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Original Article

Gli^{ko} BMSC: A potential strategy of treatment for renal fibrosis

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ABSTRACT

Objective: There are many researches on using bone marrow mesenchymal stem cells (BMSCs) in the treatment of acute kidney injury (AKI), which has certain effects, but the mechanism of action is still unclear. Previous researches show that glioma-associated oncogene homolog 1 (Gli 1) can promote the proliferation and migration of cells, which can also promote renal fibrosis. Therefore, we investigate the influence of Gli-regulated BMSCs on repairing AKI and renal fibrosis induced by limb Ischemia-Reperfusion (I/R).

Methods: The Crispr-Cas9 technique was adopted to knock out the Gli1 gene from the mouse BMSCs according to green fluorescent tracing, and the BMSCs (BMSCs-Gli^{ko}) with Gli1 gene knocked out and the BMSCs as control group were obtained. The cell proliferation, apoptosis, cycle and SHH signal pathway gene level were tested. The mice were built to the AKI model with inducing I/R injury, then the BMSCs-Gli^{ko} and BMSCs cells were injected into the mice, and their IL-1, IL-1B, TNF-a, serum creatinine (Scr) and blood urea nitrogen (BUN) levels were tested; Western blot was employed to test the expression of α-SMA, SMAD2 and SMAD4 in the renal tissues of mice. Finally, flow cytometry was used to test the content of BMSCs containing green fluorescence in the blood of mice.

Results: The BMSCs-Gli^{ko} containing green fluorescence and the mouse AKI model were built; both BMSCs and BMSCs-Gli^{ko} can reduce the damage level, and BMSCs-Gli^{ko} outperformed BMSCs in protecting renal tubules and anti-fibrosis. Our study also shows that BMSCs-Gli^{ko} stayed longer in the blood of mice, which might also be one of the reasons why BMSCs-Gli^{ko} outperformed BMSCs in preventing renal tubules and fibrosis. To sum it up, could be key target of using.

1. Introduction

Acute kidney injury (AKI) is a clinical syndrome of sudden renal injury triggered postoperative or by critical disease, which has incidence and mortality [1]. Its pathological damage process is very complicated, including damage of renal tissues and renal tubules, and it may cause renal fibrosis, severely affecting prognosis [2]. Clinically, efforts are mainly made to provide symptomatic treatment, optimize postoperative management and find alternative treatment for kidney disease. However, after AKI has developed to phase 2–3, the 5-year mortality rate of patients is still higher than 50%, which consumes a lot of social and medical resources [3]. Therefore, there is an urgent need to look for new methods and targeted therapeutic drugs for AKI.

In tissue regeneration and disease treatment, stem cell therapy has become an effective alternative therapy [4]. Bone marrow mesenchymal stem cells (BMSCs), also called pluripotent cells, are able of self-replication, proliferation and differentiation, and they can also be transformed and induced, which have been broadly used in gene therapy or immunotherapy [5]. According to clinical researches, BMSCs have strong reproducibility and differentiation ability, and exogenous transplantation of BMSCs can alleviate the degree of renal injury [6]. In treatment of renal injury using BMSCs, it not only involves a complicated mechanism, but intravenous injection of BMSCs needs to pass endothelial barrier in blood to reach the damaged part, so as to carry out the therapeutic effect [7]. This process is very complicated, and only very few BMSCs can reach kidney to carry out their effect. How to increase the residence

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time of BMSCs in blood? This problem cannot be solved by increasing their homing number.

Glioma-associated oncogene homolog 1 (Gli 1, Gli zinc finger family 1) is a key transcriptional regulatory factor of SHH signal pathway, which plays an important role in cell proliferation and differentiation [8]. Tissue damage induces the activation of Gli1 pathway in homing mesenchymal stem cells, activates downstream genes such as Cyclin D/E, PTCH1/2, Hhip1 and Myc, promotes induction and differentiation of fibroblasts, and causes fibrosis damage of tissues [9,10]. According to research findings, when BMSCs were used to treat mice with AKI, the number of $Gli1^+$ MSCs significantly increased in the renal medulla, renal cortex and around the entire renal artery. Moreover, the $Gli1^+$ cells can upregulate the expression of a-SMA and promote fibrosis development, i.e., there is the risk of promoting fibrosis during treatment of AKI using BMSCs [11,12]. However, there are no studies on how Gli1 regulates BMSCs to promote fibrosis.

In this study, we separated BMSCs with green fluorescent marker, and for culture in vitro, the Crispr-Cas9 technique was used to knock Gli1 out from BMSCs (BMSCs-Gli^{ko}). The tail vein injection of BMSCs was provided to treat the mouse AKI model, the inflammation expression of mice after treatment, the difference between BMSCs-Gli^{ko} and BMSCs in providing protection, the effect on renal fibrosis, and the residence ability of BMSCs in the blood of mice were observed, and the potential molecular mechanism of BMSCs-Gli^{KO} in the process of treating AKI was further discussed.

2. Materials and methods

2.1. Animals and reagents

Green fluorescence transgenic male C57Bl/6 mice (n = 20) and normal male C57Bl/6 mice (n = 36) 2343 bought from Gem Pharmatech Biotechnology Co., LTD (Jiangsu, China), with certificate No.320727210100567527. The DMEM high-sugar medium, fetal calf serum (FBS), penicillin-streptomycin, phosphate buffer (PBS), trypsin–EDTA were bought from Biological Industries (Israel). The Gli1 antibody was bought from Abcam (Cambridge, England). α-SMA, SMAD2, SMAD4, β-actin and goat anti-rabbit IgG II were bought from Proteintech Group Inc. (Rosemont, USA). The antibodies of CD44, CD177 and CD34 were bought from Cell Signaling Technology, Inc. (Boston, USA); TNF-a. IL-1, IL-1B, BUN and Scr ELISA kit were bought from Bluef (Shanghai, China) Biotechnology Development Co., LTD. (Shanghai, China). The apoptosis detection kit was bought from Beyotime Biotechnology (Shanghai, China).

