Original Article

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

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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 followingirradiation 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 CO_2, 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% CO_2 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 2×10^6 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% CO_2 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 5×10^3 cells/well. The cell proliferation at 12, 24, 36 and 48 hours after seeding was evaluated using the CCK8 assay (Cat No. GK10001, Glpbio, 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 1×10^6 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 ng/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 $2^{-\Delta\Delta Ct}$ formula was applied to measure the relative expression of *circ-011235*, *miR-741-3p*, *CDK6* and *CDK4* (16).

Table 1: The primer sequences used in this study

Gene	Primer sequence (5´-3´)
GADPH	F: CACTGAGCAAGAGAGGCCCTAT
	R: GCAGCGAACTTTATTGATGGTATT
circ-011235	F: AACAAGGACCGAAACTAAGAGG
	R: TGTATCCACCAGAATTACTCCC
miR-741-3p	F: TGGATGCCACGCTATGTAGAT
	R: GCGACGAGCAAAAAGCTTGT
CDK6	F: GCTGACCAGCAGTAC GAATG
	R: GCACACATCAAACAACCTGACC
cyclin D1	F: TGTTCGTGGCCTCTAAGATGAAG
	R: AGGTTCCACTTGAGCTT GTTCAC
CDK4	F: TTGCATCGTTCACCGAGATC
	R: CTGGTAGCTGTAGATTCTGGCCA

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. 5174, Cell Signaling Technology, Beverly, MA, USA). Then, the membranes were incubated at 37˚C for 60 min 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 XRQ, 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).

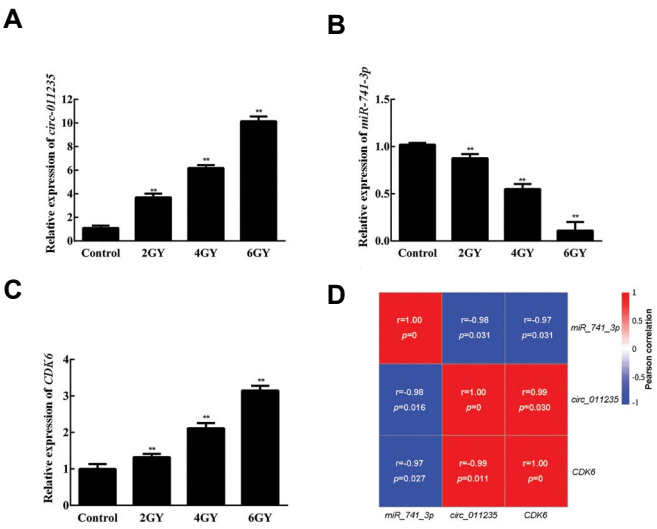

Fig.1: RT-qPCR detection of gene expression in the BMSCs after irradiation treatment with different doses (0, 2, 4, 6 GY). **A.** Relative expression of *circ-011235*. **B.** Relative expression of *miR-741-3p*. **C.** Relative mRNA expression of *CDK6* in the BMSCs. **D.** Pearson correlation analysis of the correlation among *circ-011235*, *miR-741-3p*, and *CDK6* in the BMSCs after irradiation treatment. Data were represented by mean ± SD. Independent experiments were replicated three times, **; P<0.01, vs. the control group, RT-PCR; Real time-quantitative polymerase chain reaction, and BMSCs; Bone mesenchymal stem cells.

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* mimic 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).

