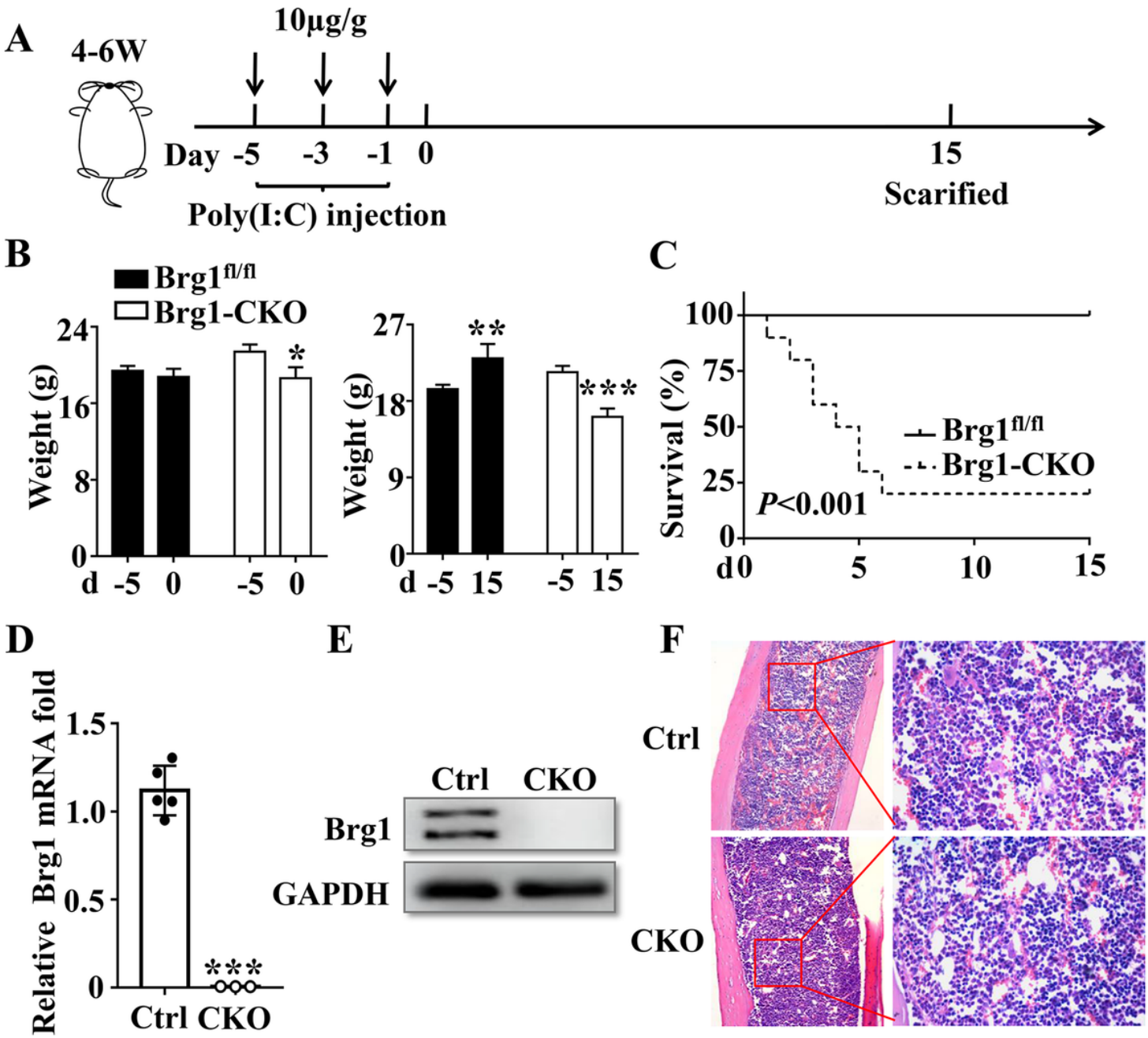

Figure 2

The poly(I: C) without other components affects the mortality of Brg1-CKO mice. A Schematic diagram of using poly(I: C) to deplete Brg1. B Bar graph showing body weight of Brg1fl/fl (n=5) and Brg1-CKO (n=4) mice. C Kaplan-Meier survival curve analysis of Brg1fl/fl (n=10) and Brg1-CKO (n=10) mice injected with poly(I: C). D The mRNA expression of Brg1 detected by q-PCR in Brg1fl/fl and Brg1-CKO mice treated with poly(I: C). (Brg1fl/fl=1.12 vs. Brg1-CKO=0.01). E The protein expression of Brg1 was measured by WB in BM from Brg1fl/fl and Brg1-CKO mice. F The representative H&E images of stained BM section of Brg1fl/fl and Brg1-CKO mice.

