Apoptosis Repressor with Caspase Domain (ARC) Promotes Bone Regeneration of Bone Marrow Derived Mesenchymal Stem Cells via Activating Fgf-2/PI3K/Akt Signaling

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Research

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Abstract

Objectives:

This study aims to investigate whether ARC could promote survival and enhance osteogenic differentiation of bone marrow derived mesenchymal stem cells (BMSCs).

Material and methods:

Lentivirus transfection method was used to establish ARC overexpressed BMSCs. CCK-8 method was used to detect cell proliferation. The BD Pharmingen™ APC Annexin V Apoptosis Detection kit was used to detect cell apoptosis. The osteogenic capacity was investigated by OCN immunofluorescence staining, ALP, ARS assay and RT-PCR analysis. Cells were seeded into CPC scaffolds, then inserted into subcutaneous of nude mice and the defect area of rat’s calvarium. Histological analysis was conducted to evaluate in vivo cell apoptosis and new bone formation ability of ARC overexpressed BMSCs. RNA-seq method was used to detect the possible mechanism of the effect of ARC on BMSCs.

Results:

ARC can promote BMSCs proliferation and inhibit its cell apoptosis. ARC can enhance BMSCs osteogenic differentiation in vitro. In vivo study revealed ARC can inhibit BMSCs’ apoptosis and increase its new bone formation ability. ARC regulates BMSCs mainly by activating Fgf-2/PI3K/Akt pathway.

Conclusions:

The present study suggested that ARC is a powerful agent to promote bone regeneration of BMSCs and provides a promising method for bone tissue engineering.

1. Introduction

Bone defect caused by tumor, trauma, inflammation or congenital deformity greatly impact patients’ quality of life. Autogenous bone graft is considered as gold standard for treatment of these defects. However, this is limited by donor site morbidity, bone volume insufficiency and incomplete integration into the defect. Bone tissue engineering strategy can provide regenerative tissues to repair bone defect without the aforementioned limitations. In these approaches, bone-forming cells are often used combined with biomaterial scaffold and growth factors. Bone marrow derived mesenchymal stem cells (BMSCs) have powerful proliferation ability and multi-lineage differentiation potential, they are easily available and are crucial for bone remodeling and repair. It has already been proved that BMSCs can enhance bone repair successfully and thus are widely used in bone tissue engineering. BMSCs can be seeded into biomaterial scaffold along with incorporated growth factors or genes, hence promoting their differentiation into osteoblasts that are capable of producing new bone matrix. Nevertheless, these implanted cells encounter hypoxia or even anoxia within the implant after they were implanted into the
defect area, which can lead to low cell survival and may even cause failure of the implant⁶,⁷. Hence, if we focus on exploring an agent to both increase cells’ ability to against hypoxia induced apoptosis and to promote its osteogenic differentiation may be a promising method to the success of these regenerative implant.

Apoptosis repressor with caspase recruitment domain (ARC) also terms NOL3, is a highly potent and multifunctional inhibitor of apoptosis⁸. It is physiologically expressed in terminally differentiated cells, including cardiomyocytes, neurons and skeletal muscle cells⁹. ARC can inhibit both intrinsic and extrinsic apoptosis and are able to protect cells from hypoxia induced cell apoptosis and death¹⁰,¹¹. It was reported cardiomyocytes isolated from neonatal ARC transgenic mice were significantly resistant to hypoxia and ARC can protect cardiomyocytes from hypoxic induced cell death by regulating its downstream, Drbp1 and pMe(2)GlyDH¹². It was also found that down-regulation of ARC protein in hippocampal neurons may eventually lead to hypoxia induced neuronal death¹³. Apart from the ability to inhibit hypoxic induced cell death, our previous study also had confirmed that ARC has the ability to promote human osteoblast osteogenic differentiation¹⁴.

Thus, ARC may be a powerful agent to enhance new bone formation in vivo after transduced into BMSCs considering its ability to prevent hypoxia induced cell death and the potential to promote osteogenic differentiation of osteoblast cells. In this study, the effects of ARC on BMSCs were detected. We established ARC overexpressed BMSCs and detect whether ARC could inhibit BMSCs’ apoptosis and enhance its osteogenic differentiation in vitro and evaluate whether ARC could promote new bone formation of BMSCs in vivo. And then further to explore its possible mechanism.

