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Original Article

MiR-126 inhibits the proliferation of myocardial fibroblasts by regulating EGFL7-mediated EGFR signal pathway

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Abstract: Objectives: The aim of this study was to investigate miR-126 for its effect on cardiac fibroblasts proliferation and regulatory mechanism. Methods: Isolation and culture of rats cardiac fibroblasts cells (CFs) were conducted and CFs in the experiment were divided into CFs, mimics, inhibitors, the NS-MiRNA group. Then relevant genetic sequences of miR-126 mimics, inhibitors and NS-the miRNA transfected into CF cells, respectively. The effect of miR-126 on the proliferation of CFs was tested by CCK8 method. And the effects of miR-126 on cell cycle of CFs were measured by flow cytometry method. Meanwhile, Immunofluorescence Tests were done for measuring the effects of miR-126 on expression of α-SMA and EDU. Finally, mRNA and protein expression levels of EGFL7, AKT, pAKT, ERK, pERK of each group of cells, respectively, were measured by fluorescence quantitative PCR (fqPCR) and protein printing. Results: The miR-126 agonist and inhibitor were successfully constructed, respectively. And results of CCK8 test showed that miR-126 mimics group inhibited effectively proliferation of CFs while miR-126 inhibitor group promoted the proliferation of CFs. Results of flow cytometry showed that compared with those of CFs group, cells of G_1 phase in the mimics group was significantly increased ($P < 0.05$), but cells proportion of S phase in the mimics group reduced ($P < 0.05$). Compared with those of CFs group, cells of G_1 phase in the inhibitors group was significantly reduced ($P < 0.05$), cell proportion of S phase in the inhibitors group increased ($P < 0.05$), accordingly. Results of immunofluorescence test indicated that compared with those of CFs group, expression levels of actin α-SMA and cell proliferation markers protein EDU in the mimics group were significantly lower ($P < 0.05$); however, expression levels of α-SMA, EDU in the inhibitors group were significantly increased ($P < 0.05$). Results of qPCR and results of western blotting manifested that compared with those of CFs group, mRNA expression of EGFL7, AKT, pAKT, ERK, pERK and protein expression levels in the mimics group significantly raise, respectively ($P < 0.05$); but mRNA expression levels of EGFL7, AKT, pAKT, ERK, Perk and protein expression levels in the inhibitor group were significantly lower ($P < 0.05$). Conclusions: MiR-126 inhibits the proliferation and differentiation of myocardial fibroblasts, possibly through increasing the expression of EGFL7 and activating AKT/ERK signaling pathway.

Keywords: MiR 126, cardiac fibroblasts, EGFL7, AKT/ERK, signaling pathways, proliferation

Introduction

Myocardial infarction (MI) or acute myocardial infarction (AMI) is a major cause of death worldwide and is defined as massive cell damage (necrosis and apoptosis) [1]. The prevalence of MI in China has happened a dramatic change as the Chinese economy developed over the last two-decade years and the mortality of ischemic heart disease has doubled, which means that more than 1 million people die of myocardial infarction each year [2]. The phenomenon appears to be more and more common for the increasing lifestyle-related stress and the growing aging population. In addition, because of coronary artery disease, particularly coronary atherosclerosis, other recognized risk factors include smoking, obesity, diabetes and so on lead to myocardial infarction [3]. Due to the regeneration capability of cardio-myocytes, the current AMI therapies mainly focus on cardiovascular remodeling to prevent further impairment of the myocardium and to restore cardiac functions [4].

Table 1. PCR primers design

miRNA	Sequence
miRNA 126-mimic	5'-UCGUACCGUGAGUUAAUGCG-3'
miRNA 126-inhibitors	5'-CGCAUUAUUACUCACGGUACGA-3'
NS-miRNA	5'-UUCUCCGAACGUGUCACGU-3'