Figure 3

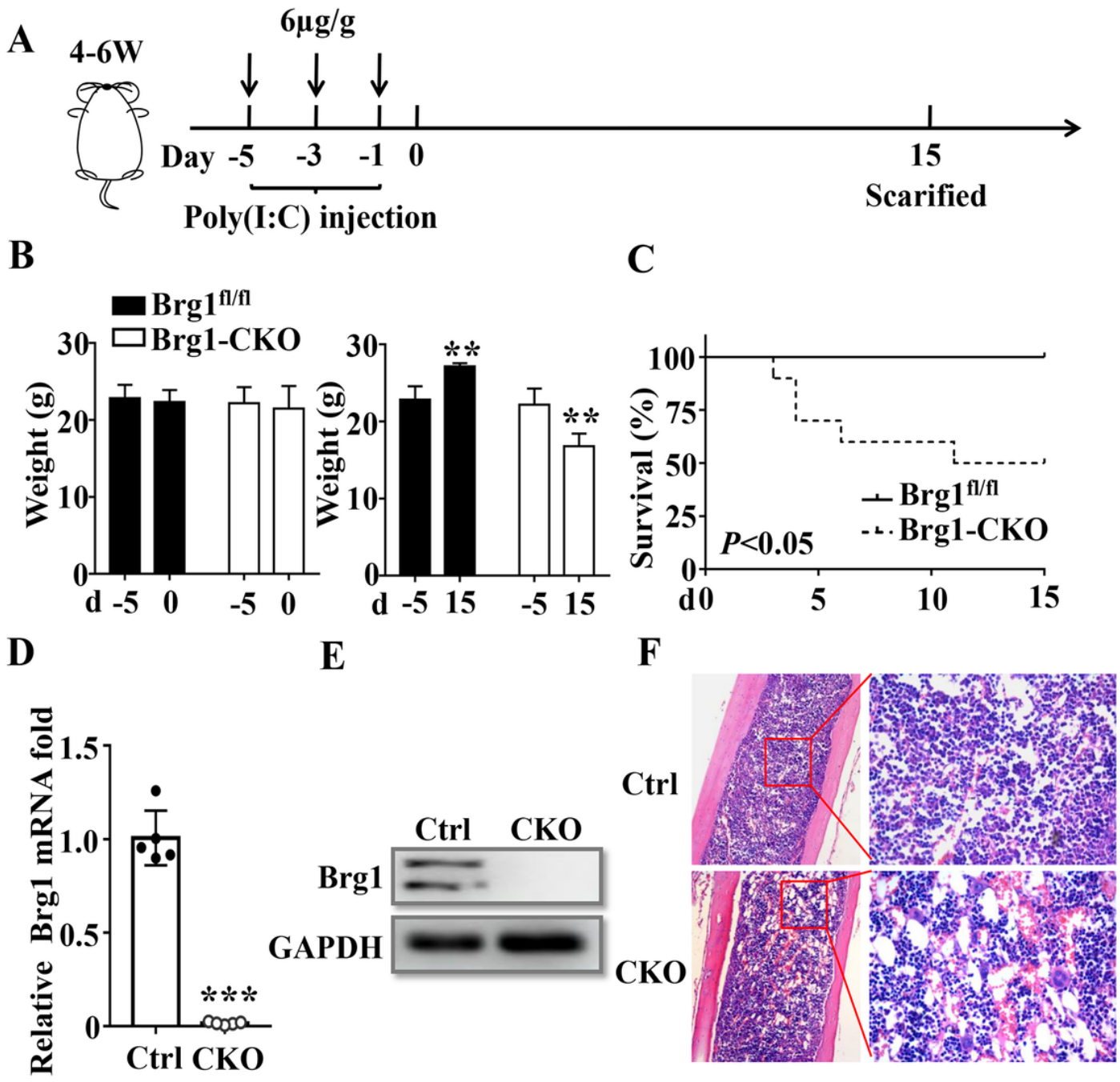

Figure 3

Multiple low-dose poly(I: C) without other components injections affect the rate of knockout and mortality in Brg1-CKO mice. A The schematic diagram of new injection protocols with poly(I: C). B The bar graph representation averages of body weight in a different time (Brg1fl/fl, n=4 and Brg1-CKO, n=4). C Kaplan-Meier survival curve analysis of Brg1fl/fl (n=10) and Brg1-CKO (n=10) mice injected with triple low-dose (6μg/g) poly(I: C). The mRNA (D) and protein (E) expression, detected by Real-time PCR (D) and WB (E) analysis showing Brg1 mRNA (Brg1fl/fl =1.01 vs. Brg1-CKO =0.02) and protein expression in Brg1fl/fl and

Brg1-CKO mice treated with poly(I: C). F The representative BM H&E images from Brg1fl/fl and Brg1-CKO 15 days after induction.