2. Methods

2.1 BMSCs isolation and culture

BMSCs from rat were isolated and cultured according to previously described protocols¹⁵. Briefly, the femurs and tibias were blunt dissected aseptically after the rats were sacrificed by cervical dislocation. The bone marrow within was then flushed quickly with DMEM which contained 10% fetal bovine serum (FBS, Hyclone, Logan UT, USA), 100 U/mL penicillin, 100 U/mL streptomycin and 200 U/mL heparin (Sigma, St Louis, MO, USA). The primary cells were re-suspended with DMEM after centrifugated at 2000 rpm for 10 min and then cultured at 37 °C in a humidified atmosphere of 5% CO2. Twenty-four hours later, the non-adherent cells were removed. The DMEM was refreshed every 3 days and the cells at passage 2 or 3 were used for future experiment. This study was conducted under the approval of the Animal Research Ethical Committee of the Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

2.2 lentiviral packaging and BMSCs transduction
Human embryonic kidney 293T cells (HEK293T) were used for lentiviral packing. The cells were cultured in 10 cm dishes for 2–3 days until it reaches 90–95% confluence. Recombinant virus plasmid pLV-nol3-EGFP which encodes whole length of nol3 and control vectors pLV-EGFP, together with packaging plasmids (pLP1, pLP2 and pLP/VSVG), were cotransfected into HEK293T cells using Lipofectamine™ 2000 (all from Genechem Co., Ltd., Shanghai, China). Subsequent to 48 h of transduction, the lentiviral particles contained in the supernatant of 293T cells were harvested and then concentrated by passing through a 0.45 µm filter. The concentrated solutions were then added into cultured BMSCs at a variety of multiplicity of infections ranging from 0100. After 72 h, the transduction efficiency was assessed via fluorescence microscopy and qRT-PCR.

2.3 Cell proliferation detection

The cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used to detect cell proliferation. Cells were seeded on 96-well plates at a density of $1.5 \times 10^3$ cells/well and the proliferation ability was detected for consecutive 72 hours. 10 µl cck-8 reagent with 100 µl DMEM were added into each well and then incubated at 37°C for 2 hours. The absorbance was then measured with microplate reader (Bio-Tek, Winooski, VT, USA) at the wavelength of 490 nm according to manufacturer's instruction.

2.4 Cell apoptosis detection

Cells were seeded on 6-well plates at a density of $1 \times 10^5$ cells/well and then cultured for 72 h. The supernatant and adherent cells were collected and then centrifuged at 1500 rpm for 10 min at 4°C. To detect cell apoptosis, the BD Pharmingen™ APC Annexin V Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) was used according to the manufacturer's protocol. Briefly, cells were washed with cold PBS for twice and re-suspended in 500µL binding buffer. Then 5µL Annexin V-APC solution was added into the solution and incubated in the dark for 15 min at 37°C. Finally, 1µL propidium iodide (PI) solution was added for the subsequent flow cytometry cell apoptosis analysis.

2.5 Immunofluorescence staining

Cells were seeded at a density of $2.5 \times 10^4$ cells/well on 24well plates. After corresponding treatment, cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at 4°C and then were permeabilized with 0.5% PBST (PBS containing 0.5% Triton X-100). Following that, cells were blocked with 5% bovine serum albumin for 1 hour at room temperature and then incubated with anti-OCN antibodies for 1 hour at 37°C. Cells were washed twice with PBST and incubated with secondary antibody for 1 hour at room temperature. After washing again twice with PBST, the nuclei were stained with DAPI (Invitrogen) for 5 min. Photographs were visualized under a light microscope (Olympus Corporation, Tokyo, Japan).

2.6 Alkaline phosphatase activity (ALP) detection and Alizarin red staining (ARS)
Cells were seeded at a density of $2.5 \times 10^4$ cells/well on 24well plates and were cultured for 7 days. For ALP staining, cells were washed twice with PBS and fixed with paraformaldehyde for 15 min at 4°C. Then paraformaldehyde was removed and cells were washed again twice with PBS and then the BCIP/NBT solution were added into each well and were incubated in the dark for 30 minutes. All the procedures were according to the protocols of ALP staining kit (Beyotime Institute of Biotechnology, Shanghai, China). For ALP activity detection, the BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used and the ALP levels were normalized to total protein content and were described as percentage of total protein.

