Study on the Mechanism of Mirna-21 Affecting the Differentiation of Bone Marrow Mesenchymal Stem Cells into Cardiomyocyte-Like Cells By Targeting Ajuba / Isl1 Axis

Wengang Yang (ywgada@163.com)
Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine
Song Xue
Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine
Hui Zheng
Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine
Jianggui Dan
Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine
Lei Zhao
Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine
Chenxi Wang
Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine

Research Article

Keywords: miRNA-21, ajuba/ Isl1, BMSC, ischemic heart disease

Posted Date: September 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-895514/v1

Abstract

Purpose: To study the mechanism of miRNA-21 targeting ajuba/ Isl1 axis to affect BMSC differentiation to cardiac myoid cells.

Methods: BMSC was cultured and miRNA-21 was constructed to infect BMSC. The miRNA-21 was directly regulated by luciferase reporter gene system. The expression of cTnI, ajuba and Isl1 was detected by RT-qPCR and WB. The expression of cTnI, ajuba and Isl1 was detected by RT-qPCR and WB, and miR-21 was detected by RT-qPCR; The differentiation ability of BMSC in all groups was detected by RT-qPCR and WB; To evaluate the effect of ajuba and Isl1 on the differentiation ability of BMSC.

Results: BMSC was cultured successfully, BMSC was successfully constructed by mir-21-OE, mir-21-KD, ajuba-OE and ajuba KD slow virus; RT-qPCR and WB were used to detect the high expression of cTnI in mir-21-OE and ajuba KD groups, and the expression of miR-21 was increased, the expression of ajuba was inhibited and Isl1 expression was enhanced.

Conclusion: The expression of ajuba was inhibited by up regulation of miRNA-21 target, and the expression of Isl1 enhanced to promote BMSC differentiation, that is, miRNA-21 regulated the axis of ajuba/ Isl1 to influence the differentiation of BMSC to cardiomyocyte-like cells.

Introduction

MiRNA-21 is abnormally expressed in many cardiovascular diseases [1]. It has been found that miRNA-21 plays an important role in the occurrence and development of cardiovascular diseases through its loss of function or enhancement of function. MiRNA-21 is involved in myocardial protection. It was found that injection of exogenous synthetic miRNA-21 reduced the size of myocardial infarction by 64% [2]. When mice were treated with miRNA-21 antagonists, the protective effect formed by miRNA-21 completely disappeared. The changes of many apoptosis related genes caused by miRNAs intervention were determined by microarray analysis. MiRNA-21 is involved in the proliferation, apoptosis and differentiation of a variety of cells. In the first part of this subject, on the premise of up regulating the expression level of miRNA-21, it was found that the differentiation ability of BMSC was improved, and the proliferation and apoptosis were accelerated, but the exact mechanism was not known, which prompted us to further study.

In this part of the study, we found that miR-21 can target and regulate ajuba expression through bioinformatics prediction. MiR-21 inhibited the translation of ajuba by targeting, resulting in the down-regulation of ajuba. The down-regulation of ajuba attenuated the inhibitory effect on Isl1, thus promoting the enhancement of Isl1 activity, and the enhancement of Isl1 activity led to the differentiation of bone marrow mesenchymal stem cells into cardiac myoid cells. To study the mechanism of miR-21 targeted regulation of ajuba / Isl1 axis on the differentiation of BMSC into cardiac myoid cells.

Materials And Methods

Laboratory animals:

Healthy SD rats (Sprague Dawley rat), weighing 250-300g, were provided by the animal experiment center of Medical College of Shanghai Jiaotong University. Factory license No.: scxk (Shanghai) 2011-0001, use license No.: syxk (Shanghai) 2011-0121. This study was supported by the ethics committee of Shanghai Jiaotong University.

Reagents and instruments:

L-dmem medium (hyclone); Super grade fetal bovine serum (FBS, GIBCO); 0.25% trypsin and EDTA (GIBCO); Ficoll (GE) with density of 1.077; Xylene (GIBCO); Antigen repair solution (trisodium citrate buffer) (GIBCO company); 4% paraformaldehyde (GIBCO); 0.01% Triton-X (GIBCO); first antibody α Some antibodies such as TnI (sigma company) are the same as the first part of the experiment; Enzyme labeling detector (thermo MK3 type); Annexin V-FITC / PI Kit (Bebo biology 401006), 75% ethanol (Wuxi prospect 64.17-5), BCA protein quantitative Kit (Kaiji); PH = 6.8 electrophoretic buffer (EBS); 10% SDS (EBS); 10% ammonium persulfate (IBOs); TEMED (ABOS Biology); 4 * protein loading buffer (IBS); Protein pre staining marker (thermo); Pgmlv-ma2 expression vector kit, emgfp and other related reagents were purchased from Qiagen; Triton (Amresco); Primer (R & F) for PCR (Shanghai aibosi Biotechnology Co., Ltd.); Taq polymerase(NEB)； Qiagen plasma pumping Kit (Qiagen); LB or SOB or SOC(Alpha aeser)； CaCL2(Sigma)； T4 DNA ligase(Fermentas)； T4 DNA ligase buffer(Fermentas); MgSO4(Sigma); Agarose (bio RAD); DNA ladder; Positive clone sequencing (Invitrogen); BamH I and Xho I (fermentas); PCR instrument (Eppendorf); Pipette (Gilson). Trizol (Invitrogen company of the United States), reverse transcription Kit (promisga company of the United States), Taq enzyme (Takara company of Japan), primer sequence synthesis (Shanghai Shenggong biology company), BCA Kit (biyuntian company), Estherichia coli DH5 (strain (Invitrogen company), Lipofectamine 2000 (Invitrogen company), opti MEM (Invitrogen company), Trizol (Invitrogen company) , prenti6.3/v5-dest (Invitrogen company), restriction endonuclease, T4 DNA ligase, large plasmid DNA Extraction Kit (Qiagen company), vertical electrophoresis apparatus (GE company of the United States), semi dry membrane apparatus (bio rad company of the United States), nitrocellulose membrane (bio rad company of the United States), (GE company of the United States), Kodak in vivo imager, cymentre2000. Relevant surgical instruments (scalpel, hemostatic forceps, etc.)

Isolation, induction and culture of bone marrow mesenchymal stem cells (MSCs),Identification of characteristic antigens of bone marrow mesenchymal stem cells (MSCs),DAPI labeled bone marrow mesenchymal stem cells,were seed in the article by wengang yang et al(Mesenchymal stem cells were affected by up-regulation of miRNA-21 in vitro.Int J Clin Exp Pathol 2018;11(1):27-37).

Bioinformatics predicts miR-21 target genes:

Prediction of miR-21 target genes we used mirwalk software to analyze the intersection of all candidate target genes predicted by miR-21 through targetscan, mirdb and Miranda. Then, according to the negative regulatory relationship between miRNA and target genes, the genes negatively related to the expression of miR-21 were screened and expressed in MSCs cells. At the same time, the pathways related to the differentiation of MSCs into cardiac myoid cells were concerned. Finally, the corresponding molecular phenotypes were screened.

Construction of miRNA-21 expression virus vector (mir-21-OE):

MiRNA-21 sponge (mir-21-KD) was obtained

MiRNA "sponges", also known as miRNA sponges, are one of the very effective tools for miRNA function research. It can continuously induce functional inhibition against cell line miRNA. MiR-21 sponge contains the target miR-21 complementary binding site. According to the miRNA-21 sequence, miRBase access No: mi0000850. The primer sequence of miR-21 sponge is as follows: miR-21 sponge f (xhoi): ccgctcgagctcgtcaacatcagtctg and miR-21 sponge R (BamHI): ccggatccttggtgagcttatcagac. The corresponding restriction enzymes digest xhoi and BamHI. Using vector cloning kit for recombination, oligonucleotides were inserted into LV overexpression vector pcdh-egfp vector (lv-mirna-21 sponge), and E.coli DH5 cells were transformed with plasmid. Subsequently, lv-mirna-21 sponge was transfected into 293T cells, packaged with virus and its titer was determined.

Construction and virus packaging of ajuba overexpression virus vector (ajuba OE):

Ajuba sequence: nm according to access No_ 053503.1. The plasmid template was purchased from Beijing Yiqiao Shenzhou Biotechnology Co., Ltd. through subcloning, the relevant primer sequences are as follows: ajub-f (xhoi): ccgctcgagatggtggttaggggagaaag; And ajub-r (BamHI): CCG ggatcctcagatatagttggtaggggctg. The corresponding restriction enzymes digest xhoi and BamHI. The PCR product was inserted into the overexpression vector pgmlv-ma2 vector (Invitrogen) and transformed with E.coli DH5. The plasmid was extracted by Plasmid Extraction Kit, and the plasmid was named LV ajuba OE. Subsequently, LV ajuba OE was transfected into 293T cells, packaged with virus and its titer was determined.

Construction of ajuba interference virus vector (ajuba KD):

Construction of shRNA lentivirus interference vector we used plko.1-gpf-puro vector through block it ™ RNAi designer tool (https://rnaidesigner.thermofisher.com/rnaiexpress/) Design and synthesize double

stranded hairpin DNA primer sequences as follows:

name	sequence
Ajuba – shRNA-1 S	5’- CCGGGCCAGAGGCCAGAGAAGATTA. CTCGAGTAATCTTCTCTGGCCTCTGGCTTTTTG -3’
Ajuba – shRNA-1 AS	5’- AATTCAAAAA GCCAGAGGCCAGAGAAGATTA CTCGAGTAATCTTCTCTGGCCTCTGGC -3’
Ajuba – shRNA-2 S	5’- CCGGGCACCTGTATCAAGTGCAACACTCGAGTGTTGCACTTGATACAGGTGCTTTTTG -3’
Ajuba – shRNA-2 AS	5’- AATTCAAAAA GCACCTGTATCAAGTGCAACA CTCGAG TGTTGCACTTGATACAGGTGC-3’
Ajuba – shRNA-3 S	5’-CCGGGTGATGAAAGAATTACCGAATCTCGAGATTCGGTAATTCTTTCATCACTTTTT -3’
Ajuba – shRNA-3 AS	5’-AATTCAAAAAGTGATGAAAGAATTACCGAATCTCGAGTTCGGTAATTCTTTCATCAC-3’
NC-sense	5’- CCGGGCCACTTGATTCTAGAGAAGA -3’ -3’CTCGAGTCTTCTCTAGAATCAAGTGGC TTTTTG -3’
NC-antisense	5’- AATTCAAAAAGCCACTTGATTCTAGAGAAGACTCGAGTCTTCTCTAGAATCAAGTGGC -3’

The designed shRNA was synthesized by Shanghai Shenggong Bioengineering Co., Ltd. and connected according to the following system

reagent	volume
5×T4 Ligase	2
Restriction endonuclease carrier DNA	1
objective DNA	1
ddH2O	1
	3

Take an appropriate amount of ligation product, add it to the competent cells, transform it with E. coli DH5, extract the plasmid through the Plasmid Extraction Kit, and name the plasmid LV ajuba shRNA.

