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Original Article

Effect of the conditional knockout of bone marrow specific RIPK3 gene on bone marrow hematopoiesis in mice

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Abstract: Receptor-interacting serine-threonine kinase 3 (RIPk3) is a key signaling molecule in the regulation of cell apoptosis and necroptosis, it plays an important role in the pathophysiological changes of many hematologic diseases. However, the regulatory role of RIPk3 in programmed cell death (PCD) is not fully known. In this study, bone marrow-specific RIPk3 gene knockout homozygotes (RIPk3-/- mice) were established by homologous recombination. The physiological index of peripheral blood, the morphology and structure of the bone marrow, the bone marrow nucleated cells (BMNCs), the hemopoietic stem cells (HSCs), interleukin-6 (IL-6) level and the colony formation capacity of bone marrow hematopoietic progenitor cells were compared between RIPk3-/- mice and wild-type mice. The results showed that, the cell death rate of BMNCs in RIPk3-/- mice was significantly higher than that in control mice, indicated that RIPk3 gene knockout may cause damage to bone marrow cells to some extent. However, the bone marrow had normal structure and morphology in the bone marrow-specific RIPk3-knockout mice, and there were not significantly different between the two mice in most of the blood physiological indicators, and colony yields of hemopoietic stem/progenitor cells. Further study found that the bone marrow IL-6 level of the RIPk3-/- mice increased significantly, besides, the number of BMNCs and HSCs in the bone marrow of the RIPk3-/- mice increased considerably as compared with the control mice. The findings implies that bone marrow RIPk3 gene knockout may lead to the increase of BMNCs cell death, however, increased secretion of hematopoietic cytokines such as IL-6 may promote the proliferation of hematopoietic stem/progenitor cells and thus maintain the stability of bone marrow hematopoiesis. This hypothesis and the detailed mechanisms remain to be further investigated.

Keywords: Bone marrow, RIPK3, gene knockout, hematopoiesis, mouse

Introduction

Programmed cell death (PCD) is a physiological cell death process involved in the selective elimination of unwanted cells. This process is closely connected with the activation, expression and regulation of multiple genes and plays an important role in maintaining homeostasis and normal cell functions. Bone marrow is the major site of hematopoiesis. The balance between the proliferation and death of bone marrow cells is the basis of normal hematopoiesis. When the regulatory mechanism of cell death is in disorder, bone marrow hematopoiesis may be affected by excessive proliferation or death of bone marrow cells caused by PCD abnormalities. This may further lead to various hematologic diseases such as aplastic anemia, leukemia and myeloproliferative diseases [1-5]. Understanding the regulatory mechanism of bone marrow PCD can facilitate hunting for pathogenesis of hematologic diseases and provide scientific clues for the diagnosis and new therapy exploration of hematologic diseases.

PCD mainly consists of apoptosis and necroptosis, which are crucial for the development and homeostasis in organisms. Recent studies have shown that RIPk3 is a key signaling molecule in the regulation of cell apoptosis and necroptosis [6, 7]. It plays an important role in the pathophysiological changes of many diseases [8-12]. According to recent studies, RIPk3 is involved in the occurrence and progression of many hematologic diseases, including aplastic anemia (AA) [13], chronic lymphocytic leukemia (CLL) [14] and acute myeloid leukemia (AML) [15]. The role of RIPk3 in the pathogenesis of diseases has drawn wide-spread attention. RIPk3 can act as an important target in gene therapy [16-

20]. However, the regulatory role of RIPk3 in PCD is not fully known. Establishing the bone marrow RIPk3 knockout mice may facilitate the understanding on the molecular mechanism of RIPk3-mediated PCD of bone marrows and the pathogenesis of related hematologic diseases. In this study, bone marrow-specific RIP3 knockout in the mouse model was induced by homologous recombination and the influence of bone marrow-specific RIPk3 gene knockout on the bone marrow hemopoietic function was observed. The purpose was to provide experimental data for understanding about the pathological role of RIPk3 in hematologic diseases.

Materials and methods

The study protocol was approved by the ethics committee of the Institute of Tumor, Medical College of Taizhou University and conformed to the Guide for the Care and Use of Laboratory Animals published by the Chinese National Institutes of Health.

Reagents

Bacterial Artificial Chromosome (BAC) containing RIPk3 was purchased from BAC/PAC Resources Center (Children's Hospital Oakland Research Institute, Oakland, CA, USA). pL-451 Plasmid and EL-350 Strain were provided by Pengtao Liu (Welcome Trust Sanger Institute, Cambridge, UK). pSC101-BAD-γβα-A-tet was provided by Youming Zhang (Gene Bridges GmbH, Germany). pBR322-2S and pDTA were provided by Shanghai Research Center For Model Organisms. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, Taq enzyme and PCR kit were purchased from TaKaRa Biotechnology (Dalian, China) and NEB company (Carlsbad, CA, USA). Alexa Fluor 488 Annexin V and a PI kit were purchased from KeyGEN BioTECH (JiangXu, China). IL-6 ELISA kits, IMDM substratum, erythropoietin (EPO), thrombopoietin (TPO), granulocyte-macrophage colony stimulating factor (GM-CSF), fluorescent antibody PEc-kit and FITC-CD45 were all purchased from the Boster Company (Wuhan, China).

Construction and identification of the targeting plasmid

The loxp sequence was inserted to the intron 3 and 9 in the RIPk3 gene, respectively. Using pGK-Neo gene as the positive selection marker (flanked by frt sequence, with the deletion of pGK-Neo gene using the flpe enzyme) and TK gene in the targeting vector as the negative selection marker, the targeting vector for exon 4-9 knockout was constructed (by reacting with the Cre recombinase).

ES cell targeting, blastocyst microinjection and transplantation

The linearized targeting vector was transferred to the ES cells by electroporation. After that, the ES cells were screened with 200 μg/mL G418. Resistant ES cell clones were subjected to further culture. Genomic DNA was extracted from the resistance ES cells, and the positive clones were screened by long fragment PCR. The 5' homology arm was identified using the forward primer 5'-GGCAGGCTGGTTTCTGAGTTTG-3', and the reverse primer 5'-GGCCTACCCGCTTCCATTGCTC-3; the 3' homology arm was identified using the forward primer 5'-CCGTGCCTTCCTTGACCCTGG-3', and the reverse primer 5'-CATGGGCAGGCAACAGTCACA-3'. Blastocytes were harvested from female C57BL/6J mice aged 3-4 weeks, the positive ES cells were used for blastocyst microinjection after check and transplanted to the mice during spurious pregnancy.