2.2. Extraction of BMSCs

Femur and tibia were separated from 4-week green fluorescence transgenic male C57Bl/6 mice, and they were soaked in ethanol for 10 min. Then, PBS was used to repeatedly purge the marrow cavity until the cavity turned white, and the purging solution was collected. Next, the solution was adherently and slowly added to the centrifuge tube containing equal volume of Percoll (1.073 g/ml) separation medium, and was centrifugalized for 20 min under 2000 rpm. The cell-PBS mixture on stem cell level was absorbed, added to a new centrifuge tube, and centrifuge for 5 min under 1500 rpm. A complete medium was used for resuspension and precipitation, and then, it was placed in an incubator containing 5% CO_2 under 37 °C for incubation. Medium change was carried out 3.5 days layer, and after cell fusion reached 80%~90%, trypsinization was provided.

Table 1
sgRNA sequence.

Name	Sequence
sgRNA1	AGACTGGGGGGCCCACGCGG
sgRNA2	GAGAGGCCAGGGAGGAGCGG
sgRNA3	TGGGGGTAATGGGAAAAGAG

2.3. Knock out Gli1 using Crispr-Cas9

In this study, three sgRNAs were designed to knock out Gli1 using the Crispr-Cas 9 technique (see Table 1 for related sequences). It was constructed to plasmid vector pT2K-CAGGS-IRES-CFP (pT2K-CAGGS-U6-sgRNA-M5-U6-sgRNA-M7-U6-sgRNA-M9-IRES-CFP was a gift from Martine Roussel, Addgene plasmid # 114,729) using the clone and recombination method, and it was named as pT2K-CAGGS-U6-sgRNA-G1-U6-sgRNA-G2-U6-sgRNA-G3-IRES-CFP. After endotoxin-free extraction of pT2K-CAGGS-U6-sgRNA-G1-U6-sgRNA-G2-U6-sgRNA-G3-IRES-CFP plasmid, it worked with Crispr-Cas9 recombinant protein to co-transfect BMSC for 48 h. Then, flow cytometry was employed to sort and obtain cells with CFP marker, and after separation using limited dilution method, it had single cell clone with gene editing (Table 1 and 2).

2.4. Use Flow Cytometer for purity, apoptosis and cycle detection of BMSCs

For collected cells or whole blood of mice, apoptosis and cycle detections of some specimens were carried out according to the manuals of apoptosis kit (DOJINDO LABORATORISE, China) and cycle detection kit (Nanjing KeyGen Biotech Co, Ltd, China); for some specimens, the CD34, CD177 and CD44 flow antibodies were incubated, and Flow Cytometer was used to detect corresponding indices.

2.5. Use CCK-8 to check the cell viability

BMSCs and BMSCs-$Gli1^{KO}$ were inoculated to 96-well plates according to the cell density of 8000 cells/well. At 24 h, 48 h and 72 h of cell culture, the cells were added to CCK-8, 10 uL each well. After even mixing, the cells had reaction for 2 h under 37 °C. Then, ELIASA was used to test absorbance, which was cell vitality.

2.6. Use WB to detect the expressions of Gli1, α-SMA, SMAD2 and SMAD4

Western Blotting was employed to detect the protein change level. Cells or tissues were collected, and the RIPA lysis buffer was used to extract the total protein of cells. The BCA assay was employed to measure the protein concentration. Equivalent proteins had 10% SDS-PAGE, and then transferred to the PVDF membrane. After sealed for 1 h using 5% skimmed milk powder, the

Table 2
Primer sequences.

Primer name	Upstream primer	Downstream primer
Cyclin D	CACCAAGTTCATCAGACAAT	AGGCAAGACAGATTCAGT
Cyclin E	ACTGAGACTGAAGGTGTA	TTGACGATATTAGGGTGATTA
PTCH1	TTCTTGCTCTTGGTGTTG	TCCTCTTATTCTGTCCTGTT
PTCH2	AGTCCAAGTATCACTCTAT	AACAGCTTCTCAATCATC
Hhip1	TTGTGTTCTGCTCTTCTC	CTTCTGCCTCTTATTAGTGTA
Myc	TCGTGAGAGTAAGGAGAA	CAAGGTTGTGAGGTTAGG
β-actin	ATCTTCCGCCTTAATACT	GCCTTCATACATCAAGTT