Subsequently, LV ajuba shRNA was transfected into 293T cells, packaged with virus and its titer was determined.

The expression of related proteins was detected by Western blotting:

The experiment was carried out in groups. 300ul tissue cell lysate was added to each tissue cell sample, mixed with a gun to completely lyse it, and the lysate was moved to a new centrifuge tube. Take 10 directly μ L sample adding 10ul 2 × SDS-PAGE loading buffer, mix well, heat at 100 º C for 5 minutes, cool on ice, 12000 g centrifugation for 5 minutes to remove insoluble precipitation. The samples were separated by 10% SDS-PAGE, and the sample amount per well was 20 UL. After electrophoresis, the PVDF membrane was soaked in methanol for 1 minutes, then soaked in Transfer Buffer gel, filter paper and PVDF film soaked in methanol for 4 minutes for 10 minutes, then the transfer sandwich was prepared. Use blocking buffer to seal the transfer film at 4 º C overnight, and 1 the next day × Tbst was washed 3 times for 15 minutes each time. Add diluted primary antibody and incubate for 2 hours at 37 º C. Use 1 × Tbst was washed 4 times for 10 minutes each time. Add diluted secondary antibody and incubate for 2 hours at 37 º C. Use 1 × Tbst was washed 4 times for 10 minutes each time. Chemiluminescence detection was carried out with super GL ECL hypersensitive luminous solution, and the X-ray film was exposed. After developing and fixing, the air dried film was photographed with gel imaging analysis system. The experiment was carried out by Gel-Pro Analyzer software.

Real time PCR was used to detect the expression of related mRNA:

（1） Total RNA extracted by Trizol:

1) Bone marrow mesenchymal stem cells (MSCs) in each group were collected, 500 ml Trizol reagent was added, and immediately blown with 2 ml syringe for 5 times;

2) Then add 100ml chloroform, mix well, stand at 0 ~ 4 ° C for 5 minutes, and then centrifuge at 12000g high speed at 4 ° C for 5 minutes;

3) Carefully move the upper aqueous phase to a new 1.5ml centrifuge tube, add equal volume of isopropanol, shake and mix well. RNA was precipitated at room temperature for 5 minutes and centrifuged at 12000g at 4 ° C for 5 minutes;

4) Carefully discard the supernatant, add 1ml 70% ethanol into the centrifuge tube, shake for a moment, and centrifuge 10000g for 5 minutes;

5) Carefully discard the supernatant and let it stand at room temperature for 5-10 minutes. The RNA precipitation is just dry. Dissolve in water and store at - 70 ° C.

（2） DNaseI processes DNA in RNA:

1) Suck 1mg mRNA, add 1ml DNase I buffer and 1ml DNase I, make up to 10ml with water and mix well. Act at 37 ° C for 30min in in PCR reactor, add 1ml EDTA, act at 65 ° C for 10min, and then continue to act at 95 ° C for 5min.

2) Synthesis of the first strand of cDNA: prepare the following mixture in a centrifuge tube on ice: Total RNA 5 μ l. Oligo (DT) 18 primer (0.5mg / ml) 1ml, gently mixed and centrifuged for 3-5 seconds. Incubate at 70 ° C for 5 minutes, remove, ice bath, and centrifuge briefly. Place the centrifuge tube on ice. Add the following ingredients in order: 5 × Reaction buffer 5ml, 10mm dNTP mixture 5ml, reverse transcriptase (200 μ/ Ml) 1ml, RNase inhibitor (20 μ/ Ml) 0.5ml, mix gently and centrifuge briefly. Incubate for 5 minutes at 37 ° C, incubate for 1 hour at 42 ° C, terminate the reaction after 10 minutes at 70 ° C, and cool on ice. CDNA synthesis is complete.

（3）Take 4 ml of reverse transcription product and add it to 10 containing 5 ml × PCR reaction buffer, 1.5 ml MgCl2 (50 mm), 1 ml dNTP mixture (10 mm), 1 ml upstream primer (10 PM), 1 ml downstream primer (10 PM) and 0.3 ml Taq enzyme (5 U / ml) were added with water to make up the volume to 50 ml. Amplification was carried out according to the following procedures: pre denaturation at 94 ° C for 5 minutes, denaturation at 94 ° C for 45 seconds, renaturation at 56 ° C (mainly based on the annealing temperature after primer sequence) for 45 seconds, extension at 72 ° C for 1 minute, repeated 35 times and extension at 72 ° C for 10 minutes. Take 10ml amplification product and 2ml 6 × After the loading buffer was mixed, electrophoresis was performed on 1.5% agarose gel, and Kodak UV-2000 UV analyzer was used for observation and scanning analysis. The primers used are as follows:

name	sequence	Product size
Ajuba –S	5’-GGGAACCCTTGGGGATTGAG -3’	222bp
Ajuba –AS	5’- AAGTTCGTCCACAGGAGCAG-3’	
Isl1 –S	5’- TGCGGTAATCAAATTCACGA-3’	180bp
Isl1 –AS	5’- GCATTTGATCCCGTACAACC-3’	
Actin–S	5’-CGTAAAGACCTCTATGCCAACA-3’	131bp
Actin–AS	5’-GGAGGAGCAATGATCTTGATCT-3’	

Luciferase reporter gene detection after BMSC was infected with mir-21-oe and mir-21-kd, miR-21 directly regulated

ajuba:

(1) According to the analysis and prediction of bioinformatics methods, miR-21 was bound to the site of ajuba 3'UTR untranslated region (2). The wild-type pmirglo-ajuba-3'utr (wt-pmirglo-ajuba-3'utr) and mutant pmirglo - ajuba-3'utr mut (MUT - pmirglo-ajuba - 3'UTR) expression vectors were constructed by gene synthesis method, and the synthesized DNA fragments were inserted into luciferase reporter gene plasmid (pmirglo) Nhel (gctagc) and Sali (gtcgac) digestion sites in E.coli dha5a.

(3) Positive clones were screened and sequenced.

(4) Amplify the transcription factor plasmid and purify it for standby. Meanwhile, prepare the corresponding empty plasmid control and purify it for standby.

(5) MSCs were cultured and inoculated in 24 well plates for 10-24 hours (80% confluence).

(6) BMSC cells were infected with the virus with mir-21-oe overexpression and mir-21-kd interference, and then the reporter gene plasmid was co transfected into the cells.

(7) Cells were lysed and used for luciferase detection.

(8) The substrate was added to determine the activity of luciferase.

(9) The relative fluorescence intensity was calculated and compared with the control group.

Statistical method:

The images were analyzed by image programmer software, and the related statistical analysis was carried out by graphpad prism 5 demo and SAS v6.12 software. The data were analyzed according to the mean ± standard deviation. T-test was used between the groups, and the experiment was carried out three times.

Results

MSCs culture and cell characterization were seed in the article by wengang yang et al(Mesenchymal stem cells were affected by up-regulation of miRNA-21 in vitro.Int J Clin Exp Pathol 2018;11(1):27-37).

miRNA-21 expression virus vector (mir-21-OE) was successfully constructed:

The target genes of miR-21 were predicted by mirwalk software: the intersection of miR-21 target genes was predicted by targetscan, mirdb and Miranda software, and 10 candidate target genes were screened; According to the negative regulatory relationship between miRNA and target gene, finally, the target gene

was analyzed by KEGG, go and literature mining. We took ajuba as the most representative candidate target gene. The software predicts that miR-21 binds to 3 'UTR of ajuba gene, as shown as follows:

Construction of miRNA-21 sponge (mir-21-KD) vector and lentivirus packaging and its validation

MiRNA-21 sponge lentivirus vector was successfully constructed by gene synthesis the results are shown in Fig 1. Xhoi and bamh1 were digested and identified as 190bp, which was consistent with the expected size. Lane 1: pcdh empty; Lane 2: xhoi and bamh1 digestion identification; Lane M: DNA marker.

The constructed lentivirus vector was packaged in 293T cells. The virus titer identification results are shown in Fig 2: miR-21 sponge virus infected HEK 293T cells. Up to 60 cells expressing green fluorescence were observed in hole 6. The titer of miR-21 sponge is 60 Tu / (10×10^{-8}) ml = 6×10^{8}tu / ml

Compared with the empty vector control virus, the inhibition rate of miR-21 detected by RT qPCR was 75% ($P < 0.001$). The results showed in Fig 3 that miR-21 sponge method successfully inhibited the expression of miR-21, the virus packaging was successful, and the intracellular inhibition effect was significant.