MicroRNAs (miRNAs) are 18- to 25-nucleotide (nt) small RNA species that serve as key regulators of translation. Over the last decade or so, a serial of new discoveries have identified important roles for miRNAs in human health and disease. MiRNA expression levels can change accordingly in response to variation of inside or outside cell environment, which in turn modulates the entire regulatory pathways and fundamentally change relevant cellular function [5]. MiRNAs have a clearly significant regular role in cardiovascular function [6]. In recent years several protective regulators in cardio-myocytes which include miR-24, miR-214 and so on have been reported [7, 8]. Among these miRNAs, miR-126 is highly expressed in vascular endothelial cells and is reported to play a protective role in post-infarct remodeling and other pathophysiological processes by activating the vascular endothelial growth factor (VEGF) [9, 10]. For the more, it has been discovered that miR-126 can up regular angiogenesis processes of gastric, oral, and metastatic colorectal cancer through the up-regulation of VEGF, which suggests a potential therapeutic strategy for preventing the neoplasm growth process via antagonizing the up-regulation of miR-126 in tumor tissues [11, 12]. VEGFs are important angiogenic molecules like EGFL7, AKT, pAKT, ERK and so on and play a key role in vascular growth and regulation. Thereby, the down-regulation of miR-126 enhances cardiac self-repair in the infarcted heart and protects cardio-myocytes from ischemia injury [13]. In addition, serum miR-126 is believed to be a prospective biomarker in the AMI therapy and its down-modulation is usually observed in AMIs [14]. Related studies have provided evidences for potential angiogenesis mediated by miR-126 *in vitro* and *in vivo* experiments [15]. Therefore, we suspected that the interaction between miR-126 and VEGF is likely to have substantial influence on myocardia angiogenesis processes and may be an alternative therapeutic target for AMI.

The present study explored this potential mechanism in the treatments of miR-126 mimics transfection to assess whether miR-126 mimics could protect the cardiac tissues once AMIs occurred.

Materials and methods

Cell extraction and cell culture

SD rats with an average age of 2-3 months and an average weight of 225-350 g were obtained to construct acute myocardial infarction (AMI) models and from these rats CFs were isolated.

CFs from rats was isolated by using the density gradient centrifugation method. All cells were cultured in Dulbecco medium with 10% fetal bovine serum at 37°C in an incubator with 5% CO_2. Proliferation of CF cells was inspected using 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide assay and changes in cellular morphology were observed under a microscope.

All rat cells in the experiment were further subdivided into 4 groups: CFs group, mimics group, inhibitors group and the negative control NS-miRNA group and each group set up 3 holes in the culture plates.

Isolation and cultivation of CFs

Newborn 1-3 days SD rats provided by the laboratory of molecular biology were executed after 75% alcohol disinfection. Isolated hearts and clipped apical tissues of rats on sterile operating table. The broken apical tissues were digested and isolated by 1.25% trypsin (Solarbio biological technology co., LTD., Shanghai, China). Then isolated cells were filtered by 200 mesh sterile stainless steel mesh and thesupernatant was removed by centrifugation and the precipitate came into cell suspension. Cell suspension was inoculated on the DMEM culture medium containing 10% calf serum (Solarbio biological technology co., LTD., Shanghai, China) in the cell bottles by differential adherence method. And the culture medium was cultivated in sterile, constant temperature incubator at 37°C and the atmosphere of 5% CO_2 saturated humidity in 60-90 minutes. Removal of myocardial cells was done by differential adhesion method and the rest of the cells are CFs. When the adherence fibroblasts cells were confluence above 90%, then those were digested and went down to posterity.

Table 2. Primer sequences for luciferase reporter experiments

PCR primers sequences	Forward (5'-3')	Reverse (5'-3')
miRNA 126	ACACTCCAGCTGGGTCGTACCGTGAGTAAT	TGGTGTCGTGGAGTCG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
EGFL7	CTGCTGATGTGGCTTCTGGT	GAGGAAGGGCTGGTACACAC
AKT	CCTCTCTACCGCCGTTG	CCACACAGGGTTCTTC
ERK	GAGCACAGAGCCTCGCCTTT	ATCCT TCTGACCCATGCCCA
GAPDH	AGATGCATATTCGGACCCAC	CCTCATGTTTGTGCAGGAGA

EGFL7: epidermal growth factor-like domain 7; AKT: protein kinase B; ERK: extracellular signal-regulated kinase; GAPDH: glyceraldehyde-phosphate dehydrogenase.

Finally, P_4 cells were used for subsequent experiments.

