CircRNA-011235 Counteracts The Deleterious Effect of Irradiation Treatment on Bone Mesenchymal Stem Cells by Regulating The miR-741-3p/CDK6 Pathway

Xinhui Wen, M.D.1, Hebin Xie, M.D.2, Rong Gui, Ph.D.1, Xinmin Nie, Ph.D.3, Dongyong Shan, M.D.4, Rong Huang, M.D.1, Hongyu Deng, M.D.4, Junhua Zhang, M.D.1*

1. Department of Blood Transfusion, The Third Xiangya Hospital of Central South University, Changsha, China
2. Research and Teaching Department, Changsha Central Hospital, Changsha, Hunan, China
3. The Third Xiangya Hospital of Central South University, Changsha, China
4. Hunan Cancer Hospital and The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha, China

*Corresponding Address: Department of Blood Transfusion, The Third Xiangya Hospital of Central South University, Changsha, China
Email: jhzhangjunhua@163.com

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Abstract

Objective: The present work was aimed at uncovering the effect of circRNA-011235 (circ-011235) on irradiation-induced bone mesenchymal stem cells (BMSCs) injury and its regulatory mechanism, with a view to establish a scientific basis for its possible medical applications.

Materials and Methods: In this experimental study, after irradiation with different doses (0, 2, 4, 6 GY), the relative expression levels of circ-011235, miR-741-3p, and cyclin-dependent kinases 6 (CDK6) were detected in the BMSCs, using the real time-quantitative polymerase chain reaction (RT-qPCR). The overexpression effects of circ-011235 and CDK6 on the cell proliferation in irradiation-treated BMSCs were measured by the Cell Counting Kit-8 (CCK8) assay. And also, their effects on the cell cycle were evaluated by flow cytometry. RT-qPCR and immunoblotting were performed to detect the effects of pcDNA-circ-011235 and pcDNA-CDK6 on the expression of cyclin D1 and cyclin-dependent kinases 4 (CDK4) at the gene and protein levels, respectively.

Results: Irradiation treatment elevated the expression of circ-011235 and CDK6, but reduced miR-741-3p expression in the BMSCs with a dose-dependent effect. The proliferation of BMSCs was significantly inhibited in the irradiation treatment group, while the overexpression of circ-011235 and CDK6 effectively attenuated this inhibition. Also, overexpression of circ-011235 and CDK6 elevated the expression of cyclin D1 in irradiation-treated BMSCs, but had no significant effect on the CDK4 expression.

Conclusion: Our results demonstrated that circ-011235 up-regulated the expression of cyclin D1 via miR-741-3p/CDK6 signal pathway, thereby promoting cell cycle progression and proliferation of irradiation-treated BMSCs. This finding suggested circ-011235/miR-741-3p/CDK6 pathway exerted a protective role in the response to irradiation and will be a potential new target for future research on the mechanism involved in the resistance of BMSCs to radiation.

Keywords: Bone Mesenchymal Stem Cell, CDK6, Cell Cycle, Irradiation

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Introduction

Bone marrow transplantation (BMT) remains the fundamental treatment for various hematological malignancies, aplastic anemia, severe thalassemia, and some congenital immune deficiency or metabolic diseases (1). Total body irradiation (TBI) is one of the most important pretreatment methods for BMT in the patients with leukemia (2). While TBI pretreatment induces bone mesenchymal stem cells (BMSCs) injury, BMT may seriously interfere with the implantation and transplantation of hematopoietic stem cells (HSCs). Also, TBI pretreatment will inevitably cause other normal cells damages as well as body tissue cells in the bone marrow, and consequently, destroy the hematopoietic system (3). When the body is exposed to irradiation, a series of adaptive stress responses are triggered to repair the damage and reduce the effects of injury (4). At present, the damage mechanism in these patients before BMT radiotherapy is not fully understood. Further study on the regulatory mechanism of irradiation stress and elucidation of the molecular mechanism of irradiation biological effect will lay a theoretical foundation for the protection and treatment of irradiation injury in the TBI treated patients.