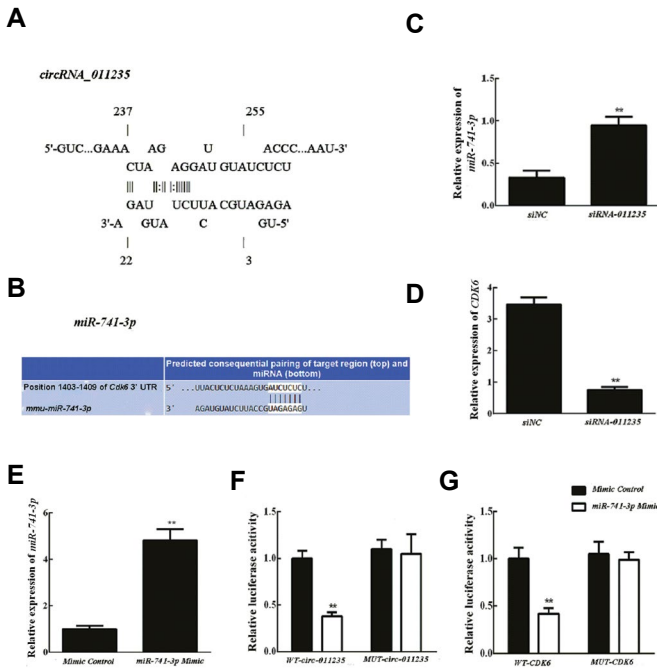

Fig.2: Circ-011235/miR-741-3p/CDK6 signal pathway. **A.** IntaRNA prediction of *miR-741-3p* as a target for *circ-011235*. **B.** TargetScan prediction of 3′-UTR of *CDK6* gene as a target for *miR-741-3p*. **C.** *Circ-011235* silencing upregulates the *miR-741-3p* expression in the BMSCs. **D.** *Circ-011235* silencing decreases the *CDK6* expression in the BMSCs. **E.** Evaluation by RT-qPCR of the transfection efficiency of *miR-741-3p* mimic. **F.** Luciferase reporter assay illustrating the interactions between *circ-011235* and *miR-741-3p.* **G.** Luciferase reporter assay illustrating the interactions between *CDK6* and *miR-741-3p* in the BMSCs. Data were represented by mean ± SD. Independent experiments were replicated three times, **; P<0.01, vs. the control group, RT-PCR; Real time-quantitative polymerase chain reaction, and BMSCs; Bone mesenchymal stem cells.

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

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).

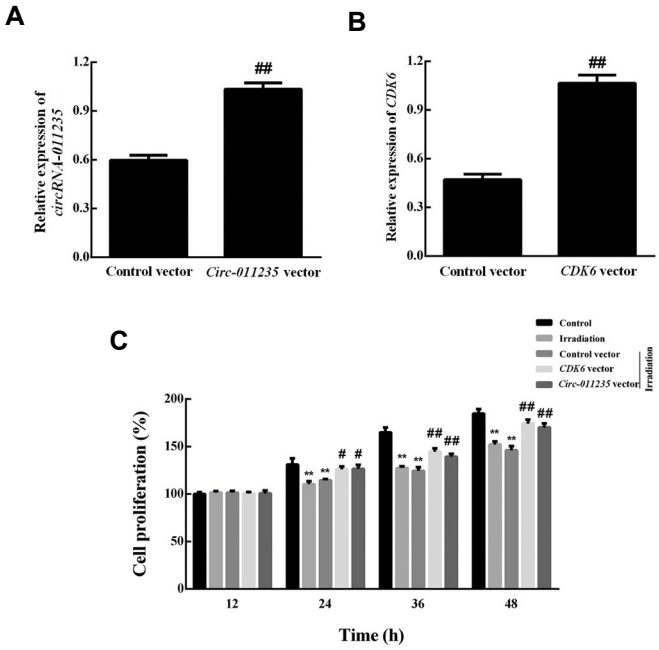

Fig.3: Effect of *circ-011235* and *CDK6* on the proliferation of irradiation-treated BMSCs. **A.** RT-qPCR detection of transfection efficiency of *pcDNA-circ-011235*. **B.** RT-qPCR detection of transfection efficiency of *pcDNA-CDK6*. **C.** CCK-8 assay detection of the effects of *pcDNA-circ-011235* 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.

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).