CFs transfection

According to miRbase database, the sequences of rats miRNA 126 mimics, inhibitors and NS-miRNA were shown in **Table 1**. The sequences were synthetized by Shanghai Yingjun company. Frist of all, CF cells in the logarithmic phase were inoculated in 6-holes culture plates and when cell fusion rate was 70% transfection was done. Secondly, 0.03 mol miRNA-126 mimics, inhibitors and NS-miRNAs reagent, separately, were mixed with 50 mul Opti-MEM serum-free medium. And then the mix reagents were still standing for 5 min. Then, 0.5 μl Lipofectamine™ 2000 reagent was joined to above mix reagents with standing for 30 min, which formed miRNA-lipofectamine compounds. Finally, the miRNA-lipofectamine compounds were transfected into CFs for 24 h and continue.

CCK8 assay

CCK8 assay was used to analyze the proliferation of myocardial fibroblasts for each group. The cell suspension after 48 h transfection was vaccinated in 96-well plates with the concentration of 100 μl per hole and incubated for 24 h. And then put culture plate in 37°C cell culture incubator. After that, each group randomly toke three holes in the 12, 24, 36, 48 and 72 h after incubation, respectively. And each hole was added 10 μl CKK reagent followed by 2 h incubation. Finally, the absorbance value at the 450 nm wave length was measured by ELIASA reader to draw the cell growth curve. The experiments repeated 3 times.

Immunofluorescent identification

Placed the logarithmic growth phase cells after transfection inoculation to the 24 hole plate glass and When the cell fusion rate was 90%, used 4% paraformaldehyde (Solarbio biological technology co., LTD., Shanghai, China) to fix cells 10 min. Then Containing 5% calf serum and 0.25% Triton X-100 (Solarbio biological technology co., LTD., Shanghai, China) sealing fluid was used to handle cells 30 min. Then Joined the rabbit anti-human α-SMA primary antibody (diluted multiples, 100), (Solarbio biological technology co., LTD., Shanghai, China) and rabbit anti-human EDU primary antibody (diluted multiples, 100), (Solarbio biological technology co., LTD., Shanghai, China), respectively. The above reagents stayed overnight in 4°C and then were rinsed by the reagent TBSB with the condition of 3 times/5 min. Added the horseradish peroxidase labeled goat anti-rabbit secondary antibody (MB005, Solarbio biological technology co., LTD., Shanghai, China) and diluted mixtures with purified water in the proportion of 1:100. Incubation was implemented at room temperature in 1 h and DAPI staining was done at room temperature and avoided light in 10 min. Finally, the reagent was fixed with glycerin and observed and taken pictures under the fluorescence microscope. The above experiments repeated by 3 times.

Flow cytometry for analysis of cell cycle

48 h after transfection cells were done with 0.25% trypsin digestion, and count 1×10^6 cells with killed by 12 h 75% ethanol treatment. And the reagent was joined the final concentration of 50 mu g/ml of RNA enzymes with reaction at room temperature for 1 h. Propidium Iodide (PI) was joined with avoiding-light staining for 30 min. Using flow cytometry instrument analyzing the cell cycle, the experiment repeated 3 times.

RNA isolation and RT-PCR

Total RNA from tissues or cells were extracted using TRIzol reagent. The Rever Tra Ace qPCR