Mice of F1 generation and genotyping

Coat color chimerism was determined in newborn mice from the female C57BL/6J mice transplanted with the blastocysts. Those with high degree of chimerism were chosen for breeding with the FLP mice to obtain the F1 generation. At 10-12 d after birth, the tail tip was harvested, and 200 μl Tail buffer and 10 μl protease K (20 μg/ml) were added. After digestion at 55°C overnight, protease K was deactivated at 95°C for 15 min and centrifuged. The supernatant was collected, amplified by PCR and sequenced. Identification of 5' homology arm was performed using the forward primer 5'-GGCAGGCTGGTTTCTGAGTTTG-3', and the reverse primer 5'-ATTCATCTCCTGAGCCCATTCCA-3'; identification of 3' homology arm was performed using 5'-CCCTCCACAGACTAAGACATCCCTAA-3', and the reverse primer 5'-CATGGGCAGGCAACAGTCACA-3'. PCR reactions were performed: 94°C for 2 min, then 98°C for 20 sec, 66°C for 20 sec and 68°C for 2.5 min, a total of 34 cycles; 68°C for 5 min.

Breeding

In theory, the breeding between RIPk3 loxp/+ and lyzcre/+ mice can lead to individuals that

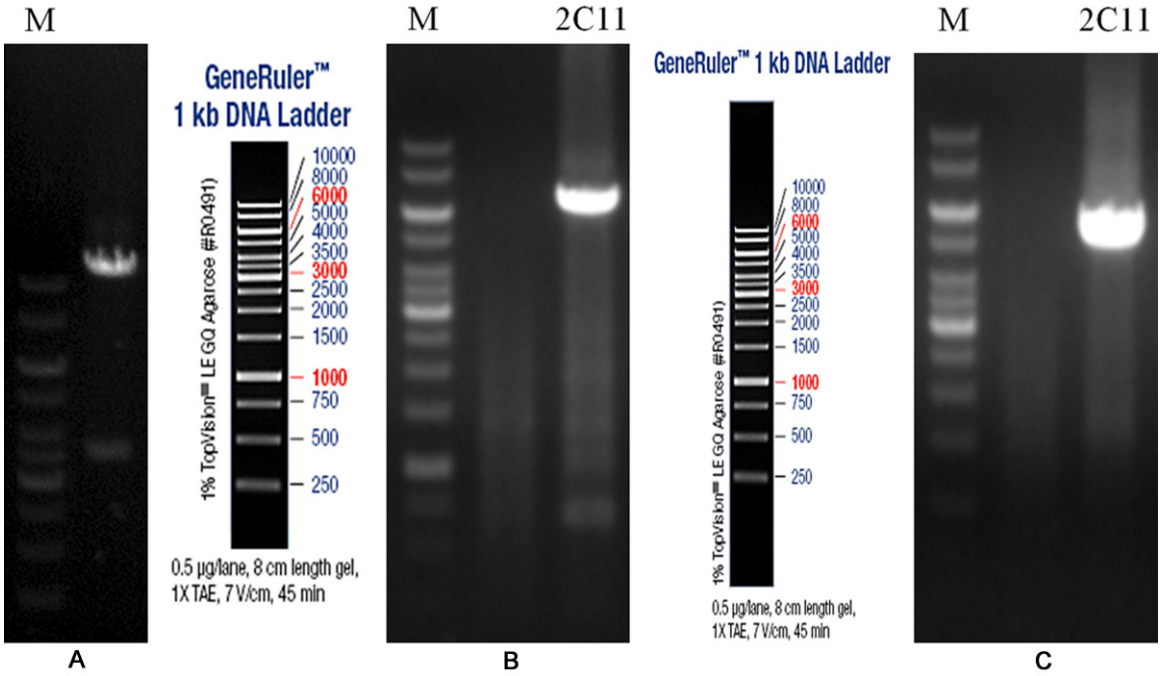

Figure 1. Analysis of the vector and ES cell clones positive for homologous recombination. A. Map of restriction enzyme analysis for the homologous recombination vector (For the digestion with SalI, the theoretical band was 3.7 kb and 14.2 kb in length, respectively; M: 1 kb DNA ladder). B, C. Electrophoretogram of PCR products for ES cell clones positive for homologous recombination (2-C11, positive clone; M, DNA marker; B. Identification result of 5' homology arm; C. Identification result of 3' homology arm).

contain loxp sites and express Cre recombinase (i.e., loxp/+_cre/+). Similarly, the breeding between RIPk3 loxp/loxp and loxp/+_cre/+ mice can lead to loxp/loxp_cre/+ individuals. Study has shown that the binding of the loxp site to Cre recombinase will result in gene knockout [21]. Therefore, the loxp/+_cre/+ individuals will be heterozygotes with bone marrow-specific knockout of RIPk3 gene (lyz-/+), while loxp/loxp_cre/+ individuals will be homozygotes.

Acquisition and identification of homozygotes

Tails were cut off from the mice reaching 2 weeks. DNA was extracted conventionally and subjected to PCR and electrophoresis. The loxp site was detected using the forward primer 5'-CTCCTTACCAGACGCCCTTCT-3' and the wild-type genomic site was detected using the forward primer 5'-CAGCGACACCTTGTGATCTCC-3'. The homozygous loxp site corresponded to a 629 bp band, and the heterozygous loxp site was split into two bands, which were 629 bp and 476 bp, respectively. The wild-type gene containing no loxp site corresponded to one 476 bp band. That is, loxp/loxp = 629 bp; wt/wt = 476 bp; wt/loxp = 476 bp + 629 bp. Cre recombinase gene was detected using the forward prime 5'-GACACGGCACTCCTTGGTAT-3'. If Cre recombinase gene was inserted into the genome, a 335 bp band would be produced; otherwise, there would be no band.

Peripheral blood and bone marrow examination

Bone marrow-specific RIPk3 gene knockout homozygotes (RIPk3-/- mice) were obtained and wild-type mice were taken as control. Routine blood examination was conducted, then three mice of each group were sacrificed, and the femurs of each mouse were taken. One femur was surgically dissected, the BMNCs suspension was prepared and number of BMNCs was counted by flow cytometer.