Construction of ajuba overexpression virus vector (ajuba OE) and lentivirus packaging and its validation

Ajuba OE lentivirus vector was successfully constructed by subclonal method. Xhoi and bamh1 were identified as 1806bp, which was consistent with the expected size as showed in Fig 4. Lane 1: pcdh empty; Lane 2: xhoi and bamh1 digestion identification; Lane M: DNA marker

The constructed lentivirus vector is packaged with 293T cells. The virus titer identification results are shown in Fig 5, and the titer is 6×10^{8}tu / ml; The cell HEK 293T was infected by ajuba OE virus. Up to 60 cells expressing green fluorescence were observed in hole 6. The titer of ajuba OE is 80 Tu / (10×10^{-8}) ml = 8×10^{8}tu / ml

Compared with the empty vector control virus, the overexpression of ajuba OE detected by RT qPCR was 75% ($P < 0.001$) . The results showed in Fig 6 that ajuba OE method successfully overexpressed ajuba, the virus packaging was successful, and the cell ajuba overexpression effect was significant. A: The expression of ajuba mRNA was significantly increased by RT qPCR; B: The expression of ajuba protein was detected by WB, and the expression of ajuba increased. β- Actin is an internal parameter*** Represents $P < 0.001$

Successful construction of ajuba interfering virus vector (ajuba KD) vector construction and lentivirus packaging and

its verification:

Ajuba SHRA was constructed into plko.1-gfp-puro vector. It was identified by EcoRI enzyme digestion whether shRNA fragment was inserted. The band size of enzyme digestion identification result was in line with the expectation, and then the shRNA sequence was confirmed to be completely correct by sequencing, as shown in Fig 7. Lane 1: plko. 1 empty; Lane 2: EcoRI digestion identification; Lane M: DNA marker.

Compared with shRNA NC control virus, the mRNA expression of ajuba was detected by RT qPCR. The results showed that the inhibition rates of ajuba shrna-1, ajuba shrna-2 and ajuba shrna-3 were 35.12%, 74.35% and 84.35% (* $P < 0.05$); The expression of ajuba at protein level was detected by WB. Shrna-2 and shrna-3 were significantly inhibited. Shrna-3 can be used to interfere with lentivirus in subsequent functional experiments, as shown in Figure 8. A: The expression of ajuba mRNA was significantly inhibited by RT qPCR; B: The expression of ajuba protein was detected by WB, and ajuba was significantly inhibited. β- Actin is an internal parameter*** Represents $P < 0.001$

Mir-21-oe and mir-21-kd infected BMSC. Luciferase reporter gene detected the direct regulatory effect of miR-21 on ajuba:

MiR-21 was overexpressed by mir-21-oe and knocked down by mir-21-kd, and then detected by double luciferase reporter gene. The results are shown in Fig 9. The results showed that compared with miRNA NC group, the reporter gene activity of wild-type expression vector transfected in mir-21-oe group decreased significantly ($P < 0.001$); It can be seen that the concentration expression of wild-type ajuba protein is negatively regulated by miR-21. It can be seen that there is a direct targeted regulation relationship between them, as shown in fig. 9A. Compared with kd-nc group, the activity of reporter gene transfected with wild-type expression vector in mir-21-kd group was significantly higher ($P < 0.001$); It can be seen that by inhibiting miR-21, the expression of wild-type ajuba protein is de inhibited, so the expression is significantly high. It can also be verified that there is a direct targeted regulation relationship between miR-21 and ajuba, as shown in fig. 9B. Wt: wild type ajuba 3'UTR; Mut: mutant ajuba 3'UTR** Represents $P < 0.01$, * * * represents $P < 0.001$.

Real time PCR was used to detect the expression of ajuba and Isl1 during BMSC differentiation after mir-21-oe and mir-21-kd infected BMSC:

MiR-21 was overexpressed by mir-21-OE, and then the mRNA expressions of ajuba and Isl1 were detected by fluorescence quantitative PCR, as shown in Fig 10. The mRNA expressions of ajuba and Isl1 in mir-21-OE group were detected at 1 week, 2 weeks and 3 weeks. The expression of ajuba did not change

significantly compared with the control group with the extension of time; The expression of Isl1 was significantly higher than that of the control group. A: There was no significant difference in ajuba mRNA expression at 1 week, 2 weeks and 3 weeks by RT qPCR; B: The expression of Isl1 mRNA was significantly increased at 1 week, 2 weeks and 3 weeks by RT qPCR. β- Actin is an internal parameter*** Represents P < 0.001, * represents P < 0.05

MiR-21 was inhibited by mir-21-KD, and then the expression of ajuba and Isl1 mRNA was detected by fluorescence quantitative PCR. The results are shown in Fig 11. The mRNA expressions of ajuba and Isl1 in mir-21-KD group were detected at 1 week, 2 weeks and 3 weeks. The expression of ajuba did not change significantly compared with the control group with the extension of time; The expression of Isl1 decreased significantly compared with the control group. A: There was no significant difference in ajuba mRNA expression at 1 week, 2 weeks and 3 weeks by RT qPCR; B: The expression of Isl1 mRNA decreased significantly at 1 week, 2 weeks and 3 weeks by RT qPCR. β- Actin is an internal parameter*** Represents P < 0.001, * * represents P < 0.01, * represents P < 0.05

Western blotting was used to detect the expression of ajuba and Isl1 during BMSC differentiation after mir-21-OE and mir-21-KD infected BMSC:

MiR-21 was overexpressed by mir-21-OE, and then the expression of ajuba protein was detected by WB. The results are shown in Fig. 12A. The expression of ajuba in mir-21-OE group was detected at 1 week, 2 weeks and 3 weeks. With the extension of time, the expression of ajuba decreased compared with the control group in 1 to 2 weeks; The decrease was most significant in 2 weeks, and there was no significant difference in 3 weeks.

Mir-21-kd was used to reduce the expression of miR-21, and then the expression of ajuba protein was detected by WB. The results are shown in Fig. 12B. The expression of ajuba in mir-21-KD group was detected at 1 week, 2 weeks and 3 weeks. The expression of ajuba increased with time, and the protein expression increased compared with the control group within 1 to 2 weeks; The increase was most significant in 2 weeks, and there was no significant difference in 3 weeks. A: WB was used to detect the expression changes of ajuba in 1 week, 2 weeks and 3 weeks after miR-21 over-expression; B: After the decrease of miR-21 was detected by WB, the expression of ajuba was different from that of the control group at 1, 2 and 3 weeks. β- Actin is an internal parameter.

MiR-21 was overexpressed by mir-21-OE, and then the expression of Isl1 protein was detected by WB. The results are shown in Fig 13A. The expression of Isl1 in mir-21-OE group was detected at 1 week, 2 weeks and 3 weeks. The expression of Isl1 increased with time, and the protein expression increased compared with the control group within 1 to 2 weeks; The increase was most significant in 2 weeks, and there was no significant difference in 3 weeks.

Mir-21-KD was used to reduce the expression of miR-21, and then the expression of Isl1 protein was detected by WB. The results are shown in Fig. 13B. In mir-21-KD group, the expression of Isl1 was detected at 1 week, 2 weeks and 3 weeks. With the extension of time, the expression of Isl1 decreased compared with the control group in 1 to 2 weeks; There were significant differences in 1-2 weeks, and there was no significant difference in 3 weeks. A: WB was used to detect the expression of Isl1 in 1 week, 2 weeks and 3 weeks after miR-21 overexpression; B: WB was used to detect the changes of Isl1 expression in 1 week, 2 weeks and 3 weeks after the decrease of miR-21. β- Actin is an internal parameter.

Western blotting was used to detect the expression of cTnI, ajuba and Isl1 during BMSC differentiation after ajuba overexpression (ajuba OE) and interference with ajuba (ajuba KD)

After ajuba OE was used to overexpress ajuba, the expression levels of ajuba, Isl1 and cTnI proteins were detected by WB. The results are shown in Fig 14A. Compared with the control group, the expression of ajuba increased, the expression of Isl1 decreased and cTnI decreased in ajuba OE group at 2 weeks. After ajuba shRNA was used to interfere with ajuba expression, the expression levels of ajuba, Isl1 and cTnI proteins were detected by WB. The results are shown in Fig. 14B. Compared with the control group, the expression of ajuba decreased, the expression of Isl1 increased and cTnI increased in ajuba KD group at 2 weeks. A: WB was used to detect the expression changes of ajuba, Isl1 and cTnI proteins after ajuba overexpression; B: WB was used to detect the expression changes of ajuba, Isl1 and cTnI proteins after ajuba interference knockdown. β- Actin is an internal parameter.

Ajuba overexpression (ajuba OE) and interference with ajuba (ajuba KD) infected BMSC. CTnI was detected by immunofluorescence to observe the differentiation ability of BMSC into cardiac myoid cells

After ajuba OE was used to overexpress ajuba, the expression of cTnI protein was detected by immunofluorescence. The results are shown in Fig 15A. Compared with the control group, cTnI in ajuba OE group decreased at 2 weeks. After ajuba shRNA was used to interfere with ajuba expression, cTnI expression was detected by immunofluorescence. The results are shown in Fig 15B. Compared with the control group, cTnI in ajuba KD group increased at 2 weeks. A: After ajuba overexpression, the expression of cTnI protein was detected by immunofluorescence; B: The expression of cTnI protein in ajuba interference knockdown group and control group was detected by immunofluorescence. The photo magnification is 200X.

Discuss

MiRNA-21 is highly expressed in breast cancer, liver cancer, bladder cancer, colon cancer and many other cancers, and is also highly expressed in the cardiovascular system. It was found that the expression of miRNA-21 in heart and blood vessels decreased in cardiovascular diseases such as heart failure, hypertrophic cardiomyopathy, proliferative vascular disease and ischemic heart disease; MiRNA-21 plays an important role in the proliferation and apoptosis of vascular smooth muscle, the formation and death of cardiomyocytes and the function of myocardial fibers; MiRNA-21 participates in the pathological process of the occurrence and development of cardiovascular diseases through the loss and acquisition of function. MiRNA-21 affects cell growth, apoptosis and invasion by regulating the expression of a variety of target protein genes; The regulation of miRNA-21 is a complex program process dependent on various transcription factors and chromosomes. Programmed cell death 4 (PDCD4), phosphatase and tensin homology deleted from chromosome 10 (PTEN), budding protein factor 1 (spry1) and budding protein factor 2 (Sprouty2, spry2) are currently recognized target genes of microRNA-21. They participate in the regulation of the cardiovascular effect of miR-21, but their target genes are far more than these. MiRNA-21 is involved in the proliferation, apoptosis and differentiation of a variety of cells [3-10], and related target genes and possible related pathways are reported [11,12,13] However, its exact mechanism is not clear. In order to improve the role of BMSC in the treatment of ischemic diseases, especially coronary heart disease, it is necessary to carry out further exploration. In the first part of this subject, on the premise of up regulating the expression level of miRNA-21, it was found that the differentiation ability of BMSC was improved, and the proliferation and apoptosis were accelerated, which encouraged our in-depth research.