For ARS measurements, cells were washed and fixed after cultured in DMEM for 21 days. And then the cells were stained with 40 mM ARS for 20 minutes in room temperature. The stain was desorbed with 10% cetylpyridinium chloride (Sigma) for 1 h and then the solution was collected and equally distributed on a 96-well plate. Finally, the spectrophotometer (Bio-Tek) was used to determine the concentration of the Alizarin Red at 590 nm wave length.

### 2.7 Real-time PCR analysis of gene expression

TRlzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total cellular RNA. Reverse transcriptions were performed by PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). Gene specific primers were synthesized commercially (Shengong Co., Ltd., Shanghai, China) and their sequences are listed in Table 1. In one reaction, 10 µl SYBR Premix Ex Taq kit (Takara Bio, Inc.) was used to amplify 1 µl cDNA (mixed with 8 µl distilled water and 0.5 µl each primer). The Bio-Rad iQ5 real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was then used to detect gene expression. All relative gene expression values were normalized to βactin based on the $2^{\Delta\Delta Cq}$ method.

| Genes | Forward primer          | Reverse primer          |
|-------|-------------------------|-------------------------|
| Actin | TGAAGTGTGACGTGGACATC    | GGAGGAGCAATGATCTTGAT    |
| ALP   | TCAAGCCAAACACAAACAGC    | GGAGCCACAATCCAGTCATT    |
| OCN   | GCGAGACATCAAGGAGAAGC    | CCAATAAGGAAGGCTGGAA     |
| Runx2 | GAAGAGGAGCGAGAAGGAGAAGAAGGAG | TCCATAGCCAGTTTGTAGC |
| Fgf-2 | CAACACTTTACCGGTACGAGGA | CCCCGTTTTGGATCCAGGTT   |

### 2.8 Western blot analysis

For western blot analysis, cells were lysed with a RIPA buffer supplemented with protease inhibitor cocktail after cultured for 7 days. The obtained protein concentrations were measured by BCA protein assay kit (Beyotime, Shanghai) according to manufacture’s instructions and then equal amounts of
protein from different samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C with the following primary antibodies: Rabbit anti-rat Akt, Rabbit anti-rat pi-Akt and Rabbit anti-rat pi-PI3K (1:1000, Cell Signaling Technology, Inc.). Finally the membranes were visualized with horseradish peroxidase-conjugated goat anti-rabbit using the ECL Plus reagents by UVItec Alliance 4.7 gel imaging system.

2.9 Ectopic new bone formation and cell apoptosis analysis in nude mice

Cells were seeded into CPC (calcium phosphate cement) scaffolds after cultured for seven days under hypoxia and normoxia condition. Then they were inserted into subcutaneous space of nude mice. Ten weeks later, the mice were sacrificed by overdose injection of ketamine, and the implanted specimens were harvested. After 4% paraformaldehyde fixation, the specimens were decalcified in 20% EDTA (PH = 7.4) for 10 days followed by paraffinembedded, sectioned and stained with HE (hematoxylin and eosin). Immunohistochemical staining was carried out with primary antibody against OCN (Cell Signaling Technology, Inc.). Cell apoptosis was detected by using Tunnel kit (Cell Signaling Technology, Inc.). Tissue slides were visualized under a light microscope (Olympus Corporation, Tokyo, Japan) and Image Pro 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to perform histomorphological analysis. New bone formation was defined as the percentage of observed new bone area in the entire implant.

2.10 Surgical procedures

Cells were loaded into the CPC (calcium phosphate cement) scaffold at a density of $2.0 \times 10^5$ cells/ml before implanted into the defect area of the rat’s calvarium. The scaffold was shaped into cylinder (5mm × 2mm$^3$) and had an average pore size of 400mm ± 50 mm and 75% porosity. A rat calvarial defect mode was created to evaluate the in vivo new bone formation ability of BMSCs. The surgical procedures were performed according to previous described protocols. Briefly, the rats were placed in a prone position after anaesthetized by intraperitoneal injection of ketamine. A 1.5 cm long sagittal incision was made on the scalp and then blunt disect the surrounding tissues to expose the calvarium. And then 5 mm diameter size defects were created bilaterally by using trephinebur (Fine Science Tools, Foster City, CA, USA). The BMSCs/CPC constructs were inserted into the defected area and the wound was closed primarily. The rats were housed in ventilated rooms with access to sterilized water and food after the surgery.