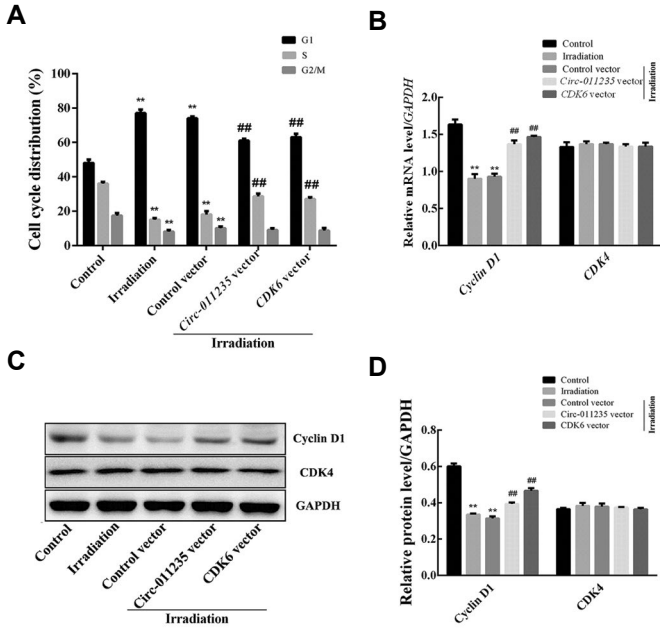

Fig.4: Effects of *circ-011235* and *CDK6* on the cell cycle in the irradiation-treated BMSCs. **A.** Flow cytometry analysis of the effects of *pcDNA-circ-011235* and *pcDNA-CDK6* on the cell cycle in the irradiation-treated BMSCs. **B.** RT-qPCR detection of the expression of *cyclin D1* and *CDK4* 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 CDK4 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.