Ajuba is a ~ 60kDa protein with three LIM domains at its C-end. The name of LIM domain comes from the acronyms of three homologous domain proteins lin-11 (Caenorhabditis elegans), Isl1 (mouse) and mec-3 (nematode) [14]. Ajuba contributes to the formation and maintenance of cell-cell connection and plays a role in cell division and cell migration. Studies on the biological function of ajuba mostly focus on mammals. Ajuba can shuttle back and forth between nucleus and cytoplasm. In the nucleus, ajuba protein mainly binds to various transcription factors and regulates the transcriptional activity of these transcription factors. In the cytoplasm, ajuba is generally combined with transmembrane proteins or signal proteins to mediate a wide range of biological functions, including cell cycle regulation, cell adhesion and connection, cell migration, cell proliferation and apoptosis and cell differentiation. Studies have shown that ajuba protein is involved in adipocyte differentiation [15,16]. Ajuba mediates a wide range of biological functions, but many functional mechanisms have not been revealed [17]. In this experiment, it was predicted by bioinformatics that miRNA-21 could target and regulate ajuba, and it was found that up regulating miRNA-21 inhibited ajuba expression, on the contrary, the expression of ajuba increased.

The differentiation of stem cells into different lineages involves a series of coordinated transcriptional changes and chromatin reorganization, which requires the coordination of tissue-specific transcription factors and epigenetic modifiers. A special type of transcription factor called pioneer transcription factor

is needed in the process of initiating cell programming and through epigenetic regulation mechanism [18,19,20,21]. During cardiogenesis, a variety of transcription factors cooperate with each other and integrate into the regulatory network to strictly control the transcription program and ensure the normal development of the heart. Isl1 is a pioneer factor in cardiomyocytes. On the one hand, Isl1 binds and regulates transcription factors, epigenetic modifiers and signal molecules, and is highly expressed in cardiac progenitor cells (CPCs). On the other hand, Isl1 binds to cardiomyocyte structure genes and cardiomyocyte function related genes in CPCs and is highly expressed in cardiomyocyte differentiation [22]. Controlling Isl1 activity may be a means to control the number of myocardial progenitor cells and mediate RA dependent restriction of SHF (second heart field) [23]. During zebrafish heart development, ajuba connects RA signal to Isl1 (ISL LIM homeobox 1), a key transcription factor of cardiac precursor cells Isl1 is a member of the islet subfamily of the LIM family of transcription factors, which plays an important role in regulating the development of the nervous system, pancreas and heart. Many animal model studies have confirmed that Isl1 is in the first heart field (FHF) and the second heart field (SHF) It is suggested that Isl1 (+) progenitor cells may be the common progenitor cells of two types of cardiac regions. Once the progenitor cells differentiate, the Isl1 expression will decrease. The latest research shows that BMSC transfected with Isl1 gene can significantly promote the differentiation of bone marrow mesenchymal stem cells into cardiomyocyte like cells [24] In this experiment, it was found that the expression of Isl1 increased during the induced differentiation of BMSC cells. Similarly, when the expression of ajuba increased, the expression of Isl1 was inhibited, showing the decline of the ability of BMSC cells to induce differentiation. Through this part of experiments, we preliminarily proposed to up regulate miRNA-21, target inhibit ajuba expression, and enhance Isl1 expression to promote BMSC differentiation, that is, miRNA-21 regulates BMSC differentiation through targeting /Isl1 axis affects the differentiation of bone marrow mesenchymal stem cells into cardiac muscle like cells, which may play a role in the regeneration and differentiation of bone marrow mesenchymal stem cells and the improvement of cardiac function by BMSC transplantation in the treatment of heart diseases.

Declarations

Acknowledgements

This study was supported by a grant from Renji Hospital Affiliated to Medical College of Shanghai Jiaotong University,Clinical scientific research innovation cultivation fund(PYII20-06).

Statement

We made a clear statement to confirm that all methods were carried out in accordance with ARRIVE guidelines 2.0.

We made a clear statement that study protocol and experimental protocols were approved by the ethics and experiment committee of Shanghai Jiaotong University.

Data availability

We confirmed that all methods used in the article were carried out in accordance with operation and experiment guidelines and regulations.

Ethics declarations

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The institutional review board of Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine,approved the use of a prospectively maintained database of animals.

Conflicts of Interest:

The authors have no conflicts of interest to declare.

References

1. Zhang C. MicroRNomics: a newly emerging approach for disease biology. Physiological Genomics. 2008; 33(2):139–147.
2. Rakesh C. Kukreja, Chang Yin, and Fadi N. Salloum.MicroRNAs: New Players in Cardiac Injury and Protection Mol Pharmaco 2011;l80:558–564
3. Fakhry M, Hamade E, Badran B, et al. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. World J Stem Cells 2013; 5: 136-148.
4. Zou DD, Tang XB. Epithelial-mesenchymal transition and cancer stem cells. Zhonghua Bing Li Xue Za Zhi 2013; 42: 62-65.
5. Zhang R, Wang D, Xia Z,et al. The role of microRNAs in adipocyte differentiation. Front Med 2013; 7: 223-230.
6. Ceppi P, Peter ME. MicroRNAs regulate both epithelial-to mesenchymal transition and cancer stem cells. Oncogene 2014; 33: 269-278.
7. Kang HY. MicroRNA-21 regulates stemness in cancer cells. Stem Cell Res Ther 2013; 4: 110.
8. Bakhshandeh B, Hafzi M, Ghaemi N, Soleimani M. Downregulation of miRNA-221 triggers osteogenic differentiation in human stem cells. Biotechnol Lett 2012; 34: 1579-1587.
9. Han J , Yang T , Gao J , et al. Specific microRNA expression during chondrogenesis of human mesenchymal stem cells. Int J Mol Med. 2010;25(3):377-84.
10. Yu Nie, Bian-Mei Han, Xue-Bin Liu, et al. Identification of MicroRNAs Involved in Hypoxia- and Serum Deprivation-Induced Apoptosis in Mesenchymal Stem Cells. Int J Biol Sci. 2011;7(6):762-8.

11. Mei Y, Bian C, Li J, et al. miR-21 modulates the ERK-MAPK signaling pathway by regulating SPRY2 expression during human mesenchymal stem cell differentiation. J Cell Biochem 2013; 114: 1374-1384.
12. SK Singh, MN Kagalwala, J Parkerthornburg, et al. REST maintains self-renewal and pluripotency of embryonic stem cells. Nature 2008,453(7192):223-7.
13. Qingling Zhou, Qiang Sun, Yongshan Zhang, et al. Up-Regulation of miRNA-21 Expression Promotes Migration and Proliferation of Sca-1+ Cardiac Stem Cells in Mice. Med Sci Monit, 2016; 22: 1724-1732.
14. Zheng QH，Zhao Y，The diverse biofunctions of LIM domain proteins: determined by subcellular localization and protein-protein interaction.Biol Cell,2007,99: 489-502.
15. Tang QQ，Lane MD， Adipogenesis: from stem cell to adipocyte.Annual ，eview of Biochem, 2012,81: 715-736.
16. MacDougald OA，Mandrup S. Adipogenesis: forces that tip the scales Trends in Endocrinology ， amp.Metabolism,2002,13: 5-11.
17. XiangYang Sun, ShuNing Liu, ZhaoYuan Hou,et al. Research progress of LIM protein ajuba.Pharmaceutical Biotechnology,2017，24(3) : 263-268，
18. Soufifi, A. et al. Pioneer transcription factors target partial DNA motifs on nucleosomes to initiate reprogramming. Cell 161, 555–568 (2015).
19. Spitz, F. & Furlong, E. E. Transcription factors: from enhancer binding to developmental control. Nat. Rev. Genet. 13, 613–626 (2012).
20. Swinstead, E. E. et al. Steroid receptors reprogram FoxA1 occupancy through dynamic chromatin transitions. Cell 165, 593–605 (2016).
21. Zaret, K. S. & Carroll, J. S. Pioneer transcription factors: establishing competence for gene expression. Genes Dev. 25, 2227–2241 (2011).
22. Rui Gao, Xingqun Liang, Sirisha Cheedipudi,et al. Pioneering function of Isl1 in the epigenetic control of cardiomyocyte cell fate.Cell Research (2019) 29:486-501.
23. Stamm C, Westphal B, Kleine HD, et al. Autologous bone-marrow stemcell transplantation for myocardial regeneration. Lancet 2003; 361: 45-46.
24. Xingxiao Li, Xiaohong Li, Qiuxiong Lin, etc.Transfection of Isl1 gene promotes the differentiation of bone marrow mesenchymal stem cells into cardiac muscle like cells. Chinese Journal of pathophysiology，2012，28(1):131-135.