2.11 Sequential fluorescence labeling

The polychrome sequential fluorescence labeling was used to characterize the mineralizing tissues. At 2, 4 and 6 weeks after operation, different fluoreschromes were injected intraperitoneally at the sequence as
follows: tetracycline hydrochloride (25 mg/kg, SigmaAldrich), alizarin red S (30 mg/kg, SigmaAldrich, USA), and calcein (20 mg/kg, SigmaAldrich).

2.12 Sample preparation

The rats were sacrificed by over-dose injection of ketamine 8 weeks after surgery. The calvarium with the implants were harvested and fixed in 10% paraformaldehyde for the following histological evaluation.

2.13 Histological evaluation

Three specimens of each group were dehydrated in ascending concentration of ethanol and then embedded in polymethylmethacrylate (PMMA). The specimens were cut into 150 mm thick sections by using a saw microtome (Leica, Hamburg, Germany) and polished to a final thickness of about 40 mm. The confocal laser scanning microscope (Leica TCS, Sp2 AOBS) was used for fluorescence labeling detection. The excitation/emission wavelengths used to observe fluorochromes were 405/580 nm, 543/617 nm and 488/580 nm for tetracycline (yellow), alizarin (red) and calcein (green) respectively. Then the sections were stained with Van Gieson's picrofuchsin for mineralized bone tissue visualization. The images were acquired by using a fluorescence microscope (Olympus, Japan). Histomorphometric analysis was conducted by Image-Pro Plus 6.0 software. New bone formation was defined as the percentage of observed new bone area in the entire implant.

2.14 Statistic analysis

Statistic analysis was performed with the GraphPad Prism 6 statistical software package. Data were expressed as means ± standard deviation (SD). Difference between two groups were analyzed by independent sample t-tests. A statistically significant difference were considered at *p < 0.05 and **p < 0.01.

3. Results

3.1 ARC promotes proliferation and inhibits apoptosis of BMSCs

Three days after lenti-virus transduction, >70% of BMSCs were EGFP-positive under fluorescence microscope (Fig. 1A). ARC expression in transfected BMSCs was detected by RT-PCR as presented in Fig. 1B, ARC mRNA expression was significantly increased in ARC overexpressed BMSCs when compared with vector control group. CCK-8 method was used to detect cell numbers from 12hrs to 72hrs, ARC overexpressed BMSCs revealed increased cell numbers in comparison with control group (*p < 0.05,**p < 0.01; Fig. 1C).
ARC is known as a highly potent and multifunctional inhibitor of apoptosis, in the current study, it was identified that ARC was able to reduce BMSCs apoptosis. Cell apoptosis was detected three days after transduction, the percentage of apoptotic cells (including the early and later stages) was 5.27%±0.6545% in BMSC-CON group and 2.833%±0.1225% in BMSC-NOL3 group, which was significantly lower in ARC overexpressed BMSCs (Fig. 1D, 1E).

3.2 ARC enhances osteogenic differentiation of BMSCs in vitro

On day 7 after lenti-virus transduction, ALP staining was decreased in BMSC-CON group compared with BMSC-NOL3 group. ARS staining on day 21 revealed the same tendency as ALP staining (Fig. 2A). The semi-quantitative analysis showed that ALP activity was lower in BMSC-CON group than BMSC-NOL3 group. The result of quantitative analysis of ARS was in consistent with ALP (Fig. 2B).

Immunofluorescence staining was used to detect the expression of intracellular OCN protein. The nuclei were stained with DAPI (Fig. 2C). The results confirmed that OCN protein expression was increased in BMSC-NOL3 group than BMSC-CON group.