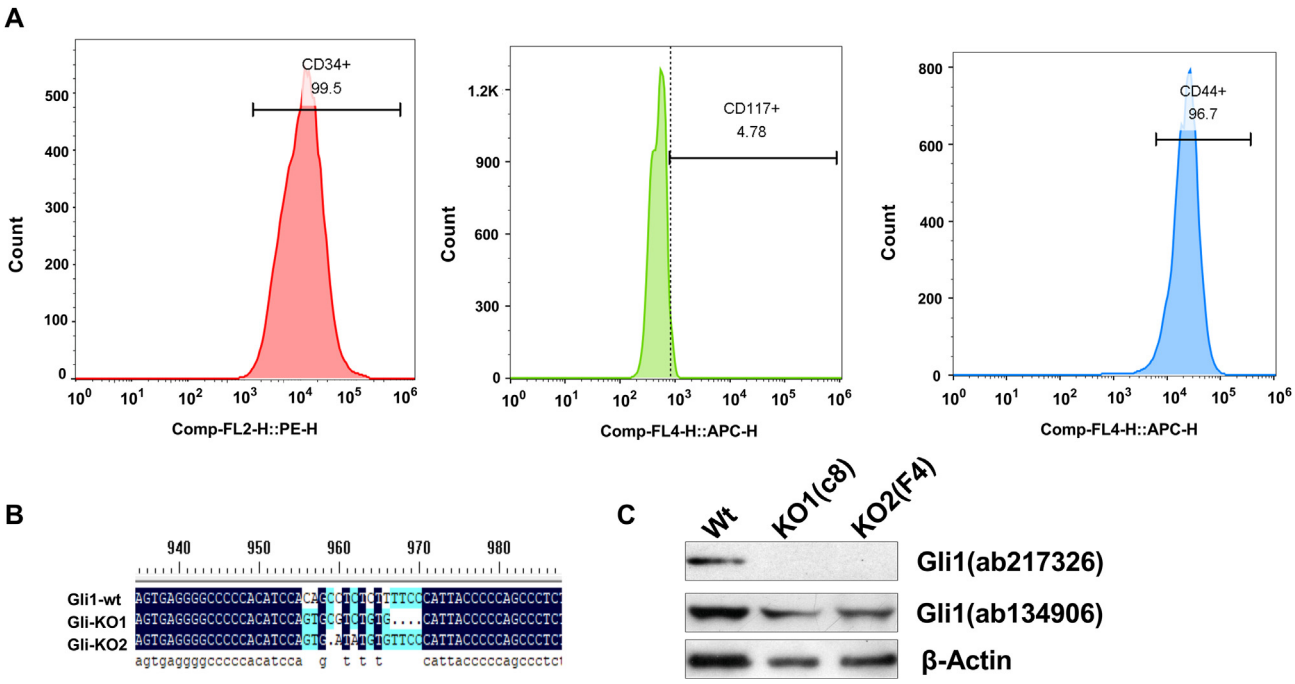

Fig. 1. BMSCs-KO identification. A. Use flow cytometry to detect the surface markers CD34+, CD117+ and CD44+ of BMSCs; B. Compare the cell line sequences of two BMSCs-Gli^{KO}; C. Test the expression levels of Gli in two BMSCs-Gli^{KO} cell lines using WB.

specimen was incubated with the Gli1, α-SMA, SMAD2, SMAD4 and Bactin antibodies under 4 °C overnight, and then, it was incubated for 1 h under room temperature with secondary antibody connected by suitable HRP (horse radish peroxidase). Next, the ECL solution was used for developing, the Biorad ChemiDoc MP System was employed for imaging, and quantitative analysis of target band was carried out.

2.7. Use qPCR to detect the target genes of downstream SHH pathway of Gli1

Cells or tissues were collected, the Trizol kit was used to extract total RNA, and the miRNA extraction kit was used to extract miRNAs in cells. The reverse transcription kit (TaKaRa, China) was used to reversely transcript 1 μg total RNA into cDNA, and the stem-loop method was utilized to reversely transcript miR-1. SYBR Green PCR Master Mix and specific primers were used to carry out real-time RNA quantification in ABI 7500 Fast fluorescence ration PCR instrument, and the expressions of Cyclin D, Cyclin E, PTCH1, PTCH2, Hhip1 and Myc mRNA were detected respectively. The annealing temperature was set at 60 °C, and 40 cycles were amplified. At the end, according to the Ct value, the $2^{-\triangle\triangle Ct}$ method was used to calculate and analyze the value, and data was exported for statistical analysis. The primer sequences are as follows (Table 2):

2.8. Mouse AKI model

The 8-week male C57BL/6 mice with SPF breeding were randomly divided into the four groups of normal control (NC) group, Ischemia-Reperfusion (I/R) group, BMSC group and BMSC-Gli^{KO} group (each group n = 10). The mice were processed as follows. All rats were intraperitoneally treated with sodium pentobarbital (40 mg/kg) and kept at 37 °C. For the NC group: the femoral artery in the right leg was exposed and the incision was closed without inducing I/R injury; for other groups rather than the NC group, by referring to the method of Tao [13]. In the I/R group, the right femoral artery was exposed and then occluded the right hind femoral artery with rubber band, thus inducing limb perfusion dificits for 3 h, followed by band release to allow reperfusion for 4 h. Then, for the BMSC group and BMSC-Gli^{KO} group, exposure to rubber band application to limb ischemia for 3 h was in combination with intravenous injection of 1.5 × 10^6/ml BMSCs or BMSCs-Gli^{KO}, 0.4 ml for each mouse; the block was then released for 4 h to allow reperfusion. According to different points in time, 1% pentobarbital sodium was used to anesthetize mice, and blood was collected from eye socket and placed under 4 °C overnight; at 14 d, the kidney was collected, after peeling off capsule, some tissues were fixed using 10% formaldehyde and had paraffin embedding, and RNA and proteins were extracted from the rest tissues.

2.9. Use ELISA to test the renal function of mice and the contents of Scr, BUN, TNF-a, IL-6 and IL-B

After blood was collected, it was centrifuged for 10 min under 3000 rpm. The serum was transferred to a new EP. Based on the serum creatinine (Scr) and blood urea nitrogen (BUN) of mice as well as the inflammation TNF-a, IL-6 and IL-B, the Scr and BUN levels and the contents of TNF-a, IL-6 and IL-B were tested according to the manual of ELISA assay kit.

2.10. Mouse kidney HE staining

The 1% pentobarbital sodium was used to anesthetize mice, and kidney was removed. The renal tissues were fixed using 10% formalin and had paraffin embedding, the tissues were cut into 5 μm thick slices, and then, they were deparaffinized and rehydrated using xylene and ethanol. Next, the tissues were stained using HE stain (Solarbio, China) according to the manual of kit, and the images were captured by an optical microscope (Nikon, Japan).