Figure 4

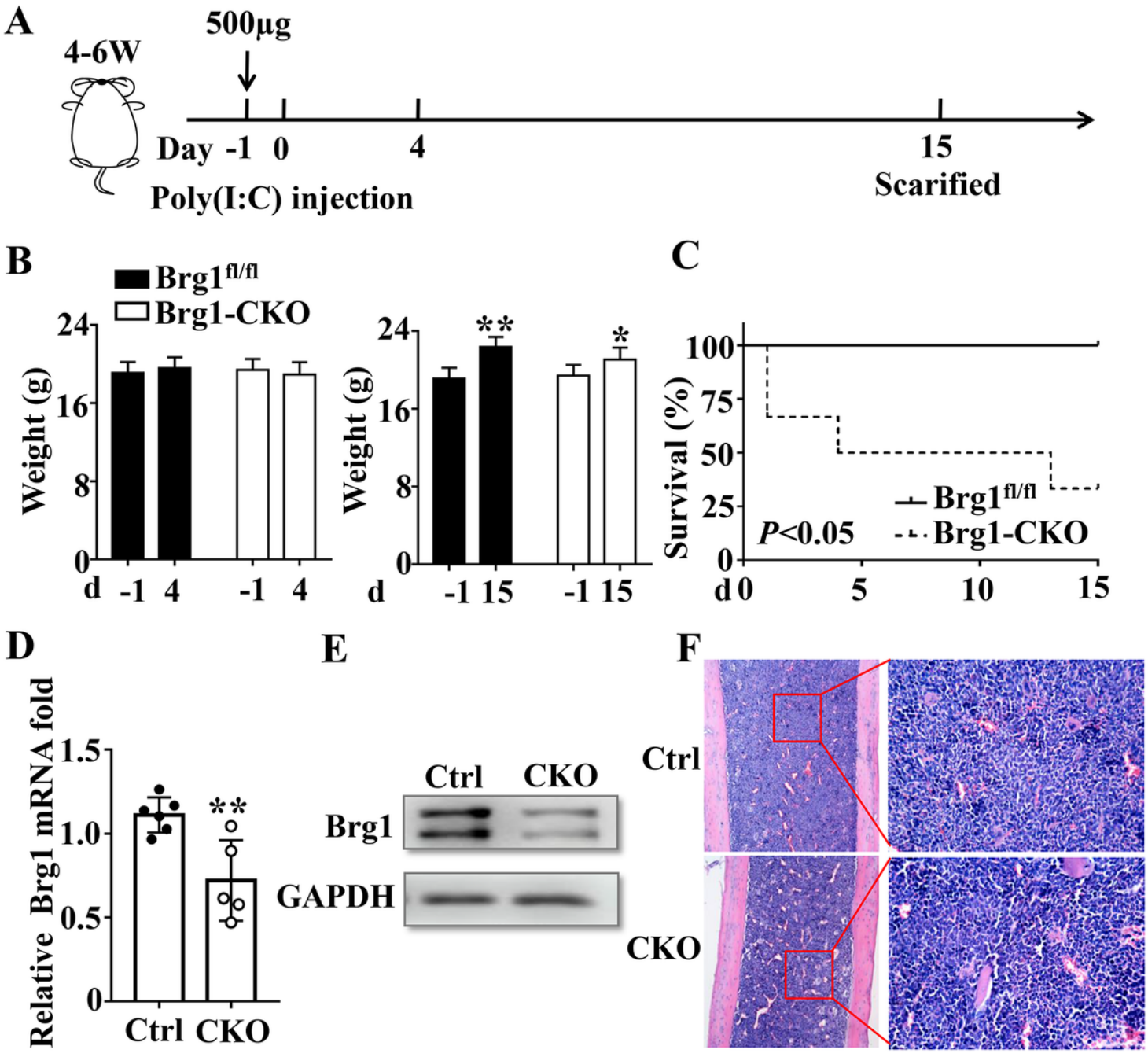

Figure 4

A single high-dose P poly(I: C) injection will reduce knockout efficiency in Brg1-CKO mice. A Schematic diagram of using poly(I: C) to delete Brg1. B The body weight of Brg1fl/fl (n=4) and Brg1-CKO (n=4) mice. C Kaplan-Meier representation of overall survival in Brg1fl/fl (n=6) and Brg1-CKO (n=6) mice injected with poly(I: C). D Detection of Cre-mediated deletion of the Brg1. (Brg1fl/fl=1.11 vs Brg1-CKO=0.72) E Brg1

protein expression in Brg1fl/fl and Brg1-CKO mice treated with poly(I: C). F The representative BM H&E images from Brg1fl/fl and Brg1-CKO after induction.