To detect osteogenic related genes, RT-PCR was performed on day 7. Levels of osteogenic related genes including ALP, OCN and Runx2 were obviously different between these two groups. Expression of ALP, OCN and Runx2 were significantly increased in BMSC-NOL3 group in comparison with BMSC-CON group (Fig. 2D).

All theses results proved that ARC can enhance osteogenic differentiation of BMSCs in vitro.

3.3 Histological analysis of ectopic new bone formation and cell apoptosis detection in nude mice

At 10 weeks after implantation, new bone formation was observed in each group (Fig. 3A). Percentage of new bone formation was 15.14 ± 0.3369% in BMSC-CON group, 20.13 ± 0.3311% in BMSC-NOL3 group. Immunohistochemical analysis of newly formed tissue was positive for OCN protein. There was statistic difference between these two groups (**p < 0.01, Fig. 3B). These results proved ARC can enhance BMSCs new bone formation in vivo.

Immunofluorescence staining was carried out to detect cell apoptosis. Nuclei was stained with DAPI. Cell apoptosis was detected by Tunnel kit and was stained into red. ARC protein was stained into pink. In ARC overexpressed group, ARC protein expression was significantly increased even when the cells were implanted into the subcutaneous of nude mice for 8 weeks. Tunnel staining showed cell apoptosis was significantly decreased in ARC overexpressed group.

3.4 Histological, Fluorochrome labeling and histomorphometrical analysis of new bone regeneration
The undecalcified specimens were used to evaluate the new bone formation ability of BMSCs. Under light microscopy, the percentage of new bone area was 5.135 ± 0.2263% in CPC group, 22.36 ± 1.413% in BMSC-CON group, 31.64 ± 1.530% in BMSC-NOL3 group. There were statistic difference between BMSC-CON group and CPC group, BMSC-CON group and BMSC-NOL3 group (Fig. 4A and 4B,**p < 0.01). The percentage of remnant scaffold was 30.00 ± 1.033% in CPC group, 28.75 ± 0.8619% in BMSC-CON group, 31.46 ± 1.961% in BMSC-NOL3 group, no significant difference was found between these groups (Fig. 4C).

New bone formation and mineralization were determined by histomorphometrical analysis by TE, CA and AL fluorescent quantification, which represent the mineralization level at different time period (Fig. 4D). At 2 weeks, the percentage of TE labeling was 3.020 ± 0.2707% in BMSC-CON group, 5.003 ± 0.3584% in BMSC-NOL3 group. At 4 weeks, the percentage of CA labeling was 6.443 ± 0.2571% in BMSC-CON group, 8.853 ± 0.3667% in BMSC-NOL3 group. At 6 weeks, the percentage of AL labeling was 6.470 ± 0.2022% in normoxia BMSC-CON group, 9.700 ± 0.3485% in normoxia BMSC-NOL3 group. Statistic analysis for both TE, CA and AL fluorescent quantification, results showed statistic difference between BMSC-CON group and BMSC-NOL3 group (Fig. 4E,*P < 0.05,**P < 0.01).

### 3.5 ARC regulates BMSCs by activating Fgf-2/PI3K/Akt pathway

RNA-seq and Bioinformatics results showed 533 differentially expressed genes were detected between BMSC-CON and BMSC-NOL3 group, 233 genes were up-regulated while 322 genes were down-regulated in BMSC-NOL3 group (Fig. 5A,5B). Enrichment results of KEGG pathway of differentially expressed genes showed TNF-α signal pathway was inhibited in BMSC-NOL3 group, which was in accordance with previous reported data\(^\text{11}\). Differentially expressed gene-protein interaction (ppi) network diagram showed Fgf-2 gene was up-regulated in BMSC-NOL3 group, the RT-PCR result confirmed this (Fig. 5C). Fgf-2 is a stimulator of osteogenesis of human MSCs\(^\text{17}\). In order to further prove that ARC regulates BMSCs’ osteogenesis differentiation via activating Fgf-2 signal pathway, we add BGJ398 (0.5 µM for 2 hours, from Apexbio), an FGFR inhibitor, into the transfected cells, then RT-PCR was carried out to detect the osteogenic related genes, the results showed that ALP, OCN and Runx2 were down-regulated in BMSC-NOL3 group after BGJ398 treatment (Fig. 5D).