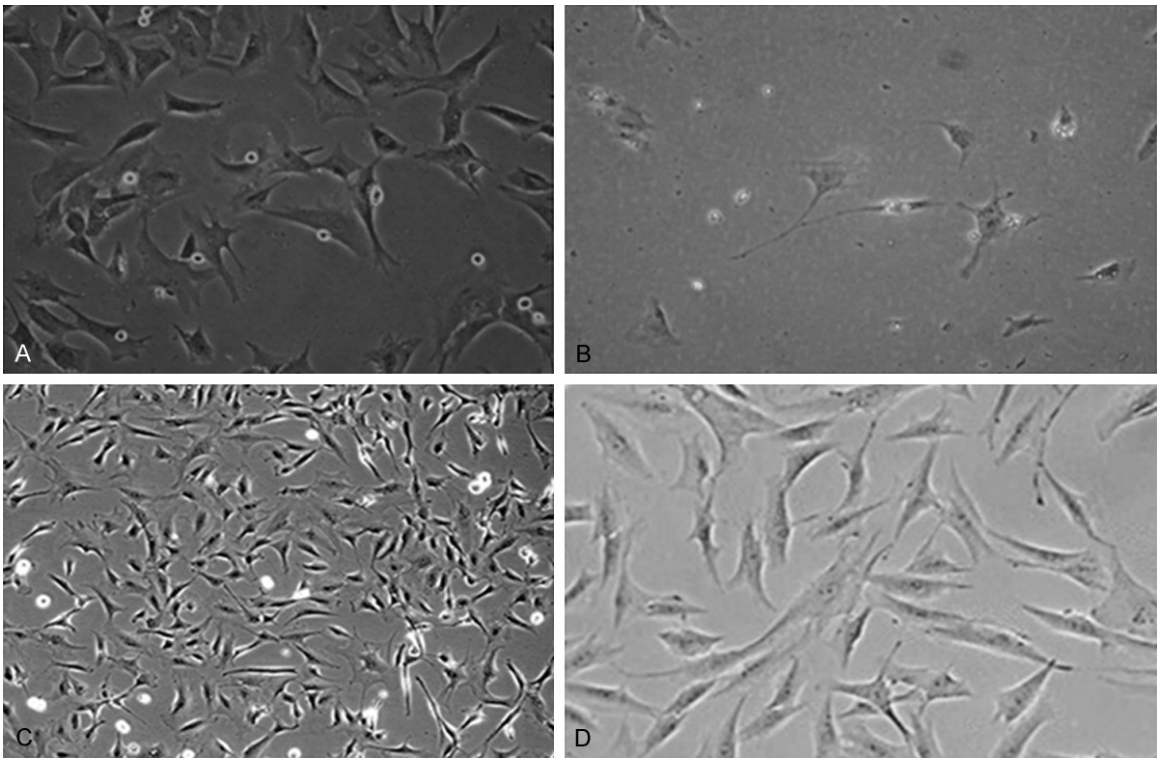

Figure 1. The morphological changes of CFs (*100). A: CFS, B: Mimics group, C: Inhibitors group, D: NS-miRNA group.

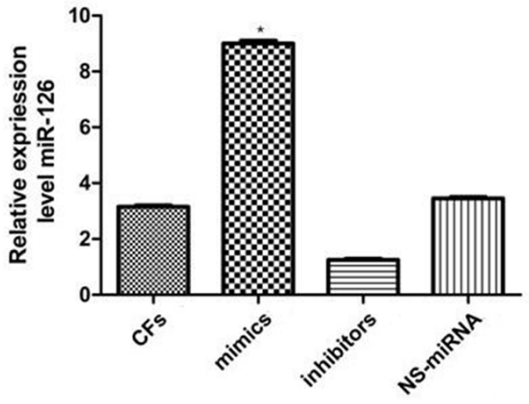

Figure 2. miR-126 expression in the RMSCs by QRT-PCR. Note: *P < 0.05, compared with CFs group.

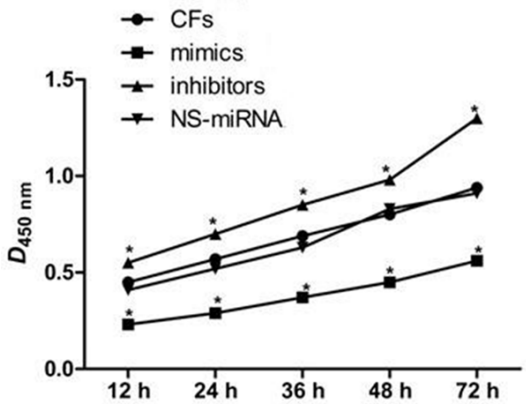

Figure 3. Effect of miR-126 on proliferation of CFs by CCK8. Note: *P < 0.05, compared with CFs group.

RT Kit was used to reversely transcribe total RNA into cDNA and real-time PCR (RT-PCR) was performed using THUNDERBIRDSYBR® qPCR Mixand the CFX96 Touch RealTime PCR Detection System. The relevant primers are listed in **Table 2**. Target gene expression levels were normalized to those of the control gene (GADPH) and were calculated using the method of $2^{-\Delta\Delta}$CT.