60 µl of the BMNCs suspension was centrifuged, then washed with PBS and stained twice with PE-c-kit and FITC-CD45, bone marrow HSCs were counted by flow cytometer. 100 µl BMNCs suspension was used for cell death rate evaluation by annexin V and PI staining, and 100 µl suspension was used in ELISA for

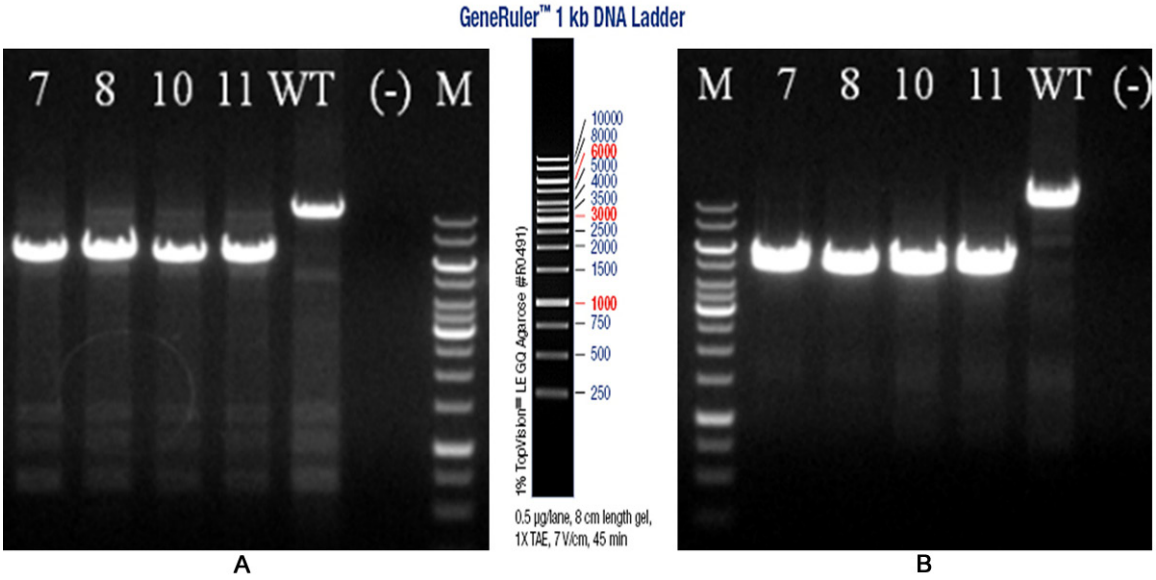

Figure 2. Electrophoretogram of PCR products for the 5' and 3' homology arm of mice of F1 generation with Neo deletion. Number, serial number of mice of F1 generation with Neo deletion; wt, wild-type control; M, DNA marker, 1 kb DNA ladder. A. Identification result of 5' homology arm; B. Identification result of 3' homology arm.

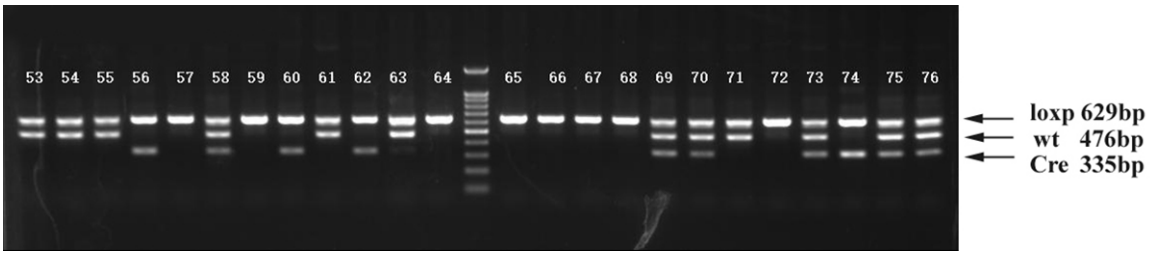

Figure 3. Electrophoretogram of PCR products of Rip3 floxed mice. (Number, serial number of mouse) Mice numbered 57, 59, 64, 65, 66, 67 and 68 were homozygous for loxp; those numbered 56, 60 and 62 were homozygotes with bone marrow-specific knockout of the RIP 3 gene (RIPk3-/-). A total of 6 homozygous were obtained after breeding.

the measurement of levels of IL-6. All operations step by kits instructions.

Morphological evaluation of the bone marrow

Another femur were dissected and fixed in 10% formalin solution for histopathological evaluation. The femur was further decalcified in 5% nitric acid solution for 7~12 h. Then, paraffin-embedded sections were prepared routinely for hematoxylin and eosin (HE) staining and histopathological evaluation.

Hemopoietic progenitor cell culture in vitro

BMNCs were adjusted to a concentration of 5×10^5 cells/ml, cells were cultured at 37°C with 5% CO_2 and saturated humidity. Three days later, CFU-E was counted under the microscope, and 7 days later, BFU-E, CFU-Meg and CFU-GM were counted.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). The *t*-test was used to evaluate the difference between groups. A *P* value less than 0.05 was considered statistically significant.

Results

Identification of the homologous recombination vector using restriction enzyme cutting

The map of restriction enzyme analysis for the homologous recombination vector is shown in **Figure 1A**. The fragments obtained by restric-

Table 1. Peripheral blood and bone marrow examination ($\bar{x} \pm s$)

Items	RIPk3-/-	Control
WBC ($\times 10^9$/l)	10.40±2.02	10.92±2.17
RBC ($\times 10^{12}$/l)	10.33±0.59	10.24±0.48
HGB (g/l)	161.67±6.28	159.33±5.75
HCT (%)	50.50±2.42	48.70±1.41
MCV (fl)	48.90±0.68	47.60±0.93
MCH (pg)	15.67±0.29	15.57±0.19
MCHC (g/l)	320.33±3.61	327.00±3.58
PLT ($\times 10^9$/l)	1385.00±72.21	1377.00±121.00
RDW-SD (fl)	30.10±0.50	29.37±0.36
RDW-CV (%)	20.47±0.81	20.77±0.85
PDW (fl)	7.40±0.24	7.23±0.26
MPV (fl)	6.77±0.23	6.73±0.29
P-LCR (%)	4.73±0.81	4.67±1.56
PCT (%)	0.94±0.81	0.93±0.12
NEUT ($\times 10^9$/l)	1.20±0.15	1.36±0.32
LYMPH ($\times 10^9$/l)	8.95±2.09	9.26±1.91
MONO ($\times 10^9$/l)	0.08±0.03	0.11±0.06
EO ($\times 10^9$/l)	0.17±0.01	0.18±0.01
BASO ($\times 10^9$/l)	0.00±0.00	0.01±0.01
RET ($\times 10^9$/l)	781.37±184.51*	624.50±31.70

*$P < 0.05$, compared with control group.

tion enzyme digestion were of the same size as expected, DNA sequencing also verified that the vector was successfully constructed (see Supplementary File 1).