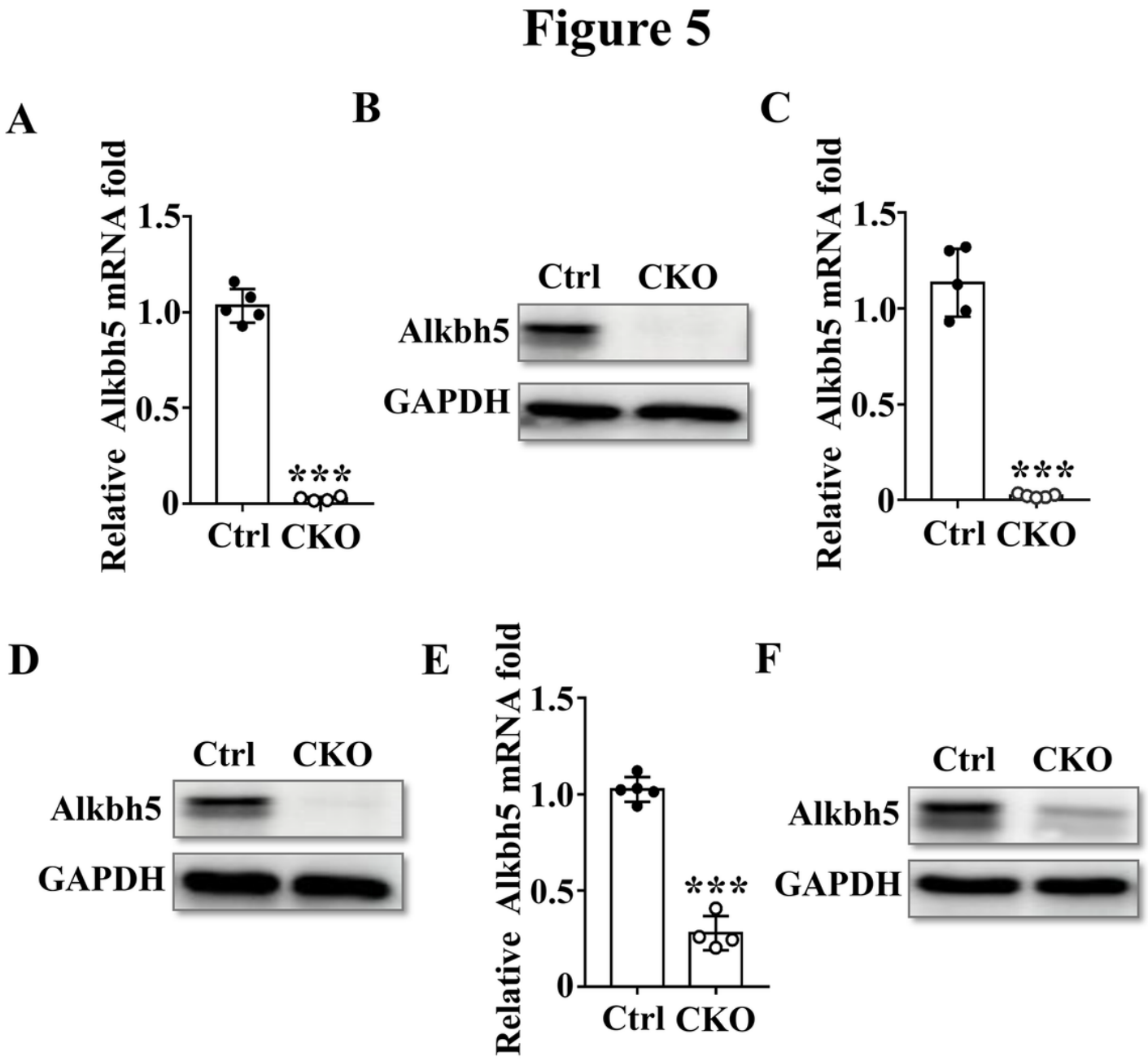

Figure 5

Multiple media or low-dose Poly(I:C) injections can ensure complete depletion of Alkbh5-CKO mice with non-lethal phenotype. A The expression of Alkbh5 in Alkbh5fl/fl and Alkbh5-CKO mice treated with triple media-dose (10μg/g) poly(I: C) injections. (Alkbh5fl/fl=1.03 vs Alkbh5-CKO=0.02) B Western blot shows loss of Alkbh5 protein in BM of triple media-dose (10μg/g) poly(I: C) treated Alkbh5-CKO mice. C Expression changes of Alkbh5 mRNA transcripts in Alkbh5fl/fl and Alkbh5-CKO mice treated with triple low-dose (6μg/g) poly(I: C) injections. (Alkbh5fl/fl=1.13 vs Alkbh5-CKO=0.02) D The Alkbh5 protein levels in two groups of mice treated with triple low-dose (6μg/g) poly(I: C) injections. Real-time PCR (E) and WB

(F) analysis showing a decrease of Alkbh5 expression after mice treated with a single high-dose poly(I: C) injection (Alkbh5fl/fl=1.02 vs Alkbh5-CKO=0.28).

Supplementary Files

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- supplFigure1.tif
- Table.tif