Figures

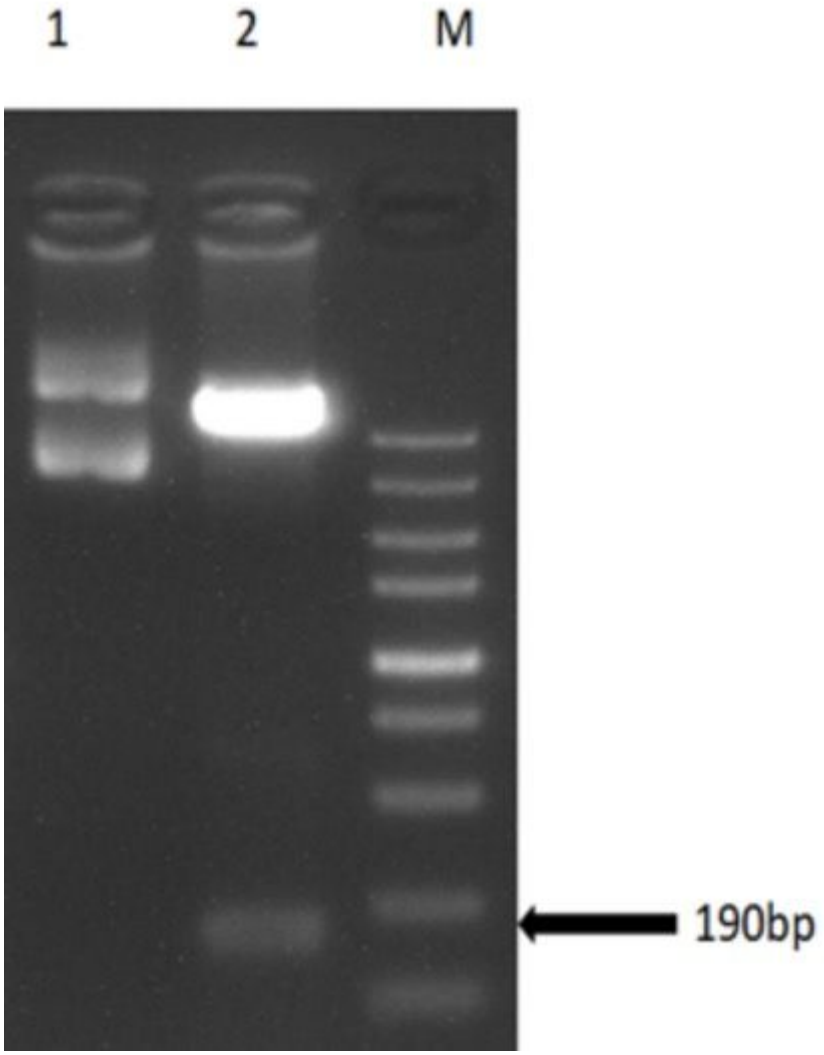

Figure 1

results of digestion and identification of miRNA-21 sponge vector

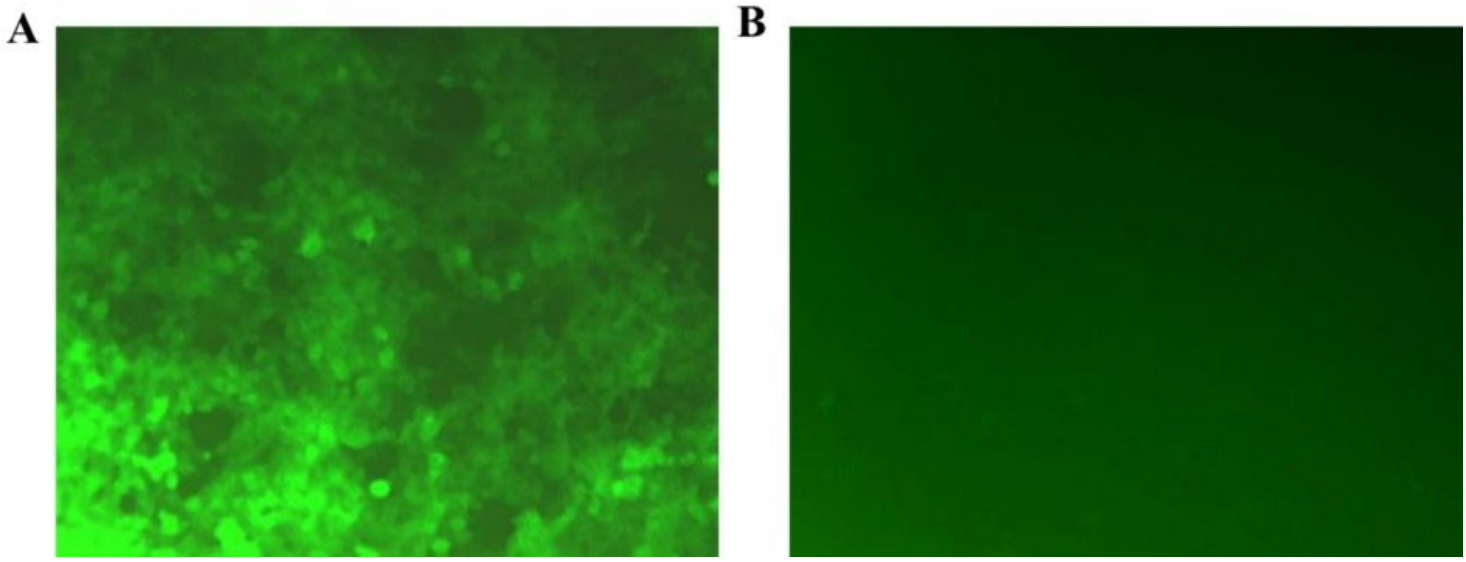

Figure 2

titer determination of lentivirus stock solution

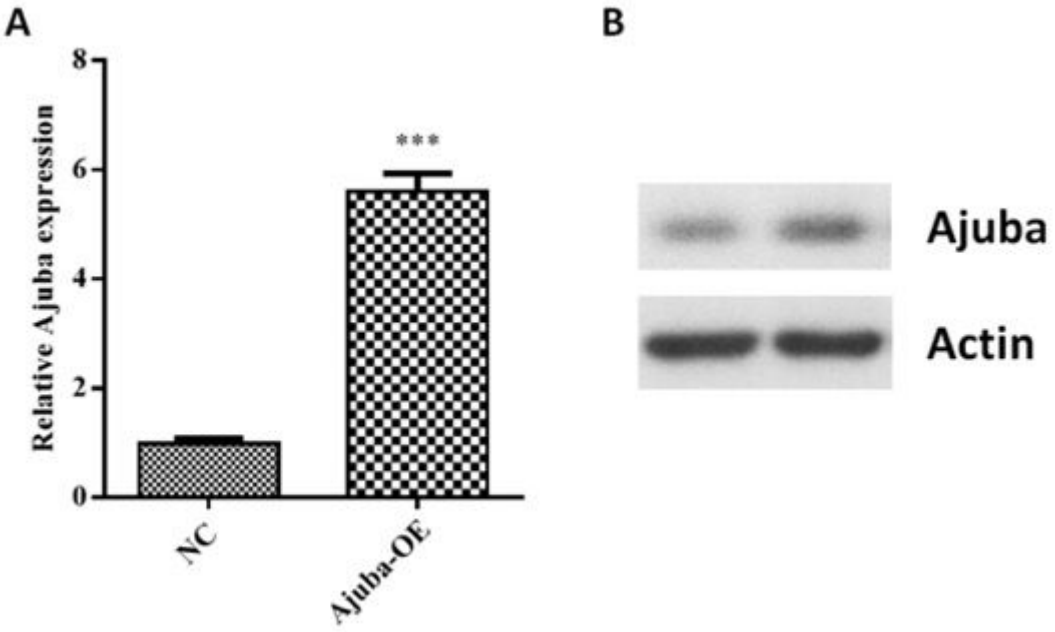

Figure 3

miR-21 sponge significantly inhibits miR-21 expression in BMSC cells

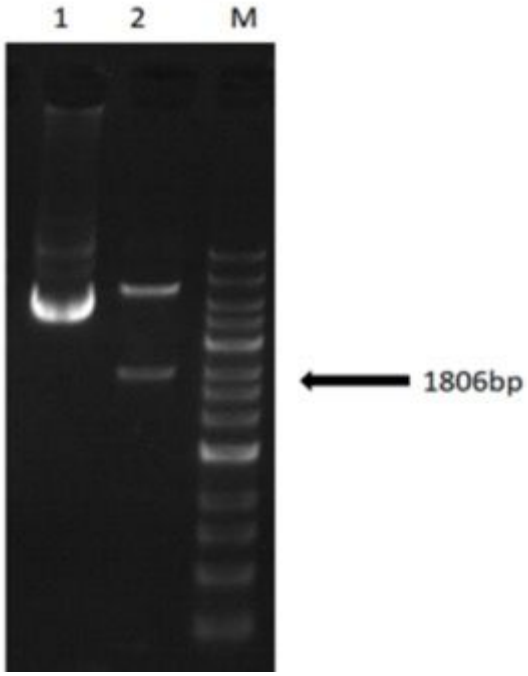

Figure 4

results of enzyme digestion of ajuba OE vector

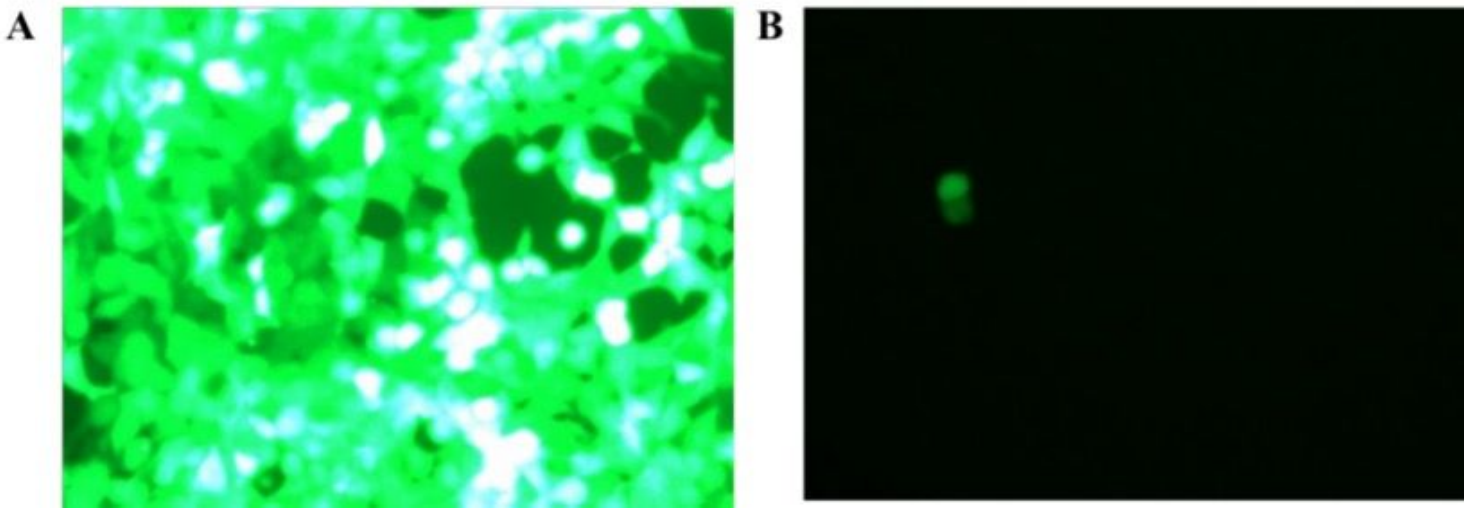

Figure 5

titer determination of lentivirus stock solution A:Hole 1: contains 10×10-3ml lentivirus solution; B:Hole 6: contains 10×10-8ml lentivirus solution.