Western blot

Tissues and cells were harvested and lysed by radio immunoprecipitation assay (RIPA) buffer. Total protein was separated and evaluated by the Bradford method [20]. Then, the total protein was denatured in boiling water and transferred onto polyvinylidene fluoride (PVDF) membranes when sodium dodecyl sulfate-poly-

Table 3. Effects of miR-126 on cell cycle of CFs through flow cytometry

	G_0/G_1	G_2/M	S
CFs	43.12 ± 4.31	13.12 ± 2.31	42.12 ± 4.31
Mimics	69.12 ± 5.31*	15.12 ± 4.01	28.12 ± 5.31*
Inhibitors	29.02 ± 3.31*	14.12 ± 4.01	68.32 ± 2.12*
NS-miRNA	45.12 ± 4.11	12.02 ± 4.31	41.12 ± 4.11

*P < 0.05, compared with CFs group.

acrylamide gel electrophoresis (SDS-PAGE) was completed. The membranes were blocked in Tris-Buffered Saline Tween (TBST) with 5% skim milk for 1 h and then they were treated with primary antibodies against VEGF-A (1: 800 dilution) at 4°C overnight. After membranes were washed, they were incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-goat, 1:2000 dilutions). Samples and reduced glyceraldehydes-phosphate dehydrogenase (GAPDH) as the endogenous control were ultimately processed with enhanced chemiluminescence and quantified using Lab Works 4.5 software.

Statistical analysis

All statistical analyses were performed with SPSS 18.0 software (Chicago, IL). Data are presented in the form of mean ± standard deviation (SD). Two-tailed Student's t-test or one way analysis of variance (ANOVA) was used to assess significant different among different groups and P < 0.05 provided evidence of statistical significance.

Results

Morphological observation of CFs

After transfection cells continued to being developed after 48 h and observed microscopically CFs group and NS-miRNA group cells. As shown in **Figure 1**, the cell present long fusiform or triangle, abundant cytoplasm, big cell body. Some cells became round with lower degree of proliferation in mimics group. Cells is woven, active and cell body expand with intensive growth in inhibitors group.

The effect of miR-126 transfection CFs

After 48 h transfection of MicroRNA-126 mimics, inhibitors and NS-miRNA of CFs, the results of QRT-PCR revealed that Mimics group of miR-126 expression level increased significantly (P < 0.05) compared with CFs group. Moreover, Inhibitors group of miR-126 expression level decreased significantly (P < 0.05) and NS-microRNA group have no significant difference (P > 0.05) compared with CFs group. Therefore, miRNA-126 mimics, inhibitors and NS-microRNAs successful infection CFs and all of they can be used for follow-up study (**Figure 2**).

The influence of miR-126 on proliferation capacity of CFs

Results of CCK8 tests showed that D-value of NS-MiRNA group had no significant change (P > 0.05) compared with those of CFs group. However, D-value of Mimics groups in 12, 24, 36, 48 and 72 h point decreased obviously (P < 0.05). That of NS-miRNA group had no significant difference (P > 0.05). D value of inhibitors group of cells in 12, 24, 36, 48 and 72 h point increased significantly (P < 0.05). In three group, the more time extended, the more cell proliferation increased (**Figure 3**). Thence, MiR-126 could significantly inhibit proliferation of CFs.

The effect of miR-126 on cell cycle of CFs

The results of flow cytometry instrument detection showed that compared with those of CFs group, mimics group cells in G_1 phase increased significantly (P < 0.05) and S period cells proportion was lower (P <0.05). While G_1 phase cells were significantly reduced in the inhibitors group (P < 0.05), S phase cell proportion increased in the inhibitors group (P < 0.05). Results of NS-MiRNA group revealed that there was no obvious difference between any periods, compared with the G_1 phase cells and S period cells proportion of CFs group (P > 0.05) (**Table 3**; **Figure 4**). The results of the experiments showed that miR-126 inhibited cell cycle progression of CFs via G_1 phase blocking and the replication activity decrease of cells.

The influence of miR-126 on protein expression of α-SMA, EDU

Myocardial fibroblasts are contractile cells that express α-smooth muscle actin (α-SMA), which, in combination with the appearance of stress fibres, is a reliable marker for the myocardial fibroblast phenotype. And cell proliferation markers protein EDU was proven for tool of research of cell cycle.