CircRNAs are a particular type of non-coding RNAs characterized by a closed ring lacking a 5’ cap and a 3’ end poly (A) features. They regulate gene expression in the eukaryotes via RNA-RNA interaction at post-transcriptional levels (5). Some circRNAs are molecular sponges of their target miRNAs, which can chelate and inhibit miRNAs activity (6). The interaction between circRNAs and miRNAs implicated in numerous processes suggests their critical regulatory roles in these processes (7, 8). In our previous studies, we found that circ-011235 and circ-016901 are significantly up-regulated in the
mice BMSCs follow of TBI irradiation. Bioinformatics analysis, we concluded that circRNA-011235 (circ-011235) can be acted as a miR-741-3p sponge (9). Previous studies showed that miR-741-3p can be used as a biomarker for non-alcoholic fatty liver disease (10) and attention-deficit/hyperactivity disorder (11). However, there are limited studies concerning circ-011235 and miR-741-3p and irradiation-induced BMSCs injury.

The cell cycle is a process tightly regulated by cyclins and cyclin-dependent kinases (CDKs) (12). CDK6, a catalytic subunit of the CDK complex, drives the G1 phase process and G1/S transition of the cell cycle. CDK6 activity first appeared in the mid-G1 phase, which is precisely controlled by many regulatory subunits such as family members of inhibitor of cyclin-dependent kinase 4 (INK4) and D-type cyclins. Cyclin D1 plays an essential regulatory role in the cell cycle and is a key regulatory protein of the G1 phase (13). Cyclin D1 overexpression may lead to an uncontrolled cell proliferation (14). Studies have shown that CDK4 and CDK6 form a complex with cyclin D1 to promote G1-S phase transformation via promoting the phosphorylation of tumor suppressor retinoblastoma (Rb) and formation of CDK2/Cyclin E complex (15).

Here, we attempted to explore the potential role of circ-011235 in the proliferation of BMSC following irradiation injury. And also, we investigated the irradiation-induced self-protection mechanism of circ-011235 in the cell cycle regulation, which may provide novel clinical targets for protecting BMSCs from radiation damage.

Materials and Methods

Ethical consideration

In this experimental study, all animal procedures complied with the guidelines permitted by the Animal Care and Use Committee of XiangYa School of Medicine (No. 2019-S534).

Isolation, culture and identification of bone mesenchymal stem cells

Twenty healthy 2-month-old male BALB/c mice (21 ± 3 g) were provided from the XiangYa School of Medicine of Central South University, China.

Isolation, culture and identification of BMSCs from femurs and tibias were performed as described in our previous study (9). Briefly, after exposure to CO₂, the femurs and tibias of euthanized mice were separated and freshly isolated bone marrow cells were washed and then cultured in the Dulbecco’s Modified Eagle’s medium (DMEM, Cat No. 12634010, ThermoFisher Scientific, Shanghai, China) containing 10% fetal bovine serum (FBS, Cat No. 10099133C, ThermoFisher Scientific, Shanghai, China) and incubated at 37°C in 5% CO₂ for 24 hours. The DMEM was replaced every three days. Using CytoFLEX V2-B2-R0 Flow Cytometer (model No. C09752, Beckman Coulter, Miami, FL, USA), the

cells were sorted with FITC-labeled CD34, CD45, CD90 and Sca-1 after obtaining 80% confluency. Isolated cells that were identified to be CD34(−), CD45(−), CD90(+) and Sca-1(+) were considered as BMSCs and used in subsequent experiments.

Radiation treatment

A cell suspension of BMSCs was prepared by trypsinization with 0.25% trypsin (Cat No. 25300120, ThermoFisher Scientific, Shanghai, China), and the cell density was maintained at 2×10⁶ cells/mL. Subsequently, the BMSCs were exposed to different doses of irradiation (0, 2, 4, 6 Gy) for 6 hours by using 6 MV X-rays of a 137 Cs-γ source (Sangyo Kagaku, Japan) at an irradiation rate of 0.4 Gy/minutes and a distance of 100 cm from the source.