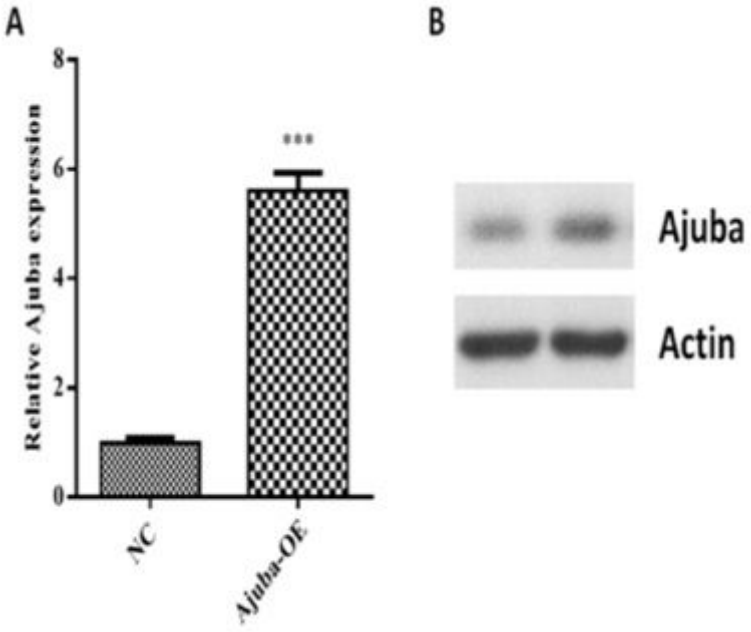

Figure 6

detection of overexpression of BMSC infected by ajuba OE lentivirus A

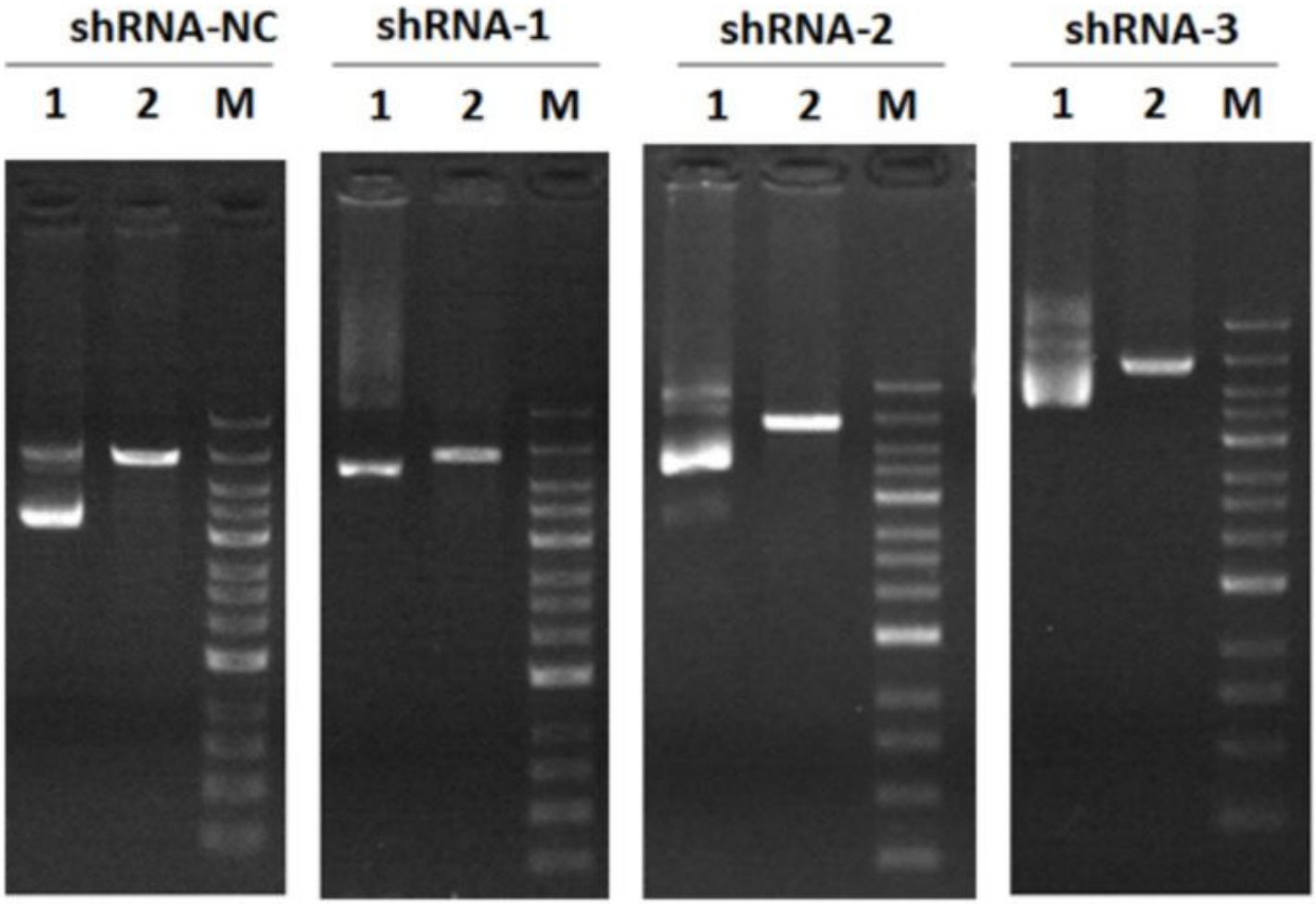

Figure 7

results of enzyme digestion of ajuba OE vector

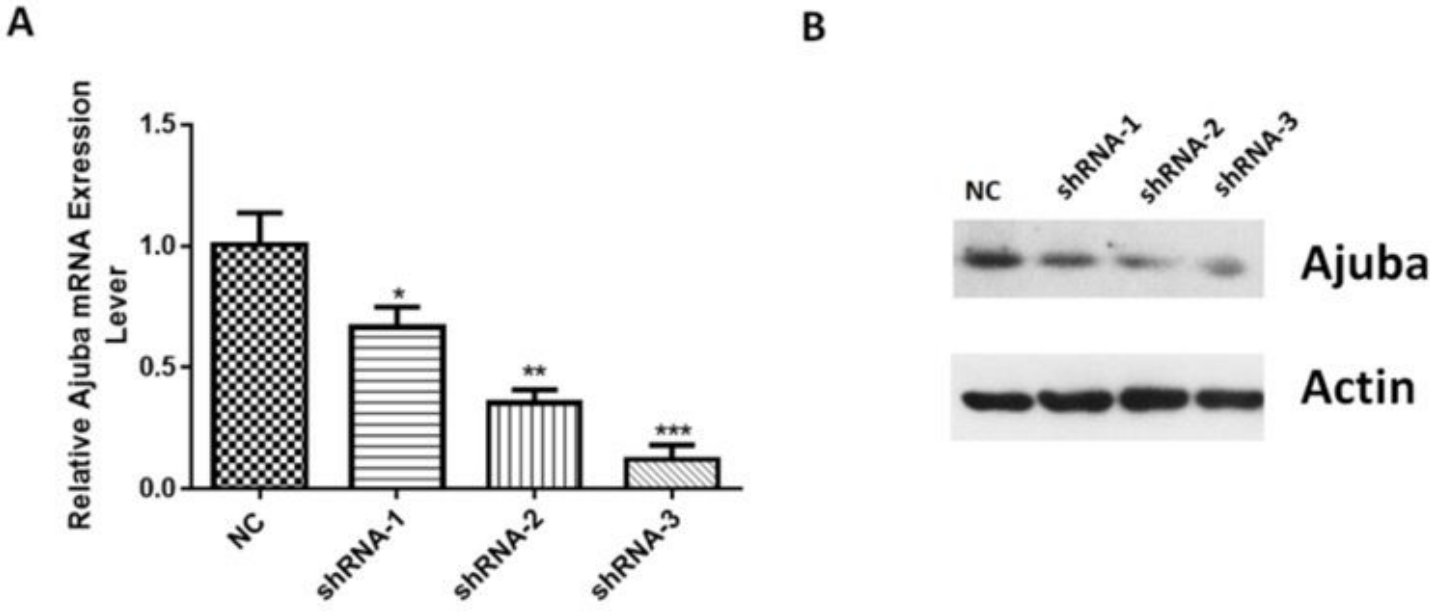

Figure 8

overexpression detection of BMSC infected by ajuba shRNA lentivirus

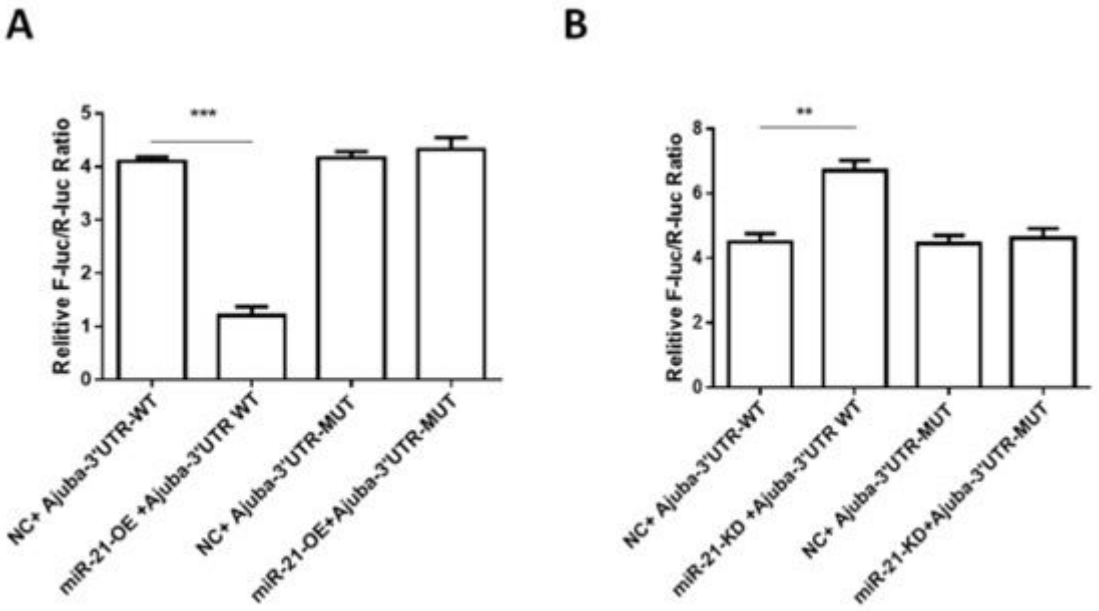

Figure 9

detection results of dual luciferase reporter gene

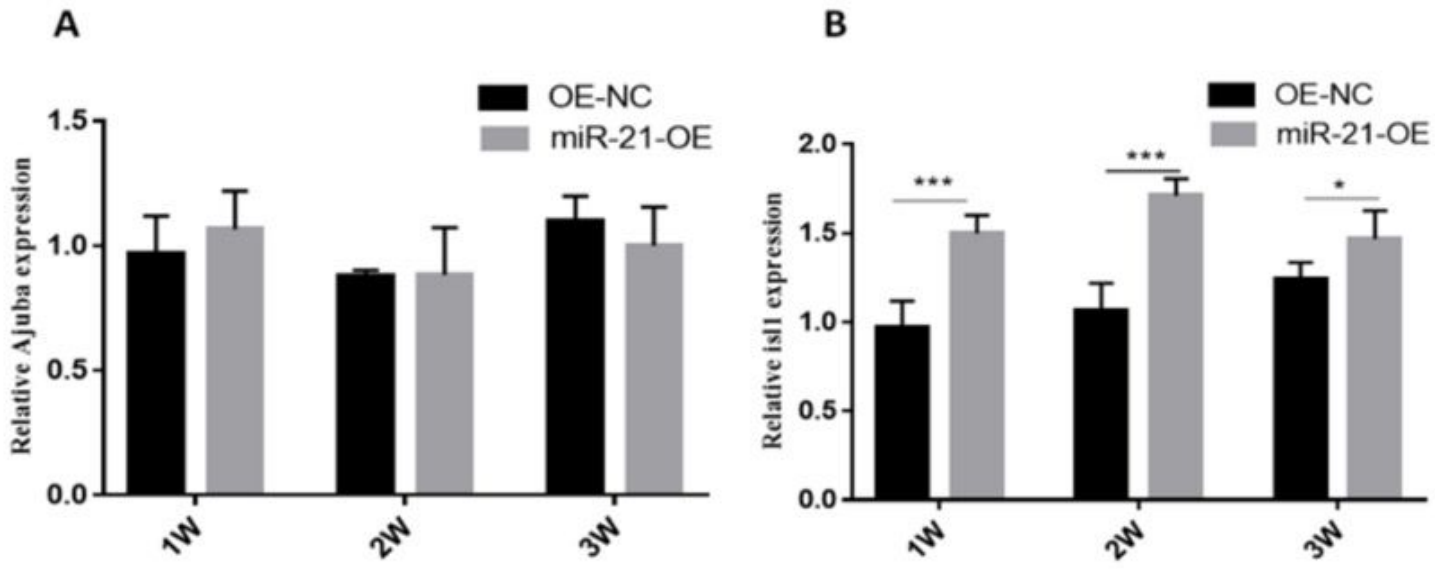