PCR identification of ES cells positive for homologous recombination

After targeting the ES cells, screening with G418 and further culture were performed. Thus a total of 96 G418-resistant ES cell clones were obtained. Genomic DNA extraction was performed for these positive clones, which were further identified using long fragment PCR and sequenced (see Supplementary File 2). One ES cell clone positive for homologous recombination was obtained (**Figure 1B** and **1C**).

Identification of mice of F1 generation with Neo deletion

Breeding between the male chimeras and FLP mice yielded F1 generation. After PCR identification (**Figure 2**) and sequencing (see Supplementary File 3), four positive heterozygous mice with Neo deletion (RIPk3 loxp/+) were obtained. They were numbered as 7, 8, 10 and 11, respectively.

Acquisition and identification of homozygotes

Breeding between RIPk3 loxp/loxp and loxp/+_cre/+ mice yielded two different individuals homozygous for loxp, namely, individuals containing only homozygous loxp sites (RIPk3 loxp/loxp) and individuals containing homozygous loxp sites and expressing Cre recombinase (loxp/loxp_cre/+), and the latters are bone marrow-specific RIPk3 gene knockout homozygotes (RIPk3-/-) (**Figure 3**).

Peripheral blood and bone marrow examination

Detection of peripheral blood indicated that RET increased considerably in the RIPk3 knockout mice, while other peripheral blood indicators were not significantly different from those of the control mice (**Table 1**). Compared with the control mice, the counts of BMNCs, HSCs and bone marrow IL-6 level in the knockout mice increased considerably. In addition, flow cytometer showed that the cell death rate of BMNCs in RIPk3-/- mice was significantly higher than that in control mice (**Figure 4**).

Morphological evaluation of the bone marrow

The bone marrow had normal structure and morphology in the bone marrow-specific RIPk3-knockout mice. Bone marrow cells proliferated actively in mice of two different phenotypes. A large number of hematopoietic cells were observed, megakaryocytes were easy to be seen (**Figure 5**). There was no sinusoidal dilatation and the sinus wall was intact.

Colony yields of CFU-E, BFU-E, CFU-GM and CFU-Meg

In vitro cell culture indicated no significant difference between RIPk3-/- mice and control mice in the colony yields of CFU-E, BFU-E, CFU-GM and CFU-Meg (**Figure 6**).

Discussion

RIPk3 is a member of the receptor-interacting protein family and exhibits specific serine/threonine kinase activity [22]. RIPk3 has been found to be an important molecular switch for PCD signaling. Excessive RIPk3 expression can induce Caspase-mediated apoptosis of cells. When Caspase activity is inhibited, overexpression of RIPk3 can promote the conversion from

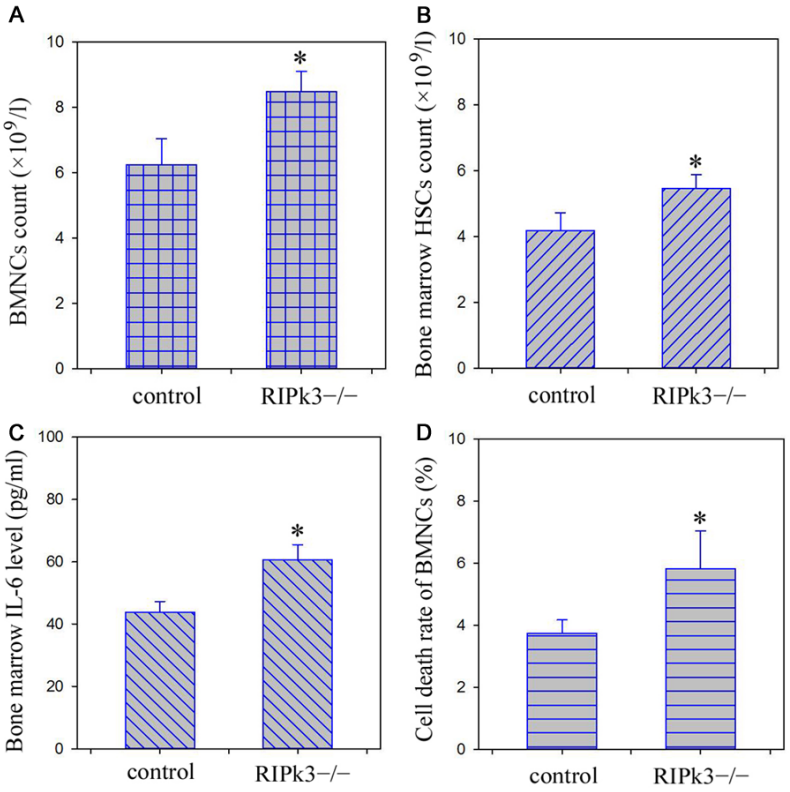

Figure 4. Bone marrow BMNCs and HSCs count, IL-6 level and cell death rate analysis. A. BMNCs count; B. HSCs count; C. IL-6 level; D. Cell death rate. *P < 0.05, compared with control group.

cell apoptosis to non-Caspase-medicated PCD [23]. RIPk3 is involved in the pathogenesis of many hematologic diseases [24, 25]. Through preliminary experiments, it was found that RIPk3-medicated PCD was involved in the cyclophosphamide- and busulfan-induced aplastic anemia in mice [4]. Xiao et al. reported that RIPk3-medicated PCD was related to the death regulation of hematopoietic stem/progenitor cells in Tak1-knockout mice. Moreover, it played an important role in the progression of bone marrow failure syndrome of Tak1(-/-) mice [26]. According to a recent study, defect in the RIPk3-mediated PCD is related to the pathogenesis of CLL and AML, and it is an important reason for resistance to chemotherapy [14, 15]. Thus RIPk3 is a valuable therapeutic target for hematologic diseases.

Flox modification of the RIPk3 gene was performed by targeting ES cells using homology recombination. The Cre/Loxp system was used for specific knockout of RIPk3 gene in the mouse bone marrow. Cre recombinase is derived from bacteriophage P1. Loxp is a 34 bp palindromic sequence. Cre recombinase can specifically recognize the Loxp sequence, causing recombination between 2 Loxp sequences and deletion of the sequence between them. DNA sequences in the important functional domain of the target gene are usually flanked by 1 Loxp on each side. The DNA sequence in the important functional domain is marked by Loxp in the target gene of the knockout mice. Breeding between such knockout mice and the transgenic mice expressing Cre recombinase in specific cells and tissues may lead to Cre recombinase-mediated deletion of the target gene in the cells expressing the Cre recombinase. However, since Cre recombinase is not expressed in other tissues or cells, DNA deletion will not affect these tissues and cells. Thus the target gene remains normal in the offspring. This method can prevent embryonic death or severe developmental disorder at an early developmental stage due to systemic knockout in all cells and tissues [27-30].