Results of immunofluorescence tests results revealed that compared with those of CFs gro-

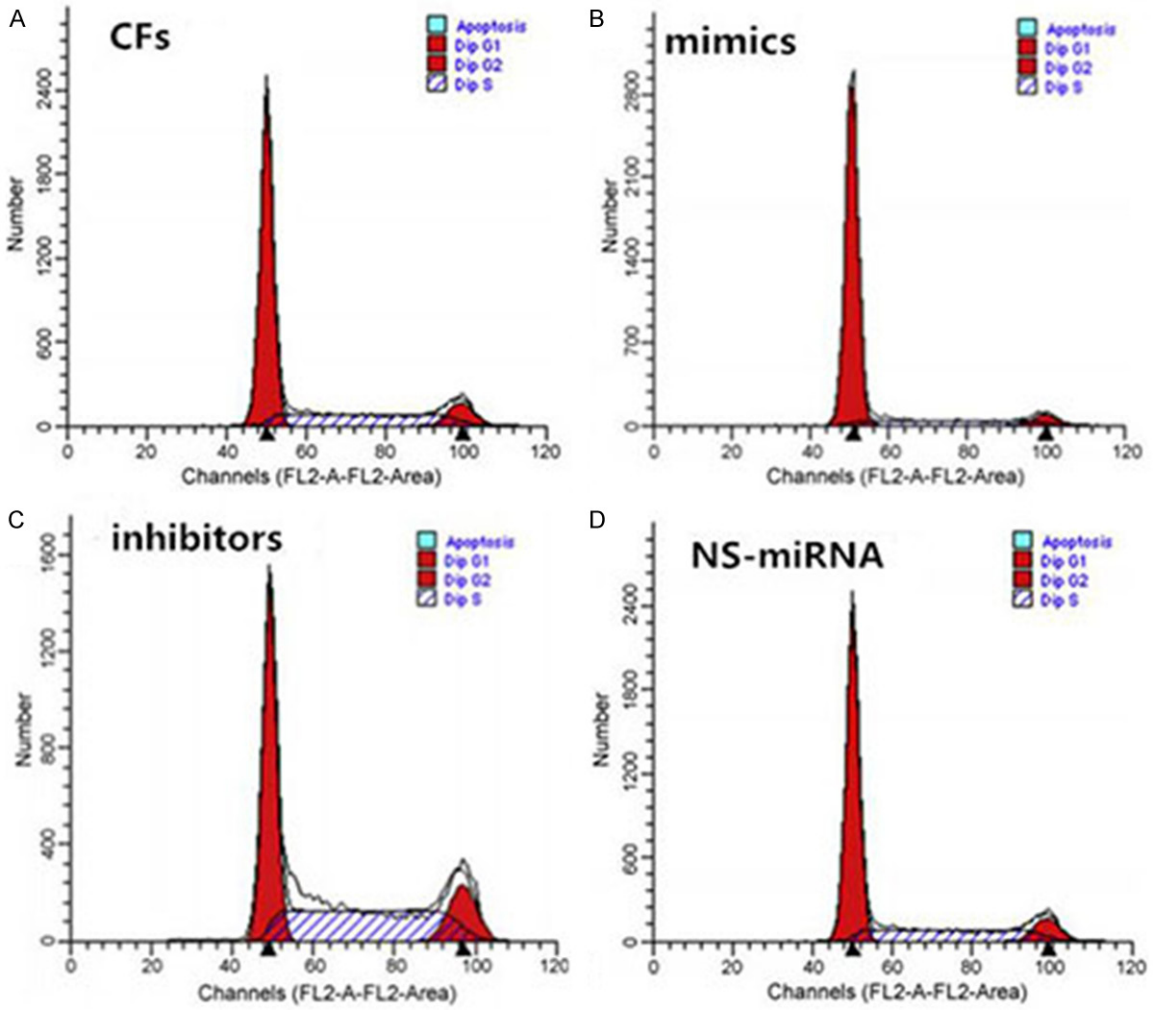

Figure 4. The effects of miR-126 on cell cycle of CFs by flow cytometry. A: CFs, B: Mimics group, C: Inhibitor group, D: NS-miRNA.

up, expression levels of α-smooth muscle, actin α-SMA, cell proliferation markers protein EDU were significantly reduced in the mimics group ($P < 0.05$). However, α-SMA, protein EDU expression levels were significantly increased in the inhibitor group ($P < 0.05$). Furthermore, α-SMA, EDU protein expression level had no significant difference in the NS-MiRNA group ($P > 0.05$). All results proved that miR-126 had proliferation inhibition effect on CFs and, to a certain extent, could inhibit the differentiation of CFs to muscle fiber cell (**Figure 5**).