Figure 10

detection of ajuba and Isl1 mRNA expression in BMSC during mir-21-oe lentivirus infection and differentiation

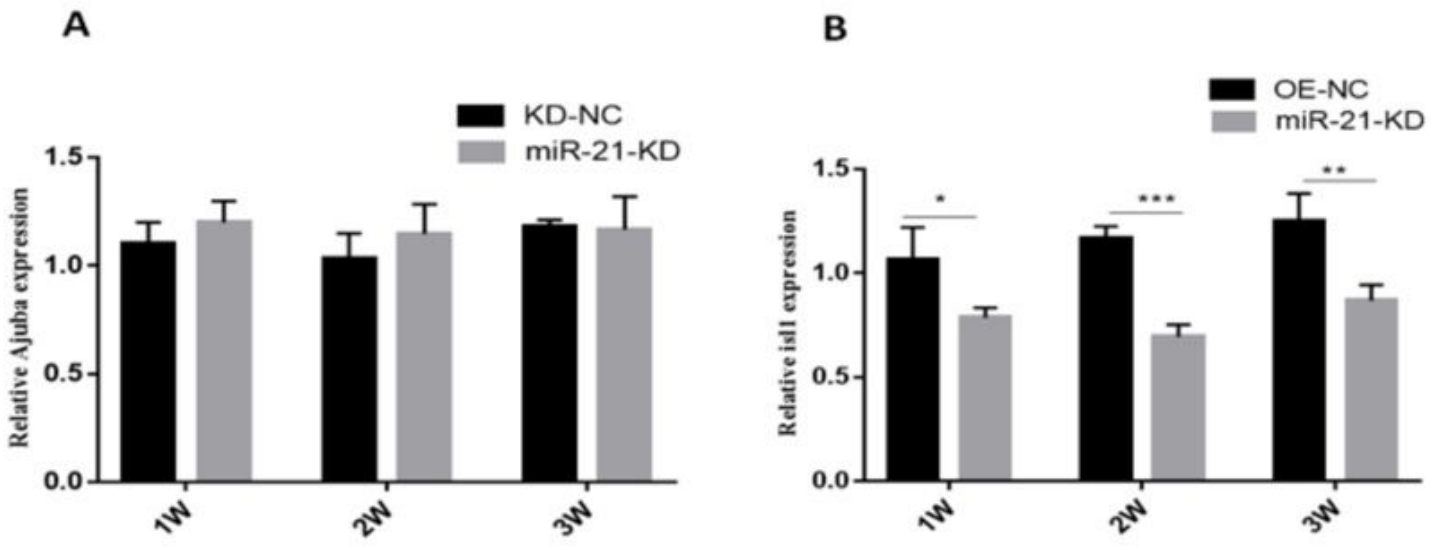

Figure 11

detection of ajuba and Isl1 mRNA expression in BMSC during mir-21-kd lentivirus infection and differentiation

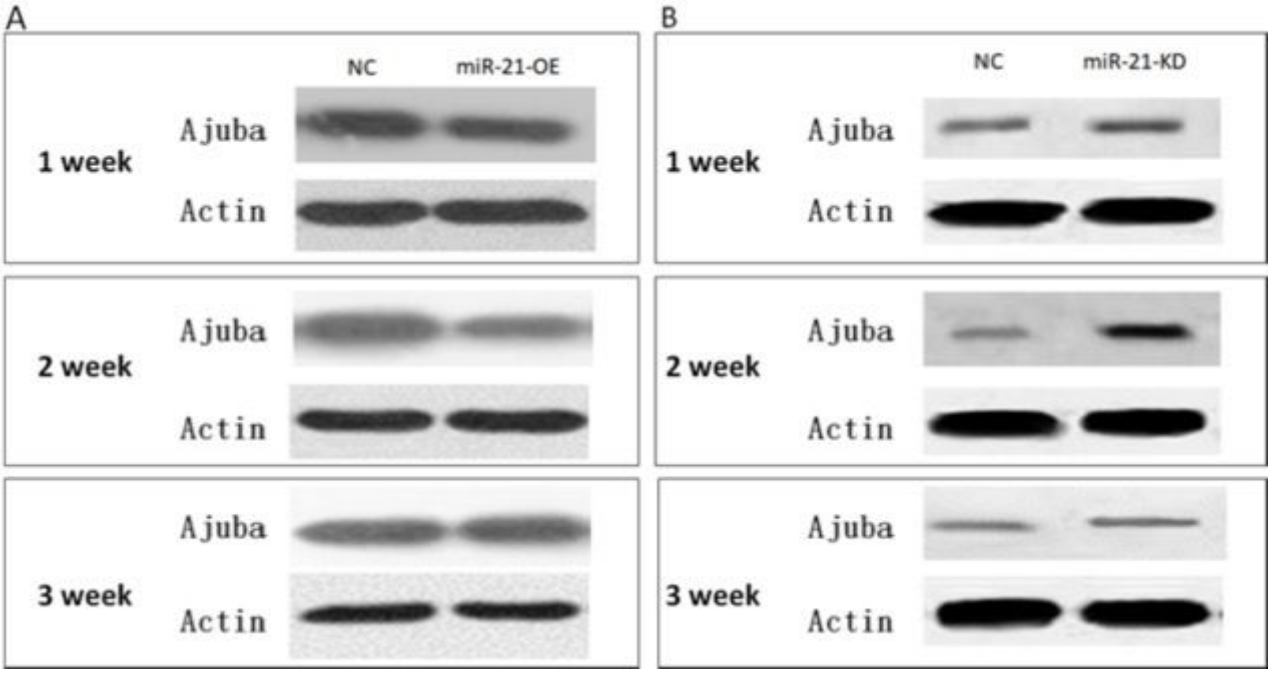

Figure 12

detection of ajuba protein expression in BMSC during lentiviral infection and differentiation of mir-21-OE and mir-21-KD