Many reports have been published on the specific knockout by the breeding between mice with specific Cre recombinase expression in the bone marrow and Loxp transgenic mice. The target gene is specifically knocked out in the bone marrow of the offspring [21, 31]. The bone marrow-specific RIPk3 knock-out mouse model was successfully established using this method. However, this does not mean that the target gene is removed from all bone marrow cells. For example, the knockout rate is only 83%-98% in the mature macrophages, and knockout may be not limited to bone marrow cells. Clausen et al. reported a 16% knockout rate in a type of dendritic cells in the spleen [21]. More researches are needed for the genotyping of bone marrow-specific homozygous and heterozygous RIPk3 knockout mice obtained in this study.

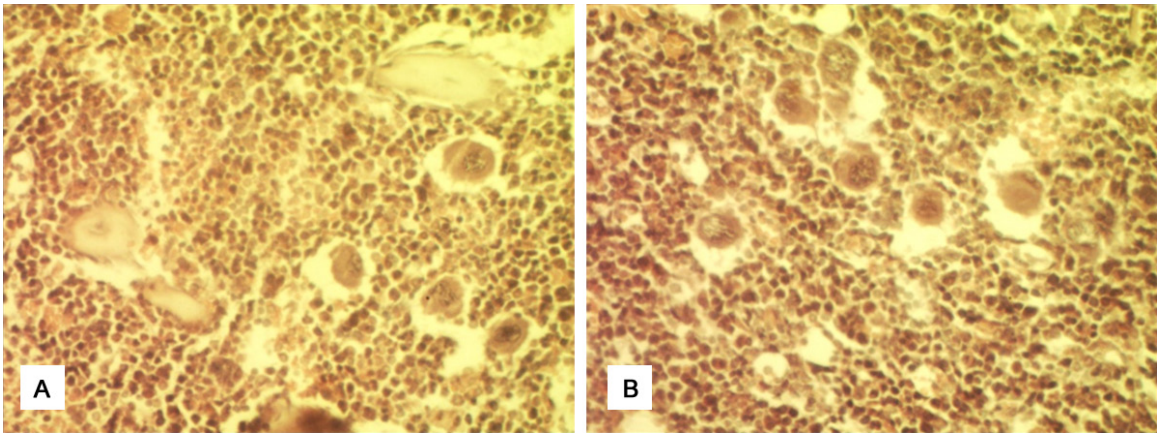

Figure 5. Histological analysis of bone marrow tissue in mice (100×), stained by HE. A. RIPk3-/- mice; B. Control.

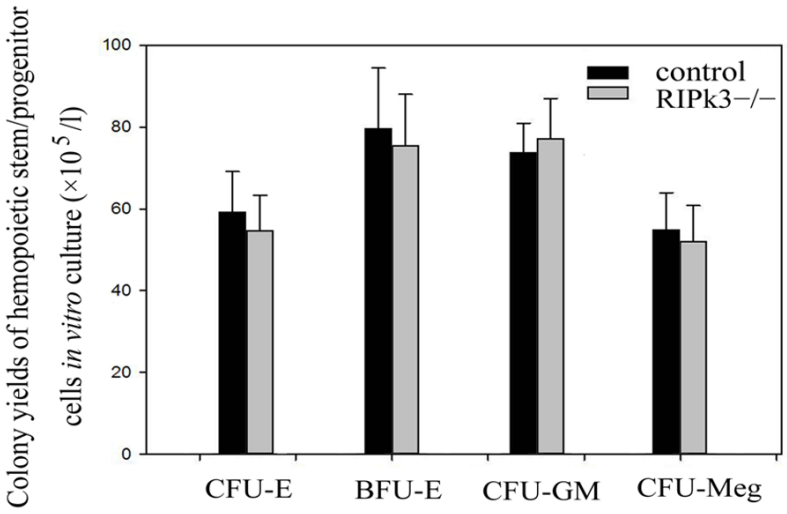

Figure 6. Colony yields of hemopoietic stem/progenitor cells *in vitro* culture.

Bone marrow is an important organ involved in hematopoiesis and immune regulation, the healthy bone marrow tissue is the structural basis for maintaining normal hematopoiesis. Our results showed that bone marrow had normal structure and morphology for bone marrow-specific RIPk3-knockout homozygotes. However, flow cytometer showed that the cell death rate of BMNCs in RIPk3-/- mice was significantly higher than that in control mice, indicated that bone marrow RIPk3 gene knockout may lead to the increase of BMNCs cell death.

Physiological value of blood can reflect the hematopoietic function of bone marrow to a certain extent and also serves as an indicator of the health status and genetic stability of animals. It is also an important basis for clinical diagnosis. Our results showed that, compared with the control mice, RET in peripheral blood increased significantly in the homozygotes, while other physiological indicators of peripheral blood did not change significantly. All primitive blood cells are derived from bone marrow hemopoietic stem/progenitor cells. Colony yields of hemopoietic stem/progenitor cells *in vitro* culture can reflect the proliferative capacity of hemopoietic cells. Our results of *in vitro* culture showed that there was no significant difference in the colony yields of hemopoietic stem/progenitor cells of three lineages between the two mice. This findings indicated that the stability of the bone marrow hemopoiesis can be maintained under RIPk3 gene knockout.

IL-6 is a kind of cytokine with a wide range of biological activities, it is involved not only in regulation of proliferation and differentiation of early hemopoietic stem/progenitor, but also in the progress of the stress reaction, autoimmune and neoplastic diseases of the body [32, 33]. In the present study, we found that the bone marrow IL-6 level in RIPk3-/- mice was increased significantly than that in control mice. In addition, flow cytometry revealed that the number of BMNCs and HSCs in the bone

marrow of the RIPk3-/- mice increased considerably, indicated that the positive hematopoietic factors such as IL-6, may involved in the promotion of proliferation, differentiation, and maturation of HSCs in RIPk3-/- mice.

Taken together, the findings of our study implies that bone marrow RIPk3 gene knockout may lead to the increase of BMNCs cell death, however, increased secretion of hematopoietic factors such as IL-6 may promote the proliferation of hematopoietic stem/progenitor cells and thus maintain the stability of bone marrow hematopoiesis. This hypothesis and the detailed mechanisms remain to be further investigated.

Acknowledgements

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Disclosure of conflict of interest

None.