References

1. Borel C, Ahmad I, Ceballos P, Desbrosses Y, Hamzy F, Ravinet A, et al. Hematopoietic stem cell transplantation ocular complications: guidelines from the Francophone Society of Bone Marrow Transplantation and Cellular Therapy (SFGM-TC). Bull Cancer. 2020; 107(12S): S116-S121.
2. Gieger TL, Nolan MW, Roback DM, Suter SE. Implementation of total body photon irradiation as part of an institutional bone marrow transplant program for the treatment of canine lymphoma and leukemias. Vet Radiol Ultrasound. 2019; 60(5): 586-593.
3. Wang Y, Zhang J, Li J, Gui R, Nie X, Huang R. CircRNA_014511 affects the radiosensitivity of bone marrow mesenchymal stem cells by binding to miR-29b-2-5p. Bosn J Basic Med Sci. 2019; 19(2): 155-163.
4. Bianchi ME, Crippa MP, Manfredi AA, Mezzapelle R, Rovere Querini P, Venereau E. High-mobility group box 1 protein orchestrates responses to tissue damage via inflammation, innate and adaptive immunity, and tissue repair. Immunol Rev. 2017; 280(1): 74-82.
5. Chan JJ, Tay Y. Noncoding RNA:RNA regulatory networks in cancer. Int J Mol Sci. 2018; 19(5) :1310.
6. Xiong DD, Dang YW, Lin P, Wen DY, He RQ, Luo DZ, et al. A circRNA-miRNA-mRNA network identification for exploring underlying pathogenesis and therapy strategy of hepatocellular carcinoma. J Transl Med. 2018; 16(1): 220.
7. Lin X, Chen Y. Identification of potentially functional circrna-mirna-mrna regulatory network in hepatocellular carcinoma by integrated microarray analysis. Med Sci Monit Basic Res. 2018; 24: 70-78.
8. Zhang Q, Zhang W, Nogales-Cadenas R, Lin JR, Cai Y, Zhang ZD. From gene expression to disease phenotypes: network-based approaches to study complex human diseases. Dordrecht: Springer; 2016.
9. Zhang J, Jiang J, Huang R, Wang Y, Nie X, Gui R. Circular RNA expression profiles are significantly altered in mice bone marrow stromal cells after total body irradiation. Leuk Res. 2018; 70: 67-73.
10. Nie J, Li CP, Li JH, Chen X, Zhong X. Analysis of nonalcoholic fatty liver disease microRNA expression spectra in rat liver tissues. Mol Med Rep. 2018; 18(3): 2669-2680.
11. Tian T, Zhang Y, Wu T, Yang L, Chen C, Li N, et al. miRNA profiling in the hippocampus of attention-deficit/hyperactivity disorder rats. J Cell Biochem. 2019; 120(3): 3621-3629.
12. Pack LR, Daigh LH, Meyer T. Putting the brakes on the cell cycle: mechanisms of cellular growth arrest. Curr Opin Cell Biol. 2019; 60: 106-113.
13. Dong P, Zhang C, Parker BT, You L, Mathey-Prevot B. Cyclin D/CDK4/6 activity controls G1 length in mammalian cells. PLoS One. 2018; 13(1): e0185637.
14. Fu X, Feng Y. QKI-5 suppresses cyclin D1 expression and proliferation of oral squamous cell carcinoma cells via MAPK signalling pathway. Int J Oral Maxillofac Surg. 2015; 44(5): 562-567.
15. VanArsdale T, Boshoff C, Arndt KT, Abraham RT. Molecular pathways: targeting the cyclin D-CDK4/6 axis for cancer treatment. Clin Cancer Res. 2015; 21(13): 2905-2910.
16. Said R, Garcia-Mayea Y, Trabelsi N, Setti Boubaker N, Mir C, Blel A, et al. Expression patterns and bioinformatic analysis of miR-1260a and miR-1274a in prostate cancer tunisian patients. Mol Biol Rep. 2018; 45(6): 2345-2358.
17. Davis NL, Stewart CE, Moss AD, Woltersdorf WW, Hunt LP, Elson RA, et al. Growth hormone deficiency after childhood bone marrow transplantation with total body irradiation: interaction with adiposity and age. Clin Endocrinol (Oxf). 2015; 83(4): 508-517.
18. Lin L, Lin H, Bai S, Zheng L, Zhang X. Bone marrow mesenchymal stem cells (BMSCs) improved functional recovery of spinal cord injury partly by promoting axonal regeneration. Neurochem Int. 2018; 115: 80-84.
19. Mohammadi S, Nikbakht M, Sajjadi SM, Rad F, Chahardouli B, Sabour Takanlu J, et al. Reciprocal interactions of leukemic cells with bone marrow stromal cells promote enrichment of leukemic stem cell compartments in response to curcumin and daunorubicin. Asian Pac J Cancer Prev. 2017; 18(3): 831-840.
20. von der Heide EK, Neumann M, Vosberg S, James AR, Schroeder MP, Ortiz-Tanchez J, et al. Molecular alterations in bone marrow mesenchymal stromal cells derived from acute myeloid leukemia patients. Leukemia. 2017; 31(5): 1069-1078.
21. Xiang Y, Wu C, Wu J, Quan W, Cheng C, Zhou J, et al. In vitro expansion affects the response of human bone marrow stromal cells to irradiation. Stem Cell Res Ther. 2019; 10(1): 82.
22. Kristensen LS, Hansen TB, Venø MT, Kjems J. Circular RNAs in cancer: opportunities and challenges in the field. Oncogene. 2018; 37(5):555-565.
23. Zhong Y, Du Y, Yang X, Mo Y, Fan C, Xiong F, et al. Circular RNAs function as ceRNAs to regulate and control human cancer progression. Mol Cancer. 2018; 17(1): 79.
24. Li X, Gong X, Chen J, Zhang J, Sun J, Guo M. miR-340 inhibits glioblastoma cell proliferation by suppressing CDK6, cyclin-D1 and cyclin-D2. Biochem Biophys Res Commun. 2015; 460(3): 670-677.
25. Molina A, Pituello F. Playing with the cell cycle to build the spinal cord. Dev Biol. 2017; 432(1):14-23.
26. Satyanarayana A, Kaldis P. Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. Oncogene. 2009; 28(33): 2925-2939.
27. Malumbres M, Sotillo R, Santamaría D, Galán J, Cerezo A, Ortega S, et al. Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. Cell. 2004; 118(4): 493-504.
28. He H, Xu J, Xie W, Guo QL, Jiang FL, Liu Y. Reduced state transition barrier of CDK6 from open to closed state induced by Thr177 phosphorylation and its implication in binding modes of inhibitors. Biochim Biophys Acta Gen Subj. 2018; 1862(3): 501-512.
29. Kaldis P. The N-terminal peptide of the Kaposi's sarcoma-associated herpesvirus (KSHV)-cyclin determines substrate specificity. J Biol Chem. 2005; 280(12): 11165-11174.
30. Kilbey A, Blyth K, Wotton S, Terry A, Jenkins A, Bell M, et al. Runx2 disruption promotes immortalization and confers resistance to oncogene-induced senescence in primary murine fibroblasts. Cancer Res. 2007; 67(23): 11263-11271.
31. Zou X, Tsutsui T, Ray D, Blomquist JF, Ichijo H, Ucker DS, et al. The cell cycle-regulatory CDC25A phosphatase inhibits apoptosis signal-regulating kinase 1. Mol Cell Biol. 2001; 21(14): 4818-4828.
32. Romero-Pozuelo J, Figlia G, Kaya O, Martin-Villalba A, Teleman AA. Cdk4 and Cdk6 couple the cell-cycle machinery to cell growth via mTORC1. Cell Rep. 2020; 31(2): 107504.
33. Sammons SL, Topping DL, Blackwell KL. HR+, HER2- advanced breast cancer and CDK4/6 inhibitors: mode of action, clinical activity, and safety profiles. Curr Cancer Drug Targets. 2017; 17(7): 637-649.