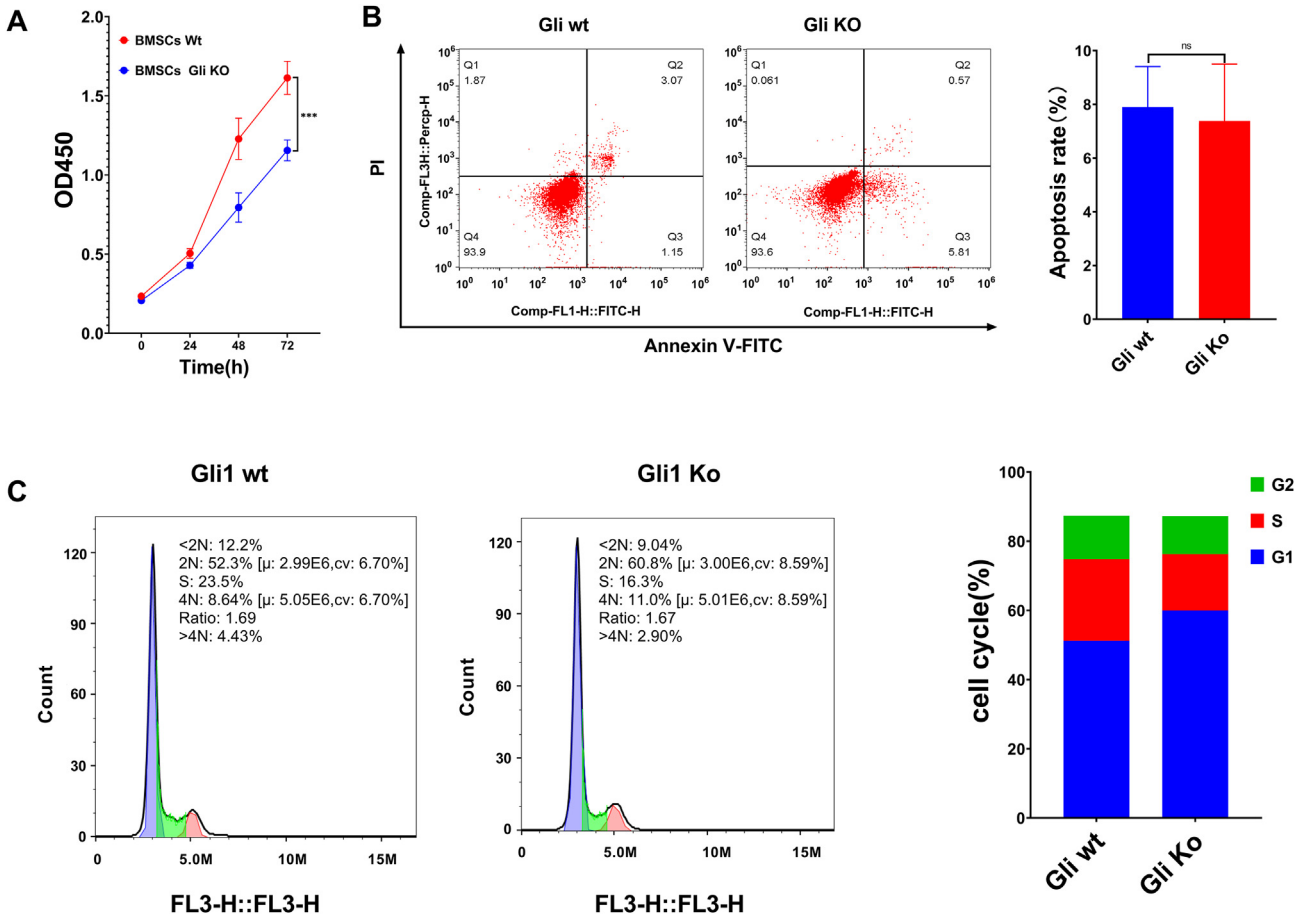

Fig. 2. Influence of Gli1 knockout on the biological functions of BMSCs. A. Use CCK-8 to test cell vitality; B. Use flow cytometry to test cell apoptosis level; C. Cell cycle test.

2.11. Statistical method

The 21.0 SPSS software was used for statistical analysis, and for all data, mean ± SD was used. One-Way ANOVA or *t* test was adopted for inter-group comparisons. When $P < 0.05$, the difference is regarded as having statistical significance.

3. Results

3.1. BMSCs-Gli^{KO} were successfully separated and built

BMSCs were separated from green fluorescence mice, and Crispr-Cas9 was employed to knock out Gli1. As shown in Fig. 1A, according to the identification of separated BMSCs using flow cytometry, we found that the CD34 positive cells were more than 98%, the CD44 positive cells were more than 95%, while the CD177 positive cells were lower than 5%, and the purity of BMSCs was good. Two Gli-KO sequences were designed for the Crispr-Cas9 experiment (Fig. 1 B), and after BMSCs were infected by lentivirus, the Gli1 protein content in cells was detected using WT. Different Gli1 protein antibodies were used to incubate SDS-PAGE, and the results are as shown in Fig. 1 C. At 18 kDa, the Gli1 protein of two BMSCs-Gli^{KO} did not have expressions. However, this protein expression can be detected at 150 kDa, but the content significantly decreased (Fig. 1 C).