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Supplementary File 1. Plasmid sequencing results

1. M0827.0479-RIP3-RIP3_C1R_F09

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2. M0827.0480-RIP3-NEO_R_G09

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3. M0827.0481-RIP3-NEO_L_H09

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Supplementary File 2. ES sequencing results

1. M0928.3699-2_5R_C11-NEO_R_A03

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2. M0928.3700-2_5R_C11-RIP3_C1R_B03

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3. M0928.3701-2_5R_H11-5RIP_111_C03

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5. M0928.3715-2_3R_C11-NEO_L_A05

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Supplementary File 3. Sequencing results of F1 generation

Results of no. 1:

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Sbjct  17     GAGTT-CAGGACAGCCAGGTCTATGCAGAGAAACCCTGTCTCGGGGGAAAAAAAAAAAA-  74

Query  12402  AAAGAAAGATTTCAGGAACTCTGAAGTGCAGCAAGGAGAGCATGTTTTCCAGCACACTCA  12461
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Sbjct  75     AAAGAAATATTTCCGGAACTCGGAAGTGCAGAATGGAAAGAGTGTTTTCCAGCCCACTCA  134

Query  12462  ACCCAAATAACTGAGCATCCTTCCAAACCCTCGCTGATGAGAGGGGTGACTCTCTTTTTC  12521
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Sbjct  135    ACCCAAATAACTGGTCATCCTTCCAAACCCCCGCTGAGGAAAGGGGGGACTCTCTTTTTC  194

Query  12522  CAAACAGCATCTGCTGTGAGCACTTCTACCTCCGAAAAGAACTAGAAGCTGGGACTCAAA  12581
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Sbjct  195    CAAACAGCATCTGCTGTGATCACTTCTACCTCCAAAAAAAACTAAAAGCTGGGACTCAAA  254

Query  12582  TAACTTCTGTACAATAATGTTCACAGCACTGTCATTTACAATGTTTGAAAGGAGAAAACC  12641
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Sbjct  255    TAACTTCTGTACAATAATGTTCACAGCACTGTAATTTACAATGTTTGAAAGGAGAAAACC  314

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Sbjct  315    AACCAATGCCCAGGATGGATGAATGGGCATAAAGGTGGGGTATGCACTGTGCTAAAATAT  374

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Sbjct  435    TTTACCTCGAAAATGTTGTGTCTGAATCCAAATGAAGTAAACCAGTCAAATAAAATAGCA  494

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Sbjct  495    TATGGTTCTTCCTCCTCCTCCTCCTCCTTTTTTTTTCTTTTTGAAACAGGGTTTCTC  554

Query  12882  TATGTAGCCCTGTCCGTAGACCAGGCTGGTCTAACTCTGCCTCCAGAGTATTGGAATTAA  12941
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Sbjct  555    TATGTAGCCCTGTCCGTAAACCAGGCTGGTCTAACTCTGCCTCCAAAGTATTGGAATTAA  614

Query  12942  TGGTGTGTCTCTTTTATAAGGCATCTGAAGTGTTCGTCAGTGAAGGTTGGGGAGTAGGAA  13001
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Sbjct  615    TGGTGTGTCTCTTTTATAAGGCATCTGAAGTGTTCGTCAGTGAAGGTTGGGGAGTAGGAA  674

Query  13002  TGAGGTGGGAATGGTGTTTTACTGGGCCTGGGAAGAAGGAATGGGGAGTAACTATGAAAT  13061
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Sbjct  675    TGAGGTGGGAATGGTGTTTTACTGGGCCTGGGAAAAAGGAATGGGGAGTAACTATGAAAT  734

Query  13062  TGCTCCAGAGTGTTAGCTTGAGGTTGGATCTTCCTACTAAGGACTGAATGCTAGGGCTGA  13121
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Sbjct  735    TGCTCCAAAGTGTTAGCTTGAGGTTGGATCTTCCTACTAAGGACTGAATGCTAGGGCTGA  794
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Query is the Target sequence (RIPK3 cko recombinated genomic DNA sequence), Subject is the result of sequencing.

Results of no. 2:

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Sbjct  1011   AGTCAAGATTCG-GAATTCGTGAATAACCCCCAGCTGTAATTCAGAAGTGTGGTCACAAC  953

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Sbjct  952    CCTGAATGGGGCAAAGGGTGAAGGTCTTCCAAAAGGCAAACCAATTTTGGCACACCCTTT  893

Query  15715  CGGCGTGTA-GGAAGAAGATATCCT-GGGAGGTGAAGGCTATGGTTAATCTTCGTAATGA  15772
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Sbjct  892    CGGTGTGTAAGGAAGAAGATATCCTTGGAAGATGAAGACTATGGTTAATCTTAGTAAAGA  833

Query  15773  GAA-CGTTCTGCTCCTG-CTGGGGGTCACTGAGGACCTCCAGTGGGACTTCGTGTCC-GG  15829
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Sbjct  832    GAAACATTTTGCTACTGGTTGGGGGTCAATGAGGACCTCCAGAGAAATTTCGTATCCAGA  773

Query  15830  GCA-GGCTCTGGTGACAAGATTCATGGAGAATGGCTCCCTCGCAGGGCTGCTGCAACCCG  15888
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Sbjct  772    GCAGGGATCTGGTGACAAGATTCAAGGAGAATGGTTCCCTCGCAAGGCTGATGCAACCCG  713

Query  15889  AGTGCCCTCGGCCCTGGCCACTCCTCTGTCGCCTGCTGCAGGAAGTGGTGCTGGGGATGT  15948
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Sbjct  712    AGTGCCATCGGCCCTGGCCACTCTTATGTCGCTTGCTGCAGGAAGTGGTGCTGGGGATGT  653

Query  15949  GCTACCTACACAGCTTGAACCCTCCGCTCCTGCACCGGGACCTCAAGCCCTCTAACATTC  16008
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Sbjct  652    GCTACCTACACAGCTTGAACCATCCGCTCCTGCACCGGGACCTCAAGCCCTCTAACATTA  593

Query  16009  TGCTGGATCCAGAGCTCCACGCCAAGGTTAGTCCATCTACAACCTAGTGTAGACTTGGGA  16068
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Sbjct  592    TGCTGGATCCAGAGCTCCACGCCAAGGTTAGTCCATCTACAACCTAGTGTAGACTTGGGA  533

Query  16069  CCATGTTGAGTACCTGTCCCTCTGAATACAAGACTACACACATGACAAGACCCAACTAGG  16128
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Sbjct  532    CCATGTTGAGTACCTGTCCCTCTGAATACAAGACTACACACATGACAAGACCCAACTAGG  473

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Sbjct  472    AGTTTTATTTCTAGTCATACCAGGTGACCCTCCTTACCAGACGCCCTTCTTAACCCCCTG  413

Query  16189  GCTCAGTTCCACAGGCCTAATGCACCCTCACGGACCCAGTCGACGAATTCCTGCAGCCCA  16248
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Sbjct  412    GCTCAGTTCCACAGGCCTAATGCACCCTCACGGACCCAGTCGACGAATTCCTGCAGCCCA  353

Query  16249  ATTCCGATCATATTCAATAACCCTTAATATAACTTCGTATAATGTATGCTATACGAAGTT  16308
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Sbjct  292    ATTAGGT  286
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Query is the Target sequence (RIPK3 cko recombinated genomic DNA sequence), Subject is the result of sequencing.