Circ-011235 siRNA interference assay

Small interfering RNA (siRNA) targeting circ-011235 (siRNA-011235) and scrambled siRNA negative control (siNC) were commercially available from KangChen Bio-tech (Shanghai, China). Using the Lipofectamine 2000 kit (Cat No. 11-668-500, Invitrogen, Carlsbad, CA, USA), BMSCs in the logarithmic phase were transfected with siRNA-011235 and siNC, according to the protocols provided by the manufacturer. Briefly, the siRNAs were mixed for 20 min with Lipofectamine 2000 to prepare the Lipofectamine 2000/siRNA complexes. Meanwhile, the BMSCs were incubated with DMEM medium at 37°C for 24 hours in 96-well plates. After that, the medium was refreshed and Lipofectamine 2000/siRNA complexes were added dropwise to the BMSCs while gently shaking the plate. Next, after incubation in 5% CO₂ incubator for 5 hours, the transfection efficiency was examined by Reverse-transcription quantitative polymerase chain reaction (RT-qPCR).

Dual luciferase reporter assay

The potential negative regulatory relationship between circ-011235 and miR-741-3p was found by Bioinformatics analysis. Also, predicted databases, Targetscan and miRBase, were used to identify a binding site between the CDK6 gene and miR-741-3p. The respective fragments of circ-011235 and CDK6 harboring miR-741-3p binding sites, miR-741-3p mimic, and miRNA negative control (mimic control) were synthesized by GenePharma (Shanghai, China). Subsequently, the fragments were subcloned into the psiCHECK-2 Renilla (Cat No. C8021, Promega, Madison, WI, USA) luciferase reporter vector following the manufacturer’s instructions. BMSCs were cotransfected with circ-011235/CDK6 wild type (WT) vectors or circ-011235/CDK6 mutated (MUT) vectors with miR-741-3p mimic via the Lipofectamine 2000 (Cat No. 11-668-500, Invitrogen, Carlsbad, CA, USA) transfection approach. The Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was employed for assessing renilla luciferase activity.
Circ-011235 and CDK6 overexpression assay

Sequences of circ-011235 and CDK6 gene were synthesized by KangChen Bio-tech (Shanghai, China) and cloned into the expression vector pcDNA3.1 (Cat No. V79020, Invitrogen, Carlsbad, CA, USA) to generate the overexpression recombinant plasmids pcDNA-circ-011235 and pcDNA-CDK6, and the empty vector pcDNA3.1 was used as a negative control. The recombinant plasmids were identified by RT-qPCR and DNA sequencing. Then, recombinant plasmids were transfected into the logarithmic phase BMSCs by Lipofectamine 2000 kit (Cat No. 11-668-500, Invitrogen, Carlsbad, CA, USA) based on the manufacturer’s protocol. RT-qPCR was performed to detect the transfection efficiency 48 hours after transfection.

Cell proliferation assay

Following of 6 GY of irradiation treatment, transfected cells were cultured in 96-well plates and the cell concentration was maintained at 5\times10^5 cells/well. The cell proliferation at 12, 24, 36 and 48 hours after seeding was evaluated using the CCK8 assay (Cat No. GK10001, Gpbio, Montclair, CA, USA). In brief, we added 10 μL CCK8 solution to each sample and incubated for 2 hours. Subsequently, the absorbance was detected by the spectrophotometric approach at 450 nm wavelength.

Cell cycle analysis

Twenty four hours after transfection, BMSCs were harvested by centrifugation at 1200 rpm for 5 minutes. The number of BMSCs was calculated using a haemocytometer, and the final density of cells was maintained at 1\times10^5 cells/mL with phosphate buffer saline (PBS, Cat No. C791P76, Thomas Scientific, Swedesboro, NJ, USA). Subsequently, the cells were fixed with 70% ethanol (3 mL) at 4˚C overnight, and collected by centrifugation at 1200 rpm for 5 minutes. Next, 200 μL of RNase (Cat No. AM2288, ThermoFisher Scientific, Shanghai, China) was added, followed by incubation at 37˚C for 30 minutes. Afterwards, 800 μL of propidium iodide (PI, 20 mg/mL, Cat No. P1304MP, ThermoFisher Scientific, Shanghai, China) was added, followed by incubation for 30 minutes at 25˚C in the dark. The distribution of cells was detected by flow cytometry (Beckman, Los Angeles, CA, USA).