Effects of miR-126 on EGFL7, AKT ERK expression

Compared with those of CFs group, expression levels of EGFL7, AKT, pAKT, ERK, pERK mRNA and protein in the mimics group were significantly increased ($P < 0.05$). The expression levels of EGFL7, AKT, pAKT, ERK, pERK mRNA and protein were significantly reduced ($P < 0.05$) in the inhibitor group. The relevant expression levels had no significant difference in the MiRNA group ($P > 0.05$) (**Figure 6**). Thence, MiR-126 could increase EGFL7 expression level, and activate AKT/ERK pathway and inhibit cardiac fibroblasts proliferation.

Discussion

This study investigated the mechanism of miR-126 in the regulator of myocardial infarction. The results revealed that MiR-126 could alleviate myocardial infarction damage through EGFL7 expression up-regulation. Epidermal Growth Factor like domain 7 (EGFL7) also known as Vascular Endothelial-statin (VE-statin)

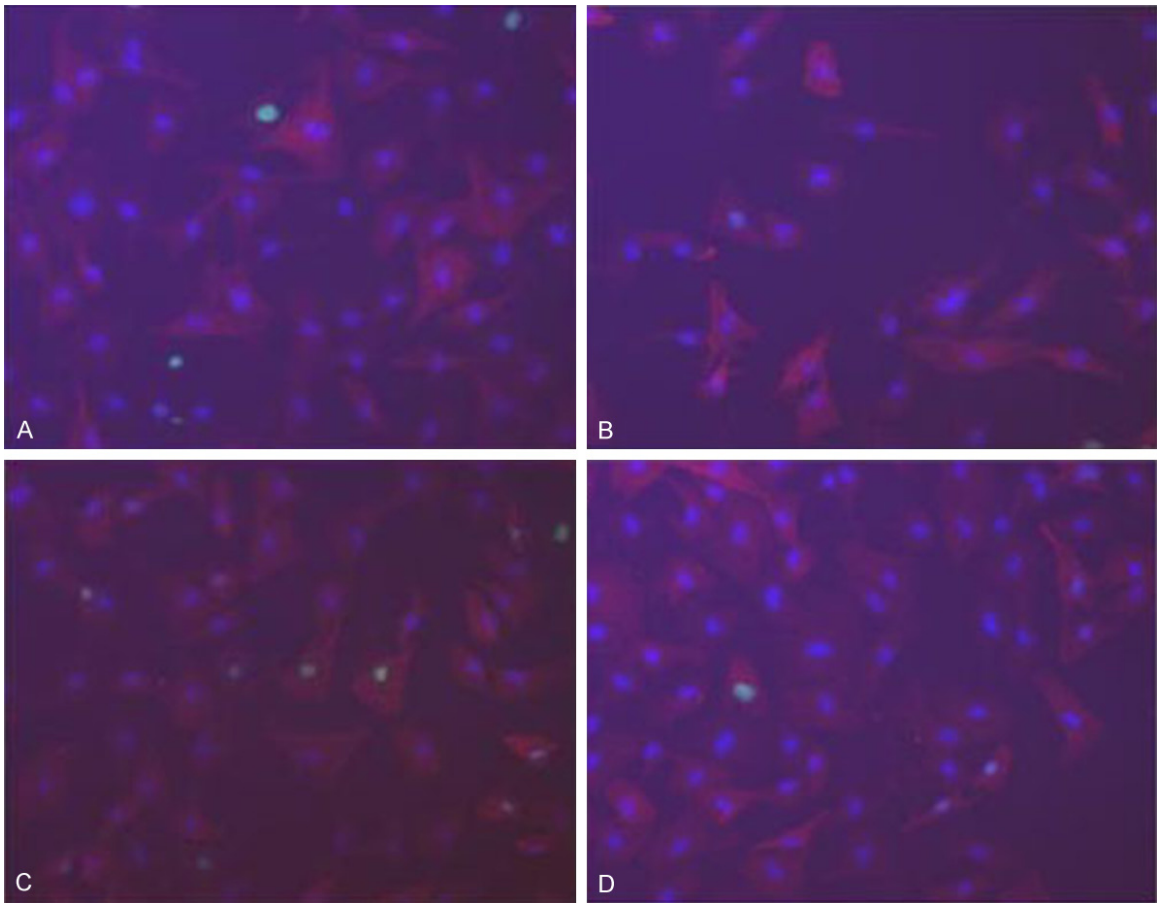

Figure 5. α-SMA, EDU protein expression by immunofluorescence. A: CFs, B: Mimics group, C: Inhibitor group, D: NS-miRNA. Notes: blue as the nucleus DAPI, green as cell proliferation EDU, red as α-SMA.

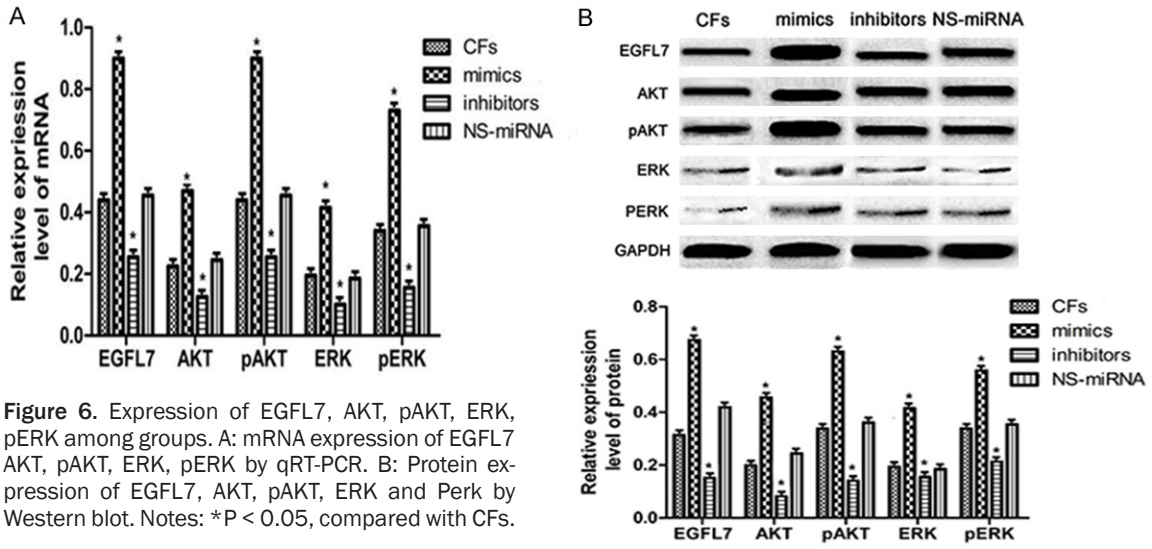