Results of no. 3:

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Sbjct  779    TTCCTGGAAAATGTCCTGAGAGGCAAGCACAGGACACATCAGTTGGGCCTGCCACACCAG  720

Query  17815  CAAGG-ACATCTTCTGACCCCGTGGCTGGCACTCCTCAGATTCCACATACTTTACCCTTC  17873
              |||||  |||||||||||||||||||||||||||  ||||||||||||||||||||||||||
Sbjct  719    CAAGGGACATCTTCTGACCCCGTGGCTGGCACTCATCAGATTCCACATACTTTACCCTTC  660

Query  17874  AGAGGCACAACACCTGGGCCAGTCTTTACTGAGACTCCCGGTCCTCACCCCCAAAGGAAT  17933
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  659    AGAGGCACAACACCTGGGCCAGTCTTTACTGAGACTCCCGGTCCTCACCCCCAAAGGAAT  600

Query  17934  CAGGTGAGACACTGGCAAGACTAGAGCACACCCTCTTACCCAGGAGAACTGCACGGTGAT  17993
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  599    CAGGTGAGACACTGGCAAGACTAGAGCACACCCTCTTACCCAGGAGAACTGCACGGTGAT  540

Query  17994  GAGATTAGGCTCTTGAATAGAAAGCCTGAGAGCTCTGTGTTGTTTGCAGGGAGATGGAAG  18053
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  539    GAGATTAGGCTCTTGAATAGAAAGCCTGAGAGCTCTGTGTTGTTTGCAGGGAGATGGAAG  480

Query  18054  ACACGGCACTCCTTGGTATCCCTGGACCCCACCGAATCCAATGACAGGTACCCATTCTTC  18113
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  479    ACACGGCACTCCTTGGTATCCCTGGACCCCACCGAATCCAATGACAGGTACCCATTCTTC  420

Query  18114  ATTCTCTCTTGCTCTCCCCGCTCAGCTCTCCCATCACCTTCCTCCTCAGCACTTATCTAT  18173
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  419    ATTCTCTCTTGCTCTCCCCGCTCAGCTCTCCCATCACCTTCCTCCTCAGCACTTATCTAT  360

Query  18174  AGACATGAGTTACTCTGGAGGTGGGAAGGAATGACTAGAGATCATCTAACCCGATCTGCC  18233
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  359    AGACATGAGTTACTCTGGAGGTGGGAAGGAATGACTAGAGATCATCTAACCCGATCTGCC  300

Query  18234  TCGTTTAAGACGAGGAACATGACATCCACTAGGAGAGGATCCCACTGAGGTCACACAACG  18293
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  299    TCGTTTAAGACGAGGAACATGACATCCACTAGGAGAGGATCCCACTGAGGTCACACAACG  240

Query  18294  AAAGAAAGTCGGGATTGGCTGACTTTTCCCAGCACAGAGTCTGTGAGCCCCCACAGTTCC  18353
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  239    AAAGAAAGTCGGGATTGGCTGACTTTTCCCAGCACAGAGTCTGTGAGCCCCCACAGTTCC  180

Query  18354  TTTATTGATAAGCAAGTCCAGAAGGTCTCCCATCAAAGACACCCAAACAGCAGAATTCCG  18413
              |||||||||||||||||||||||||||  ||||||||||||||||||||||||||||||||
Sbjct  179    TTTATTGATAAGCAAGTCCAGAAGGTCTTCCATCAAAGACACCCAAACAGCAGAATTCCG  120

Query  18414  AAGTTCCTATTCTCTAGAAAGTATAGGAACTTCATCAGTCAGGTACATAATATAACTTCG  18473
              ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  119    AAGTTCTTATTCTCTAGAAAGTATAGGAACTTCATCAGTCAGGTACATAATATAACTTCG  60

Query  18474  TATAATGTATGCTATACGAAGTTATTAGGTGGATCCCCCTCCACAGACTAAGACATC  18530
              |||||||||||||||||||||||||||||||| ||||||||||||| ||  |  ||||
Sbjct  59     TATAATGTATGCTATACGAAGTTATTAGGTGG-TCCCCCTCCACAGATTATGTCATC  4
```

Query is the Target sequence (RIPK3 cko recombinated genomic DNA sequence), Subject is the result of sequencing.

Results of no. 4:

Range 1: 1 to 674 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1210 bits(655)	0.0	670/676(99%)	5/676(0%)	Plus/Plus

```
Query  18542  GGGCTCAG-GAGATGA-ATGGCACTACTCTTTGAGCTTCTACAAGACTAATTCTGAAGAG  18599
              ||| ||||  ||| || ||||||||||||||||||||||||||||||||||||||||||
Sbjct  1      GGG-TCAGCTAGA-GAGATGGCACTACTCTTTGAGCTTCTACAAGACTAATTCTGAAGAG  58

Query  18600  GCACAGCTCATACTGGGACGCAGTAGCTAGCAGGTGAAGACCCTGCATTTGCATTCCCCA  18659
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  59     GCACAGCTCATACTGGGACGCAGTAGCTAGCAGGTGAAGACCCTGCATTTGCATTCCCCA  118

Query  18660  GGGAGATCACAAGGTGTCGCTGCCCCTTTCCCCAAAGTGCTTGTCAACTTCATCTTTCTA  18719
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  119    GGGAGATCACAAGGTGTCGCTGCCCCTTTCCCCAAAGTGCTTGTCAACTTCATCTTTCTA  178

Query  18720  ACCTCTGAACCCTCTCACCATCACCTCCTCCTTTCCTCTTAAAGGGCCACCGGCTCTCGT  18779
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  179    ACCTCTGAACCCTCTCACCATCACCTCCTCCTTTCCTCTTAAAGGGCCACCGGCTCTCGT  238