Real time-quantitative polymerase chain reaction

After transfection, total RNA of BMSCs was extracted by TRIzol Reagent (Cat No. 15596018, ThermoFisher Scientific, Shanghai, China), based on the manufacturer’s directives. The isolated total RNA (20 μg/μL) was employed to synthesize the cDNA by reverse transcription using the GoScript reverse transcriptase (Promega, Charbonnières-les-Bains, France). For circ-011235, the RNase R digestion reaction was performed at a ratio of 3U enzyme/1μg RNA before reverse transcription. The cDNA library was amplified using the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The reaction was started at 95˚C for 2 minutes and then subjected to 40 cycles of 95˚C for 10 seconds and 60˚C for 60 seconds. The primer sequences were synthetically produced by KangChen Bio-tech (Shanghai, China). The primer sequences were displayed in Table 1. The 2^(-ΔΔCt) formula was applied to measure the relative expression of circ-011235, miR-741-3p, CDK6 and CDK4 (16).

| Gene         | Primer sequence (5’-3’)       |
|--------------|-------------------------------|
| GADPH        | F: CACTGAGCAAGAGAGGCCCTAT     |
|              | R: GCAGCGAAGTTTATGTGATTTT    |
| circ-011235  | F: AACAAGGACCGAATAAGAGG       |
|              | R: TGTATCCAGCAGAATCTCCC       |
| miR-741-3p   | F: TGGATGCCACGCTATGTGATAG    |
|              | R: GCGACGAGAAAGACTTGTTT       |
| CDK6         | F: GCTGACCAGCGATGAAATG       |
|              | R: GCACACATACAAACACTGACC      |
| cyclin D1    | F: TTTCGAGGGCTCTAAGATGAAG    |
|              | R: AGGTTCCCAGAGCTGTTG         |
| CDK4         | F: TTGGATCTGGTCACGAGATC       |
|              | R: CTGGTACGCTGTGATAGTTCCGCA  |

Protein extraction, purification and western blotting

Radio-immunoprecipitation assay (RIPA) lysis buffer (Cat No. 20-188, Merck, Shanghai, China) was used to extract the total cellular RNA while this contains an inhibitor cocktail mixture of phosphatase and protease (Cat No. ab201119, Abcam, Waltham, MA, USA). Next, protein concentration of the collected supernatant was measured by Pierce BCA assay kit (Cat No. 23225, ThermoFisher Scientific, Rockford, IL, USA). The extracted proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Cat No. IPVH00010, Merck, Shanghai, China) after purification on 10% SDS-PAGE (Cat No. MT-46040CI, Fisher Scientific, Loughborough, USA), and then, 5% skim milk (Cat No. 100518-0201, Medallion Milk Co., Winnipeg, MB, USA) was used to block the membranes. Next, the membranes were reacted with our primary antibodies at 4˚C overnight. Our primary antibodies were included: CDK4 (1:1000; Cat No. 12790, Cell Signaling Technology, Beverly, MA, USA), cyclin D1 (1:1000; Cat No. 55506, Cell Signaling Technology, Beverly, MA, USA) and GAPDH (1:2000; Cat No. 12790, Cell Signaling Technology, Beverly, MA, USA). Then, the membranes were incubated at 37˚C for 60 minutes with the HRP-labeled secondary antibody (1:2000; Cat No. 7074, Cell Signaling Technology, Beverly, MA, USA). Subsequently, a chemiluminescence imaging system (model No. GeneGnome XQR, Syngene, Cambridge, UK) was used to examine the immunoblot signals of the target proteins, and GAPDH was chosen as a housekeeping endogenous.
control for these proteins. The analysis of protein bands was performed using the software Image J (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

The results were analysed by SPSS 28.0.1 software (IBM, Armonk, NY, USA). All data were presented as mean ± standard deviation (SD). All data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by Turkey’s Post Hoc multiple comparison test to detect the differences among the groups. Pearson correlation analysis was achieved using the R package Hmisc. Also, P<0.05 was considered for statistical significance.