3.2. Influence of Gli1 knockout on the vitality, apoptosis and cycle of BMSCs

After successfully building BMSCs-Gli^{KO}, we tested the influence of Gli1 knockout on the biological functions of BMSCs. CCK-8 was used to observe cell vitality, and the results show that the vitality of BMSCs-Gli^{KO} was significantly lower than that of BMSCs-WT at 48 h and 72 h (Fig. 2 A); flow cytometry was employed to test cell apoptosis, and the results indicate that BMSCs-Gli^{KO} and BMSCs-WT did not present significant difference in apoptosis level (Fig. 2 B); the cell cycle results show that the cell cycle of BMSCs-Gli^{KO} had arrest (Fig. 2 C).

3.3. Influence of Gli1 on SHH downstream genes

Next, the mRNA expressions of SHH downstream genes Cyclin D. Cyclin E, PTCH1, PTCH2, Hhip1 and Myc were tested using qPCR, and the results are presented in Fig. 3. After knocking out Gli1, the expressions of Gli1 downstream genes significantly declined, which indicates that the SHH pathway activation could be inhibited.

3.4. BMSCs-Gli^{KO} promote protection against AKI

Elisa was adopted to detect the BUN and Scr levels of model mice in different groups. The serum concentrations of BUN and Scr were significantly increased in the I/R group. After BMSC WT treatment, the BUN and Scr concentrations were significantly lower (0.75 and 0.74 times, respectively) than those in I/R group. Besides, concentrations of BUN and Scr in the BMSC Gli^{KO} group were also significantly lower (0.57 and 0.65 times, respectively) than those in I/R group. However, there were no significant differences in BUN and Scr concentrations between BMSC WT group and BMSC Gli^{KO} group (Fig. 4A and B). According to the test of early inflammatory markers TNF-a, IL-6 and IL-1B of model mice, we found that in the BMSC

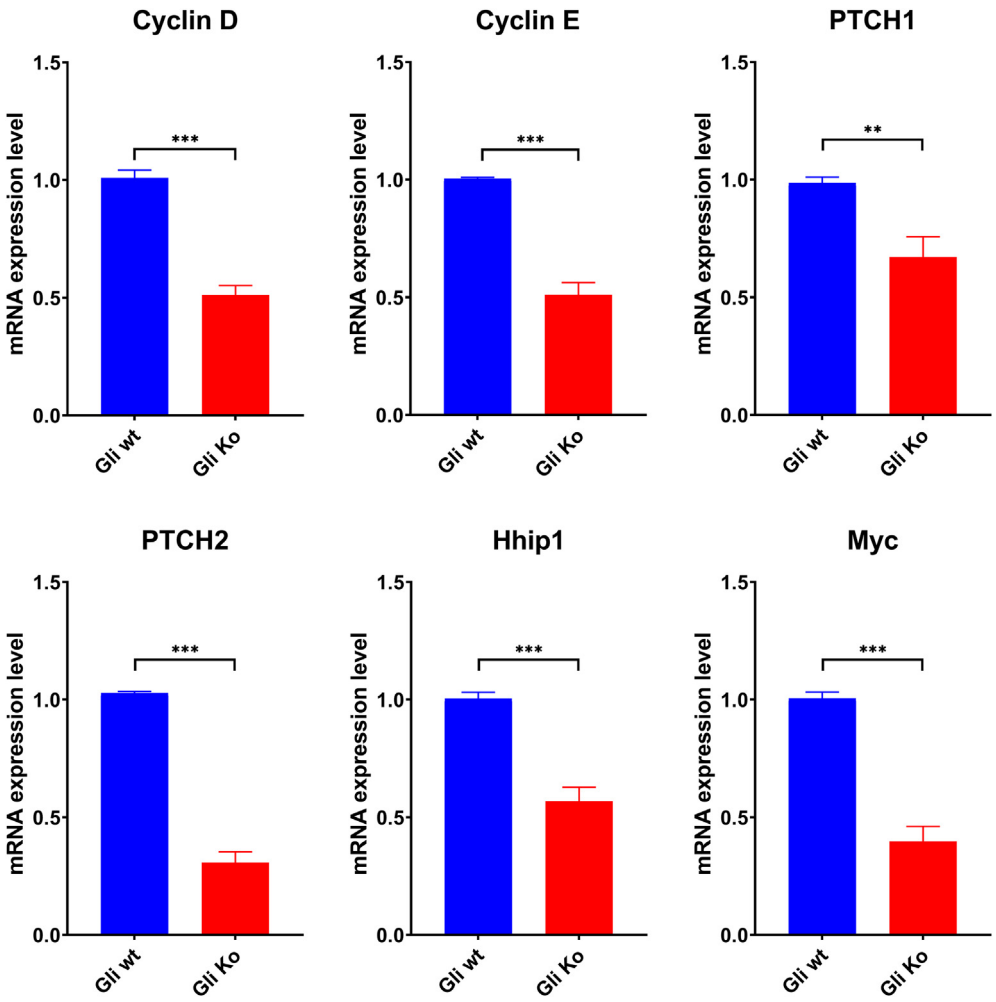

Fig. 3. Use qPCR to test the relative expressions of SHH downstream genes.

intervention group, TNF-a decreased at both 72 h and 96 h, while IL-6 and IL-1B only showed significant decrease at 96 h. Furthermore, compared to the BMSCs WT group, three inflammatory markers of the BMSCs-Gli^{KO} group all showed significant decline at 96 h (Fig. 4C–E). The HE staining results show that mice in the NC group had normal structure of renal tissues, and in the AKI model group, the renal tubule epithelial cells of mice had vacuolar degeneration, and brush border detachment was obvious. After 20 d of treatment, BMSCs WT and BMSCs-Gli^{KO} both reduced the AKI renal tubular necrosis, renal tubular dilation and formation of casts induced by I/R. In other words, in the AKI mouse model provided with BMSCs treatment, the renal tubule damages were significantly alleviated.