Query  18780  CTTCAACAACTGTTCTGAAGTGCAGATTGGGAACTACAACTCCTTGGTAGCACCACCAAG  18839
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  239    CTTCAACAACTGTTCTGAAGTGCAGATTGGGAACTACAACTCCTTGGTAGCACCACCAAG  298

Query  18840  AACTACTGCCTCAAGTTCGGCCAAGTATGACCAAGCACAGTTCGGCAGGGGTAGGGGCTG  18899
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  299    AACTACTGCCTCAAGTTCGGCCAAGTATGACCAAGCACAGTTCGGCAGGGGTAGGGGCTG  358

Query  18900  GCAGCCCTTCCACAAGTAGACTTCAGAGAATCACTGCAAGAGCCTGAAGTGTGCCATTCA  18959
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  359    GCAGCCCTTCCACAAGTAGACTTCAGAGAATCACTGCAAGAGCCTGAAGTGTGCCATTCA  418

Query  18960  GCGTGGCAATAAAAAGCACGTTTTAAGCAACCTGGACTGGCTAAGACAGTCCTTGCCACT  19019
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  419    GCGTGGCAATAAAAAGCACGTTTTAAGCAACCTGGACTGGCTAAGACAGTCCTTGCCACT  478

Query  19020  TCCTGAAGCTCACAACATTCTGTGAGGACAGTTGGACCTACACCCAAACTGACTCTTGAC  19079
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  479    TCCTGAAGCTCACAACATTCTGTGAGGACAGTTGGACCTACACCCAAACTGACTCTTGAC  538

Query  19080  CCATCTCCTTAAAGTCAATAAACATAGCATGTTAACTGTGAGAGAGTCTGAGGTGTCAAT  19139
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  539    CCATCTCCTTAAAGTCAATAAACATAGCATGTTAACTGTGAGAGAGTCTGAGGTGTCAAT  598

Query  19140  CCAGGGCATCAGGGATGGACAGCCAGGAGCTGGCTACCCTGGAggggggggTGAGGTGT  19199
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  599    CCAGGGCATCAGGGATGGACAGCCAGGAGCTGGCTACCCTGGAGGGGGGGGGTGAGGTGT  658

Query  19200  GTGTGGGGGG-AGTTG  19214
              |||||||||| |||||
Sbjct  659    GTGTGGGGGGGAGTTG  674
```

Query is the Target sequence (RIPK3 cko recombinated genomic DNA sequence), Subject is the result of sequencing.

Results of no. 5:

```
Query  22673  CGCTGCTACATCAGTGCACCATGCCTACCTTTGTCAGAAGAGGGCACTATAGCCATTGGG  22732
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  739    CGCTGCTACATCAGTGCACCATGCCTACCTTTGTCAGAAGAGGGCACTATAGCCATTGGG  680

Query  22733  AAATCAGTCAAGATTCTCTCGCGGGTACAGAAGTAAACCGAGGAGCATTCGTGGAAAACG  22792
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  679    AAATCAGTCAAGATTCTCTCGCGGGTACAGAAGTAAACCGAGGAGCATTCGTGGAAAACG  620

Query  22793  TGAATCAGAACTGGAGTAGTTTCTTGCAGCTGGAATGGGCTCAGGAGATGAATATGGAAG  22852
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  619    TGAATCAGAACTGGAGTAGTTTCTTGCAGCTGGAATGGGCTCAGGAGATGAATATGGAAG  560

Query  22853  AATAGCACTCTAGACAGAGGAAATATACATGCAAGATAGCTAGGCCTAAGCGGTCAGGTT  22912
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  559    AATAGCACTCTAGACAGAGGAAATATACATGCAAGATAGCTAGGCCTAAGCGGTCAGGTT  500

Query  22913  GGTTCCACAGCCAAGAAAGAGTCTATAGCAGAGAGCAGAGAAGGAAAGCAACCAACTCTT  22972
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  499    GGTTCCACAGCCAAGAAAGAGTCTATAGCAGAGAGCAGAGAAGGAAAGCAACCAACTCTT  440

Query  22973  CCGGATGGATGTCACAAAGTGGCTCCTATTGTTCTGAGATACCTAGGTCCTTAACCATAG  23032
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  439    CCGGATGGATGTCACAAAGTGGCTCCTATTGTTCTGAGATACCTAGGTCCTTAACCATAG  380

Query  23033  AGCCAGCTCTCCTCGACCTGTGTTTGGAACCAACTCCAGCTACACAGATAGACTCCCCCA  23092
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  379    AGCCAGCTCTCCTCGACCTGTGTTTGGAACCAACTCCAGCTACACAGATAGACTCCCCCA  320

Query  23093  CAGACTAGAGATAGGGGAGTGGTACACTTTCTAAGTCTGGCCTTTGCCCACAGTTGGCAG  23152
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  319    CAGACTAGAGATAGGGGAGTGGTACACTTTCTAAGTCTGGCCTTTGCCCACAGTTGGCAG  260

Query  23153  CAAACGCGGTATTGTTCCTGTGTGGGAACGTGGTGGGAGCGTACCACAAGGCCCTGATGG  23212
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  259    CAAACGCGGTATTGTTCCTGTGTGGGAACGTGGTGGGAGCGTACCACAAGGCCCTGATGG  200

Query  23213  AGCGAGCATTGCGGGCCACGTTCCGGGAGGCTCTTAGCTCTCTGCACTCACGCCGGAGGC  23272
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199    AGCGAGCATTGCGGGCCACGTTCCGGGAGGCTCTTAGCTCTCTGCACTCACGCCGGAGGC  140

Query  23273  TGGACACTGAGAAAAAGCACCAGGTCAGTAGGACCTAAGAGGCAGAAAGGGACAGCAGCT  23332
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139    TGGACACTGAGAAAAAGCACCAGGTCAGTAGGACCTAAGAGGCAGAAAGGGACAGCAGCT  80

Query  23333  CAGAAAGAGCAGCTTCTTAGCACCTTCTTTCTAAGTGGCTCTGGGTAATTCATTTGCCTT  23392
              ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
Sbjct  79     CAGAAAGAGCAGCTTCTTAGCACCTTCTTTCTAAGTGGCTCTGGGTAATTCATT-GCCT-  22

Query  23393  CCTCAAGTAT  23402
              ||||||||||
Sbjct  21     CCTCAAGTAT  12
```

Query is the Target sequence (RIPK3 cko recombinated genomic DNA sequence), Subject is the result of sequencing.