Results

Irradiation increased the expression of circ-011235 and CDK6

To examine the relationship between the expression of circ-011235, miR-741-3p and CDK6, the correlation of their expression levels in the BMSCs treated with irradiation was analyzed. It was found that the irradiation treatment increased the expression of circ-011235 and CDK6 in the BMSCs in comparison with the control group (0 GY) (P<0.01, Fig.1A, B) but decreased the expression of miR-741-3p (P<0.01, Fig.1C); there was a significant dose-dependent effect (2 GY<4 GY<6 GY) in the irradiation treated BMSCs. The Pearson correlation analysis found a strong correlation between circ-011235 and CDK6 (r=0.99, P<0.05, Fig.1D). Additionally, miR-741-3p expression was negatively interrelated with the expression levels of circ-011235 (r=-0.98, P<0.05) and CDK6 (r=-0.97, P<0.05, Fig.1D).

Circ-011235 regulated miR-741-3p/CDK6 pathway in irradiation-treated bone mesenchymal stem cells

Using online databases, IntaRNA, TargetScan, and miRBase, we observed that miR-741-3p have a potential binding site for circ-011235 (Fig.2A), and a unique binding site with 7 base pairs between 3’-UTR of CDK6 gene and miR-741-3p (Fig.2B). The results showed that circ-011235 siRNA significantly up-regulated the expression of miR-741-3p, but down-regulated the expression of CDK6 (P<0.01, Fig.2C, D). The transfection efficiency of miR-741-3p overexpression was examined using RT-qPCR, and the results indicated that miR-741-3p overexpression significantly upregulated the expression of miR-741-3p (P<0.01, Fig.2E). In addition, miR-741-3p overexpression significantly decreased luciferase activity in the WT-circ-011235 and WT-CDK6 transfected cells (P<0.01) but there was no significant effect on luciferase activity in the MUT-circ-011235 and MUT-CDK6 transfected cells (P>0.05, Fig.2F, G).

Overexpression of circ-011235 and CDK6 increased the irradiation-treated bone mesenchymal stem cells proliferation

To investigate the transfection efficiency of pcDNA-circ-011235 and pcDNA-CDK6, we detected the expression levels of circ-011235 and CDK6 in irradiation-treated bone mesenchymal stem cells.
BMSCs. The results showed that pcDNA-circ-011235 significantly up-regulated circ-011235 in comparison with the control group (P<0.01, Fig.3A), and pcDNA-CDK6 treatment led to the similar results for the CDK6 cell (Fig.3B). Furthermore, we also examined the overexpression effect of circ-011235 and CDK6 on the proliferation after exposure to 6 Gy irradiation. It was found that irradiation treatment hindered the proliferation of BMSCs compared with the control group (P<0.01); however, overexpression of circ-011235 or CDK6 significantly reversed this inhibitory effect after 24 hours seeding (Fig.3C).

Overexpression of circ-011235 and CDK6 affected the cell cycle of irradiation-treated bone mesenchymal stem cells

To investigate the overexpression effect of circ-011235 and CDK6 on cell proliferation, cell cycle analysis was performed. When compared with the control group, the results showed that irradiation treatment significantly increased the percentage of cells in the G1 phase (P<0.01), while the proportion of cells in the S phase was significantly declined (P<0.01, Fig.4A). However, compared with the irradiation-treated group, circ-011235 overexpression significantly reduced the percentage of cells in the G1 phase and elevated the percentage of cells in the S phase (P<0.01, Fig.4A). Similar results were found with the CDK6 overexpression.