FGFR can activate PI3K/Akt signal pathway to regulate osteogenic differentiation. In this study, expression of p-PI3K,Akt and p-Akt was evaluated by WB. The expression of p-PI3K and p-Akt was significantly increased in BMSC-NOL3 group compared with BMSC-CON group (Fig. 5E). Thus ARC promotes osteogenesis differentiation of stem cells may partially due to the activation of Fgf-2/PI3K/Akt signal pathway.

### 4. Discussion
In this study, ectopic new bone formation in nude mice and new bone formation in calvarium defect rat experiments both confirmed ARC has the ability to promote BMSCs new bone regeneration, this may due to 1) ARC reduce BMSCs apoptosis after cell seeded into scaffold and implanted into in vivo hypoxia environment. 2) ARC has the ability to directly enhance BMSCs osteogenic differentiation. Devoid of oxygen in the center of implanted tissue engineered constructs may cause massive cell death and reduced regenerative capacity. Necrotic cells are observed in the center of implanted large bone construct. ARC, known as a highly potent and multifunctional inhibitor of apoptosis, has already been proved to be capable of inhibiting hypoxia induced various cell apoptosis. In this study, in vitro results reveal that ARC can reduce BMSCs apoptosis and promote its proliferation. Immunofluorescence staining of sliced tissues showed cell apoptosis was decreased in BMSC-NOL3 group. We also detect the effect of ARC on BMSCs under hypoxia environment in vitro, results confirmed that ARC can reduce hypoxia induced apoptosis of BMSCs (Data was not shown).

The effect of ARC on bone cells was not reported yet except for our previous published work focusing on the effect of ARC on human osteoblasts. In this study, we explore more deeply about the effect of ARC on BMSCs. ALP, ARS staining, quantitatively analysis, OCN immunofluorescence staining, RT-PCR analysis both showed ARC can enhance BMSCs osteogenic differentiation in vitro. We then further created the nude mice mode and calvarium defect rat mode to evaluate the in vivo bone formation ability of ARC overexpressed BMSCs. The results also confirmed ARC overexpressed BMSCs showed increased new bone formation ability than vector control group. Both in vitro and in vivo study proved that ARC is a powerful agent to promote BMSCs' bone regeneration.

The ability of ARC to reduce cell death has already been established in various cells including skeletal muscle cells, cardiac muscle cells and neurons. ARC functions as antiapoptotic reagent mainly by binding proapoptotic molecules at its N-terminal CARD then further inactivating their proapoptotic functions. More recently, ARC was proven to inhibit the TNF-α pathway itself to block multiple downstream outcomes, including apoptosis, necrosis and NF-κB activation. Exogenous ARC was also shown to bind JNK and inhibit its activation in hepatocytes and islet β-cells. In this study, the RNA-seq analysis revealed TNF-α signal pathway was inhibited and Fgf-2 gene was up-regulated in ARC overexpressed BMSCs. RT-PCR analysis confirmed Fgf-2 was up-regulated in BMSCs-NOL3 group. Fgf-2 plays an important role in osteoblast lineage determination. Alteration in Fgf-2 signaling may cause impairment of BMSCs bone formation capacity. Fgf-2 activates several signaling pathways including MEK/ERK and PI3K/AKT. Fgf-2 mediates cell survival via the PI3K/AKT pathway in mouse models of neuronal differentiation, embryonic stem cells and primary neural stem cells. In this study, we examined the activation of Fgf-2/PI3K/AKT signal pathway in ARC overexpressed BMSCs. Our data indicate that activating of Fgf-2/PI3K/AKT pathway signaling is a major mechanism through which ARC enhances BMSCs new bone regeneration.

Gene therapy emerged as a promising approach for repairing bone defect, genetically engineered cells can release growth factors at the defect area in a sustained and precise way. But the safe matter
limits its clinical application. Protein delivery involves in application of recombined protein into the defect area and it obtains great potential for bone tissue engineering. In this study, we have already proved that ARC transfected into BMSCs can powerfully enhance its new bone formation. For clinical application, we have to further detect the effect of ARC protein on BMSCs. Cell permeable Tat-fused NOL3 protein can be obtained to use for further study.