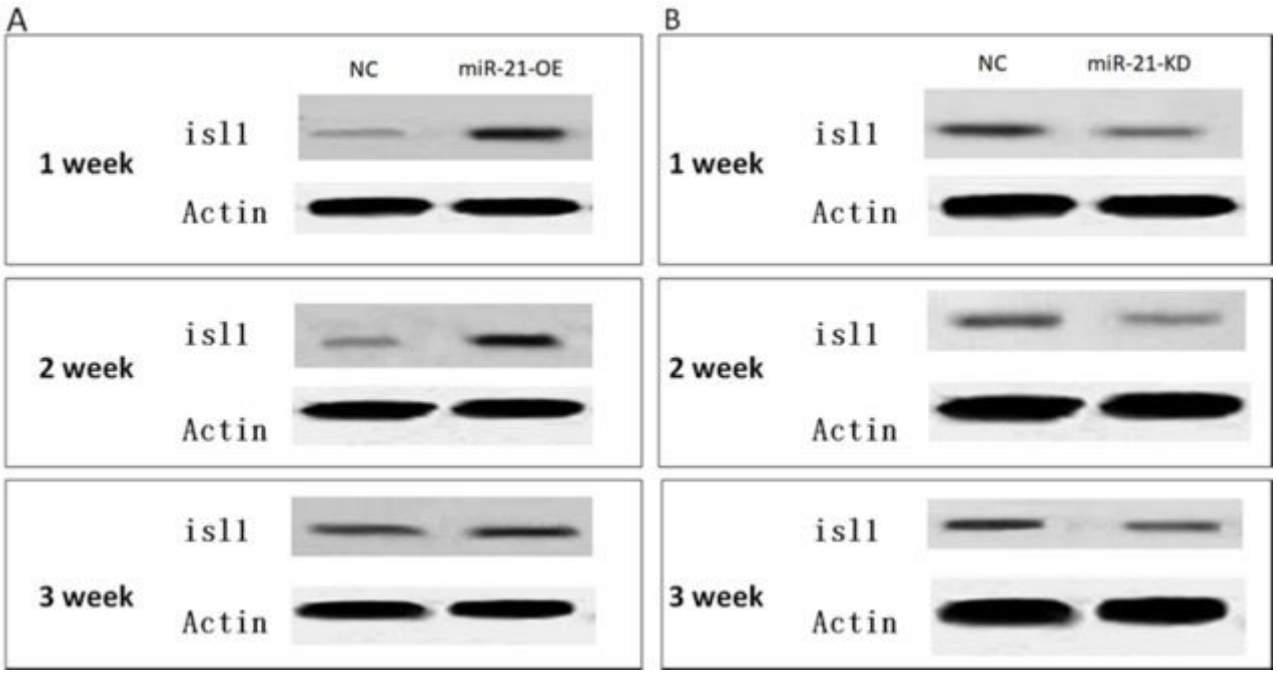

Figure 13

detection of Isl1 protein expression in BMSC during lentiviral infection and differentiation of mir-21-OE and mir-21-KD

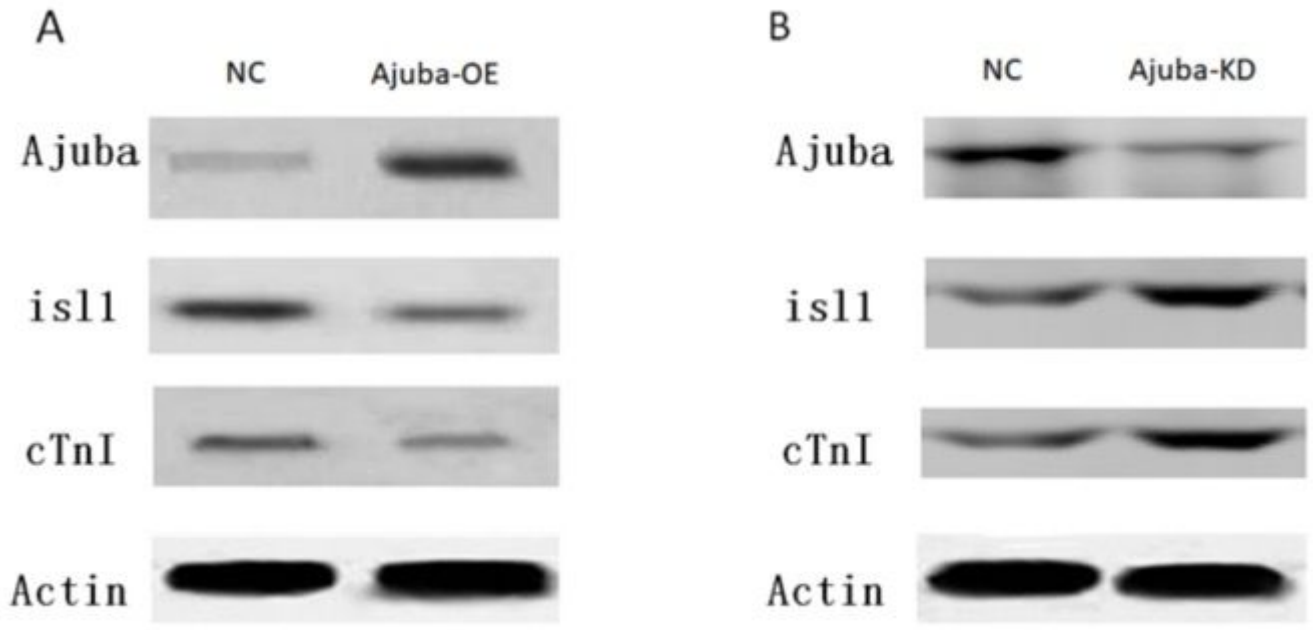

Figure 14

detection of ajuba, Isl1 and cTnI protein expression in BMSC after ajuba overexpression and ajuba interference

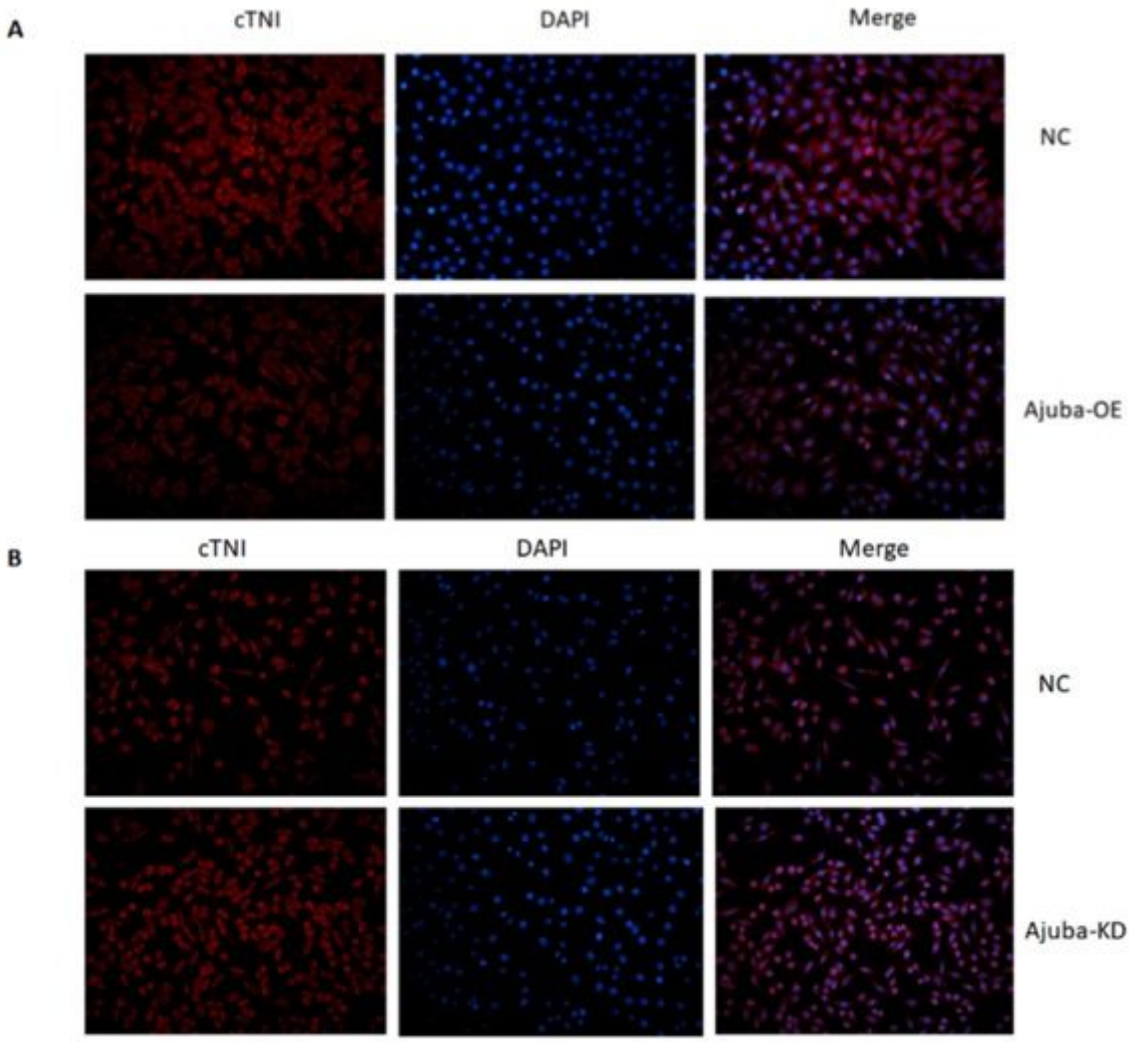

Figure 15

immunofluorescence detection of BMSC and cTnI in the process of infection and differentiation after ajuba overexpression and ajuba interference

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- schematicdiagram.png