Also, the expression analysis of cyclin D1 showed that cyclin D1 was significantly up-regulated in the circ-011235 overexpression group and CDK6 overexpression group compared to the irradiation-treated group (P<0.01), while there was no significant change in CDK4 expression (Fig.4B-D).

![Fig.3](image1) Effect of circ-011235 and CDK6 on the proliferation of irradiation-treated BMSCs. A. RT-qPCR detection of transfection efficiency of pcDNA-circ-011235. B. RT-qPCR detection of transfection efficiency of pcDNA-CDK6. C. CCK-8 assay detection of the effects of pcDNA-circ-011235 and pcDNA-CDK6 on the proliferation of irradiation-treated BMSCs. Values were represented by the mean ± SD. Separated experiments were repeated three times. **; P<0.01, vs. the control group, #; P<0.05, ##; P<0.01, vs. the irradiation treatment group and control vector group, RT-PCR; Real time-quantitative polymerase chain reaction, BMSCs; Bone mesenchymal stem cells, and h; Hour.

![Fig.4](image2) Effects of circ-011235 and CDK6 on the cell cycle in the irradiation-treated BMSCs. A. Flow cytometry analysis of the effects of pcDNA-circ-011235 and pcDNA-CDK6 on the cell cycle in the irradiation-treated BMSCs. B. RT-qPCR detection of the expression of cyclin D1 and CDK4 at the gene level. C. Western blot detection of the expression of cyclin D1 and CDK4 at the protein levels. D. Densitometry analysis of western blot bands of the expression of cyclin D1 and CDK4. Data were represented by mean ± SD. Separate experiments were replicated three times, **; P<0.01, vs. the control group, ##; P<0.01, vs. the irradiation treatment group and control vector group, RT-PCR; Real time-quantitative polymerase chain reaction, and BMSCs; Bone mesenchymal stem cells.

**Discussion**

HSC transplantation has been achieved a huge success in the treatment of blood system diseases and is currently known as a most effective cell replacement therapy. TBI is one of the necessary pretreatments for HSC transplantation (17). Whether the injury of TBI to BMSCs affects the hematopoietic function of HSCs. Although, its regulatory mechanism is not fully known, rational treatment plans will direct to high efficiency and low toxicity in the clinical phase.

The hematopoietic support of BMSCs mainly regulates the survival, self-renewal, migration and differentiation of hematopoietic stem or progenitor cells through intercellular interactions and secretion of growth factors, chemical factors, and extracellular matrix (18). Co-infusion of HSCs and MSCs heterogeneity have been observed to promote hematopoietic reconstruction (19). BMSCs with low immunogenicity and immunomodulation can avoid and alleviate host immune responses, induce the formation of specific immune tolerance, and promote the transplantation of HSCs. It repairs tissue injury
caused by pre-transplant pretreatment, which can reduce the incidence of severe GVHD and transplant-related mortality (20). Compared with HSCs, BMSCs are highly resistant to irradiation and can survive acute exposure (21), which is an important hematopoietic support cell for hematopoietic recovery after irradiation. The study of irradiation-induced injury in the BMSCs has significant implications to improve the HSCs transplantation survival rate. In the present study, the expression of circ-011235, miR-741-3p, and CDK6 was dose-dependent after irradiation for 6 h, indicating that irradiation can change the molecular profile of BMSCs.

Studies have shown that non-coding RNAs such as circRNAs and miRNAs act as gene expression regulators and have been confirmed to be involved in the regulation of the cell proliferation such as cancer cells (22). CircRNA contains miRNA response elements, which act as a competitive endogenous RNA to compete binding site between miRNA and its target gene, thereby may be acted as a eliminating agent against the inhibitory effect of miRNA on their target gene (23). In this study, our results showed that circ-011235 increases the cell proliferation and progresses cell cycle of irradiation-treated BMSCs by down-regulating miR-741-3p expression and up-regulating CDK6 expression. This suggests that circ-011235 derepresses CDK6 gene by acting as a sponge of miR-741-3p to counteract the irradiation-induced damage of BMSCs. This is the first study to explore the role and function of circ-011235 and miR-741-3p in the irradiation-induced BMSCs injury.