3.5. BMSCs-KO inhibit the fibrosis degree of AKI

The WB results (Fig. 5) of fibrosis index show that in the model group, the protein expressions of α-SMA, SMAD2 and SMAD4 all increased. In the BMSCs and BMSCs-Gli^{KO} treatment groups, the above protein expressions were significantly lower than the expressions of model group, and the BMSCs-Gli^{KO} group had even lower expressions than the BMSCs group. The above results prove that treatments with BMSCs and BMSCs-Gli^{KO} both presented anti-fibrosis effects, and BMSCs-Gli^{KO} outperformed BMSCs in anti-fibrosis treatment.

3.6. Test of residence ability of BMSCs in blood by BMSCs-KO

During test of the residence ability of BMSCs in blood, we found that the percentage of green fluorescence BMSCs in CD34 positive cells in blood decreased with time. At 96 h, the percentage of green fluorescence cells in the blood in the BMSCs-Gli^{KO} group (7.356%) was significantly higher than that in the BMSCs group (1.973%) (Fig. 6). The above results indicate that Gli1 knockout can extend the residence time of BMSCs.

4. Discussion

More and more researches prove that transplantation can provide certain repair effect for AKI, but its action mechanism is still unclear [1]. In this study, first, the BMSCs of green fluorescence transgenic mice were separated successfully for culture in vitro. Then, the Crispr-Cas9 technique was employed to knock the Gli1 gene out from the cells. It was found that the activity of KO cells declined, which had arrest effect on cycle S, but it did not affect the cell apoptosis. Moreover, it inhibited the expression of downstream genes of SHH pathway, which reveals that Gli knockout inhibited the expression of SHH downstream genes, reduced cell activity and arrested cell cycle. Secondly, the BMSCs with Gli1 knockout can significantly lower AKI and reduce renal fibrosis. Finally, according to number of green fluorescence BMSCs in mouse blood after

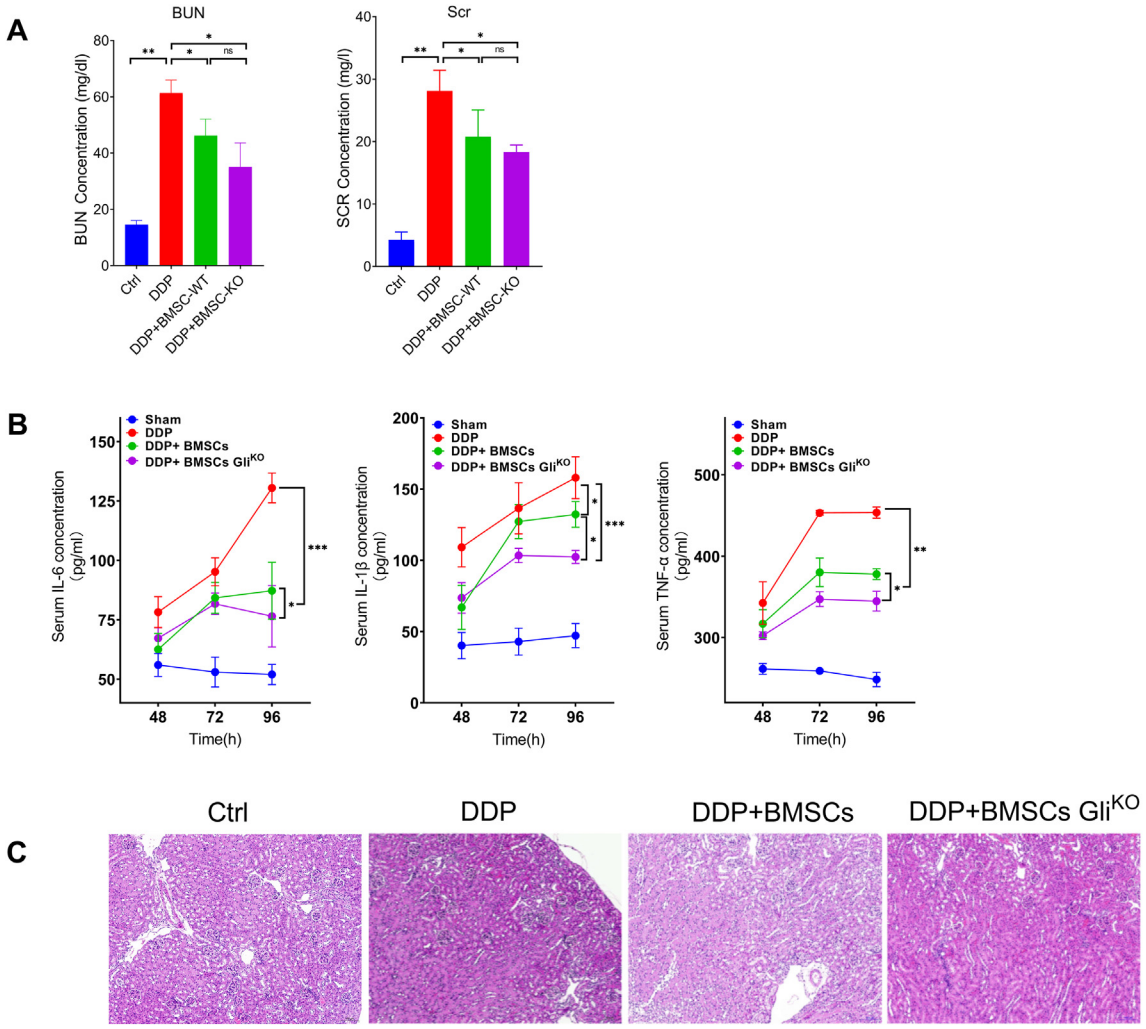

Fig. 4. BMSCs Gli^{KO} promote protection against AKI. A. Scr and BUN levels; B. Detection of inflammatory markers TNF-a, IL-6 and IL-1B; C. HE staining.

transplantation via flow cytometry, we found that Gli1 knockout can significantly increase the residence time of BMSCs in blood. Our study further proves that Gli1 knockout can inhibit the proliferation of BMSCs, but it has potential advantages in anti-inflammation, anti-fibrosis and injury repair of AKI, and it can also increase the residence time of BMSCs.