In conclusion, the present study suggested that ARC is a powerful agent to enhance BMSCs new bone regeneration and provides a promising method for bone tissue engineering. The effect of ARC protein on BMSCs warrants for future studies.

**Abbreviations**

ARC Apoptosis Repressor with Caspase Domain  
BMSCs Bone marrow derived mesenchymal stem cells  
CPC Calcium phosphate cement  
FBS Fetal bovine serum  
DMEM Dulbecco's modified eagle medium  
ALP Alkaline phosphatase activity  
ARS Alizarin Red Staining  
HEK293T Human embryonic kidney 293T cells  
CCK-8 Cell counting kit-8

**Declarations**

**Acknowledgement**

Not applicable

**Authors’ contributions**

Longwei Hu and Yang Wang performed in vitro study and wrote the manuscript, they contribute equally to this article. Chenping Zhang and Hongya Pan designed the experiments. Siyi Li and Jin wen analysed the data. Kathreena Kadir revised the manuscript.
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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All animal procedures were approved by the Animal Research Committee of the Ninth People's Hospital, Shanghai Jiao Tong University.

Consent for publication

All the authors are consent for publication of this article.

Competing interests

All of the authors declare that there are no competing interests.

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**Figures**
Figure 1

ARC overexpression analysis in BMSCs, CCK-8 and flow cytometric analysis. (A) The fluorescence microscope detection of EGFP-positive BMSCs on day 3 subsequent to lentiviral transduction. (B) ARC mRNA level was significantly increased in BMSC-NOL3 group compared with BMSC-CON group. (**p<0.0001). (C) CCK-8 method was used to detect cell proliferation from 12 hours to 72 hours. (*p<0.05, **p<0.01, BMSC-CON VS. BMSC-NOL3). (D) One of three representative images of flow cytometry was presented. (E) The percentage of apoptosis was analyzed in each group. (*p<0.05).
Figure 2

In vitro analysis of osteogenic differentiation ability of ARC overexpressed BMSCs. (A) ALP and ARS analysis. ALP staining on day 7 and ARS staining on day 14. (B) Semi-quantitative analysis of ALP activity and semi-quantitative analysis of ARS. (**p<0.01). (C) Immunofluorescence staining detection of OCN protein expression. Nuclei were stained with DAPI. (D) RT-PCR analysis of osteogenic related genes, including ALP, OCN and Runx2. (*p<0.05,**p<0.01).
Ectopic new bone formation and cell apoptosis detection in nude mice. Cells were seeded into CPC scaffolds and then were implanted subcutaneously for 8 weeks. (A) Both HE staining and OCN immunohistochemistry analysis confirmed newly formed bone tissues in each group. (B) Percentage of new bone formation area in each group. (**P<0.01). (C) Immunofluorescence staining was carried out to detect cell apoptosis. Nuclei was stained with DAPI. Cell apoptosis was detected by Tunnel kit and was stained into red. ARC protein was stained into pink.
Figure 4

Histological analysis of new bone formation area and remnant scaffold area in the calvarial defects. (A) The specimens were stained with Van Gieson’s picro fusions. (original magnification, 1.25××40×). (B) Percentage of new bone formation area in each group per 40 ×. (C) Percentage of remnant scaffold area in each group per 40 ×. (D) New bone formation and mineralization were determined by histomorphometrical analysis using TE, CA and AL fluorescent quantification, which represent the mineralization level at different time period. (E) Percentage of fluorochrome stained bone area in each group.
Figure 5

(A) Geometric mean-centered, hierarchical clustering heat map from microarray data. The 533 differentially expressed genes were detected between BMSC-CON and BMSC-NOL3 group, 233 genes were up-regulated while 322 genes were down-regulated in BMSC-NOL3 group. (B) Differentially expressed gene-protein interaction (ppi) network diagram, blue round node for up-regulated gene expression, green diamond node for down-regulated gene expression. (C) RT-PCR analysis of Fgf-2 gene expression between BMSC-CON and BMSC-NOL3 group. (D) RT-PCR analysis of osteogenic related genes including ALP, OCN and Runx2 after BGJ398, an FGFR inhibitor treatment. (E) WB analysis of p-PI3K and p-Akt protein expression between BMSC-CON and BMSC-NOL3 group.