As an oncogene, CDK6 can promote the cell proliferation and play a regulatory role in the occurrence and development of various cancers, such as bladder cancer, glioma, and medulloblastoma (24). Cyclin D-associated kinases inhibitors such as CDK4 and CDK6 can be used as a potential cancer therapeutic targets. The function and regulation mechanism of CDK6 in the BMSCs are still not clearly explained. CDK6, together with CDK4, acts as a switching signal in the G1 phase that, directing cells towards the S phase (25). CDK6 is an important driving factor in the shift of the cell cycle from G1 to S stage. However, a previous study showed that the cell cycle is regulated by complex regulatory pathways, and CDK6 is not necessary for the proliferation of every cell type (26). In addition, CDK4 or CDK2 are protein kinases that compensate the effects of CDK6. Moreover, CDK6 is primarily associated with cyclins proteins such as cyclins D1, D2, and D3 (27). The positive activation of CDK6 can be achieved by phosphorylation of the 177th conserved threonine residue by CDK activated kinase (CAK) (28). In addition, Kaposi’s sarcoma-associated herpesvirus can phosphorylate and overactive CDK6, and cause uncontrolled cell proliferation (29). In the present study, our results demonstrated that circ-011235 and CDK6 were activated by irradiation and their expression associated with a dose-dependent increase effect. Similarly, a previous study in mice found that the cell viability was reduced by ultraviolet light C (UVC) treatment, but loss of Runx2 could counteract UVC induced cell death by increasing the expression of cyclins and related CDK activities (30). Moreover, Zou et al. (31) showed that the Cell Division Cycle 25A (CDC25A), an activator of G1 CDKs in the nucleus, could inhibit the oxidant-triggered gamma irradiation induced apoptosis via diminishing the activation of the oxidative stress kinase cascades. Furthermore, the complex of cyclin D1 with CDK4 and CDK6 is involved in the regulation of the cell cycle, which phosphorylates the Rb protein, thereby promoting the cell cycle from G1 to S stage (32). Previous studies showed that CDK6 activity elevates in the cultured mouse astrocytes without alteration of CDK4 activity (33). Also, we found that overexpressions of circ-011235 and CDK6 both promoted the proliferation and cell cycle through increasing the expression of cyclin D1 in the irradiation treated BMSCs, which suggested its vital role in the self-protection mechanism of BMSCs in response to irradiation. We did not same results in the overexpression of CDK4.

Finally, our study presents some limitations. In cell cycle analysis, only cyclin D1 and CDK-4 protein and mRNA expression were detected, and further cyclins such as cyclin E, CDK2, and p27 kipl are required to increase the confidence of our findings. Moreover, animal experiments are needed to explore the potential role of circ-011235, miR-741-3p, and CDK6. However, further measurement methods are still needed to confirm our findings.

**Conclusion**

In this study, we uncovered the regulatory function of circ-011235 on the cell cycle in the irradiation-treated BMSCs. Our results showed that circ-011235 increases the irradiation-treated BMSCs proliferation and promotes cell cycle progression through down-regulating miR-741-3p and up-regulating CDK6. Also, Circ-011235 and CDK6 overexpression could effectively reverse the inhibitory effect of irradiation on the proliferation and cell cycle arrest of the BMSCs through promoting the expression of cyclin D1. This is the first study to demonstrate the protective role of circ-011235/miR-741-3p/CDK6 axis, especially circ-011235 and miR-741-3p, against irradiation-induced damage of BMSCs. The circ-011235/miR-741-3p/CDK6 axis may be a probable therapeutic target in the clinical application of TBI-induced BMSCs injury.

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Authors’ Contributions

X.W., H.X., R.G., X.N., D.S., R.H., H.D.; Experiment performance, data collection and interpretation. X.W., H.X., R.G., X.N., D.S., R.H., H.D., J.Z.; Study design, data collection, evaluation, participation in manuscript preparation, data analysis and manuscript drafting. All authors read and approved the final manuscript.

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