Gli1 is a transcription factor, which regulates the Hedgehog (HH) transmembrane receptor and downstream pathway proteins in the "classic" regulation of HH pathway. It transfers the transduction signal to the nucleus via cytoplasm, combines with specific DNA sequence 5′-GACCACCCA-3′, and regulates the transcription of specific genes during normal development process [14]. Gli1 carries out its effect in the development of central nervous system and gastrointestinal system, which can regulate downstream genes Cyclin D, Cyclin E, PTCH1, PTCH2, Hhip1 and Myc, and it can regulate cell proliferation, differentiation and angiogenesis [9,10]. This phenomenon was also observed in our study. As Gli1 was knocked out, the expression of downstream genes of SHH pathway was inhibited, the vitality of BMSCs decreased, the cell cycle was arrested, but it did not have influence on the apoptosis of BMSCs. This further proves Gli1's influence on cell proliferation and differentiation.

The experiments prove that BMSCs can reduce the AKI degree. However, some articles mentioned significant increase of Gli1+ MSCs in the medulla, cortex and around the entire renal artery in AKI caused by DDP and tamoxifen, most Gli1+ cells up-regulated the expression of α-SMA and GLI1+ BMSC accumulate around blood vessels after injury, which increased the risk of fibrosis [11,12]. Moreover, in the study of using BMSCs to treat pulmonary fibrosis, it was also found that the degree of pulmonary fibrosis can be inhibited by reducing Gli1. In our study, after transplanting BMSCs with Gli1 knockout to the AKI mouse model, it can significantly increase protection of kidney, reduce early inflammation level, and reduce the degree of renal fibrosis during later phase of AKI, which is consistent with the results of Gli + enrichment and increased fibrosis degree after treatment using BMSCs. Our initial hypothesis was that GLI knockout would inhibit signaling by Hedgehog signal pathway and prevent BMSC from differentiating into myofibroblasts, thereby inhibiting fibrosis. However, the actual result was that no labeled BMSCs or other cells were detected around the injury site, but the retention time of Gli1^{KO} BMSCs in peripheral blood was increased. We hypothesized that these are caused by cytokines secreted by long-retained Gli1^{KO} BMSCs, such as exosomes. These results also further prove that Gli1 can promote fibrosis and differentiation of BMSCs, and indicate that Gli1 knockout can better reduce the risk of fibrosis.

There are some literatures have reported that BMSC have both pro-inflammatory and anti-inflammatory effects [15,16], but Gli1^{KO} BMSCs showed significant anti-inflammatory effects in this study, which was unexpected. About its possible anti-inflammatory

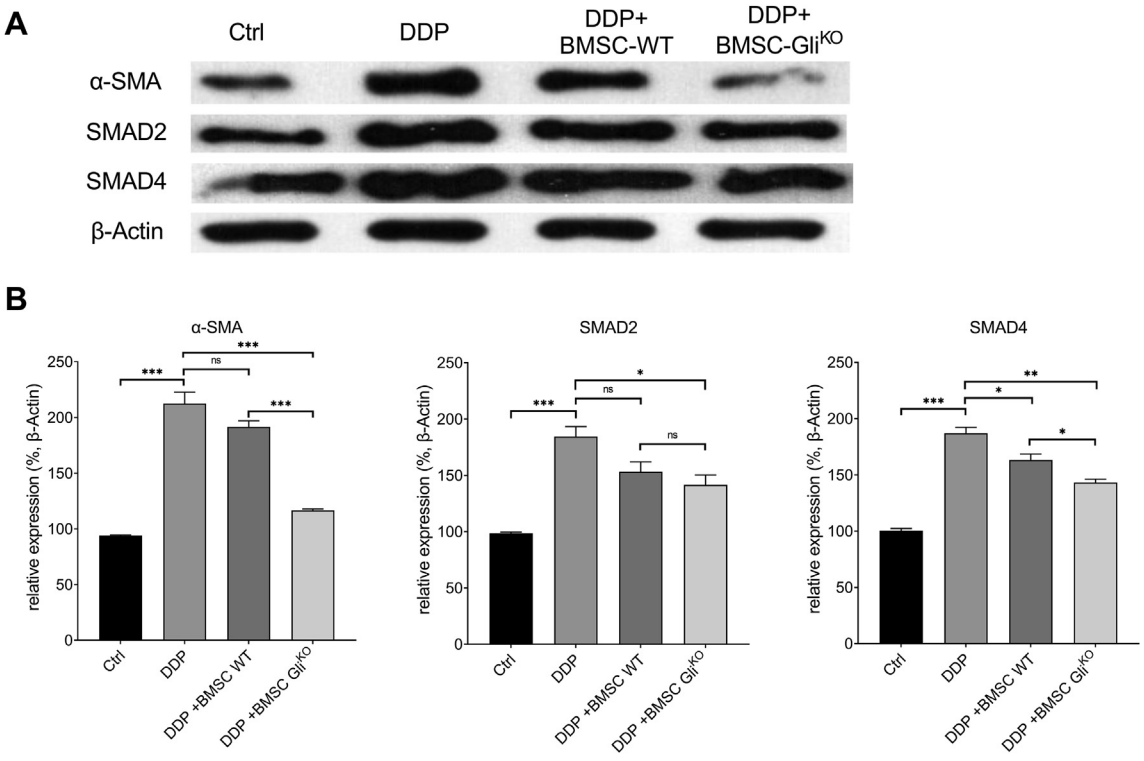

Fig. 5. BMSCs and BMSCs-Gli^{KO} inhibit fibrosis degree of AKI. A. Test α-SMA, SMAD2 and SMAD4 using WB; B. Statistical results of WB.

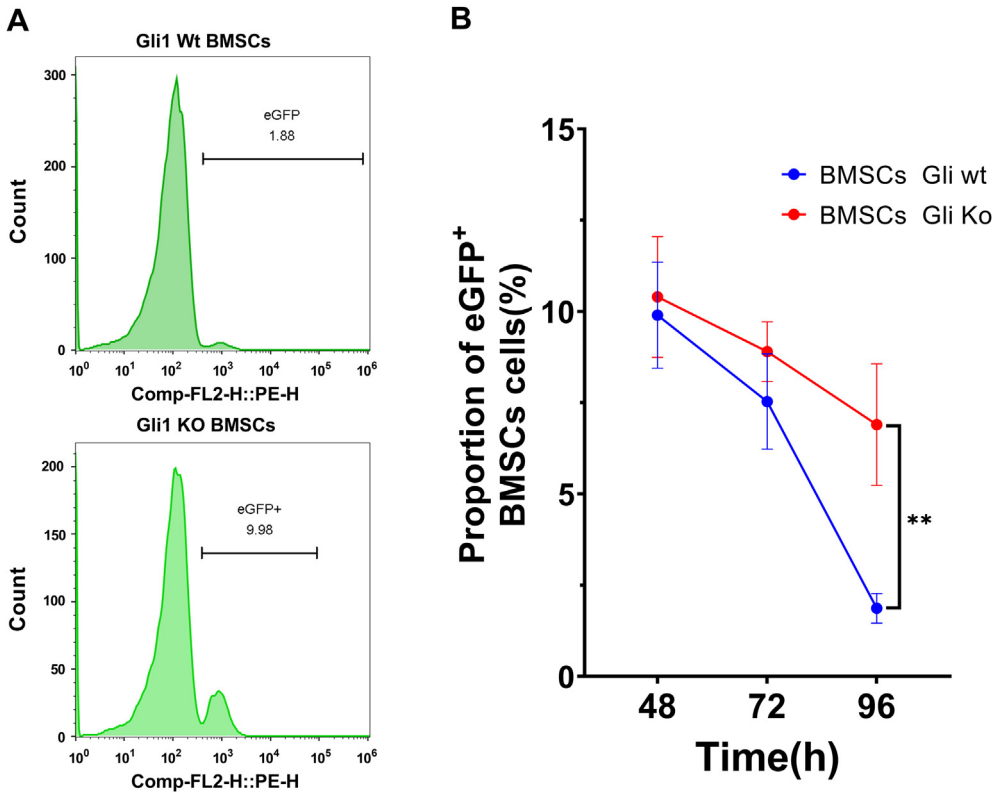

Fig. 6. Test of residence ability of BMSCs in blood by BMSCs-KO. A: CD34+ with green fluorescent markers at 48 h, 72 h and 96 h of flow cytometry; B: Statistical results of proportion of eGFP^{+} BMSCs cells.

mechanism, there are two hypotheses we are currently testing, one is mechanism of exosome regulation, and the other is the effect of $Gli1^{KO}$ BMSC cells on monocytes in the blood. These will be the focus of Our subsequent studies. In addition, the homing speed and number serious affect the treatment effect of tissue damage after transplantation of BMSCs [17]. When BMSCs are transplanted into organs via tail vein injection or other measure, they pass endangium to reach the damaged part, and carry out the anti-inflammation and repair effects [18]. The specific path and action mechanism of BMSC homing are still unclear [7]. In this study, by checking the percentage of green fluorescence BMSCs in BMSCs in mouse blood, we found that after Gli1 knockout, the residence ability of BMSCs in blood could be significantly improved, so as to provide conditions for BMSCs with Gli1 knockout to reach the damaged part, better reduce inflammation, and improve their repair ability. Even though the green fluorescence BMSCs were not tested after homing, which was related to the experimental conditions and scheme design, the extended residence time is a new finding after Gli1 knockout, and its action mechanism deserves further investigation.

Our results have provided a research idea for using BMSCs to repair AKI and reduce risk of renal fibrosis. The results also prove that BMSCs with Gli1 knockout played an important role in the transplantation treatment induced by I/R. Although the reason for extended residence time cannot be completely determined, BMSCs with Gli1 knockout can become a new research direction for AKI treatment.

5. Conclusion

Gli1 knockout can inhibit cell vitality, arrest cycle development, reduce fibrosis and differentiation degree, extend residence time of cells in blood after transplantation, and alleviate AKI induced by I/R. The regulation of Gli can provide a new research idea and important theoretical basis for AKI treatment and reducing fibrosis level.

Ethics approval and informed consent

The study was carried out by strictly following The Guide for Care and Use of Laboratory Animals formulated by National Institutes of Health (NIH). The animal use plan was approved by the Animal Care and Use Committee of The Second Hospital of Hebei Medical University, and authorization was obtained from the Ethics Committee of The Second Hospital of Hebei Medical University.

Consent for publication

All authors agree to publish.

Availability of data and material

All data generated or analysed during this study are included in this published article (and its supplementary information files).

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Declaration of competing interest

The author reports no conflicts of interest in this work.

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