Figure 6. Expression of EGFL7, AKT, pAKT, ERK, pERK among groups. A: mRNA expression of EGFL7 AKT, pAKT, ERK, pERK by qRT-PCR. B: Protein expression of EGFL7, AKT, pAKT, ERK and Perk by Western blot. Notes: *P < 0.05, compared with CFs.

codes for a gene mostly expressed in endothelial cells [16-18]. Vascular Endothelial Growth Factor (VEGF) is an important neovascularization regulator which can effectively enhance the establishment of collateral circulation in ischemic myocardium [19]. The main mecha-

nism of VEGF related to angiogenesis is its specific effects on vascular endothelial cells, inducing endothelial cell proliferation, sprouting, migration, and luminal formation [20]. Animal and clinical trials have demonstrated that the expression of VEGF was increased in myocardial infarction [21].

MiR 126 is specifically expressed in vascular endothelial cells and vascular smooth muscle cells and has an important role in the process of angiogenesis through regulation of cell proliferation, differentiation, and apoptosis [22]. In our research, we transfected miR-126 into CF cells to assess the relationship between miR-126 and VEGF *in vivo*. The injected miR 126 inhibitors contributed to an increased expression of α-smooth muscle actin α-SMA, cell proliferation marker proteins EDU but miR 126 mimics could effectively inhibited α-smooth muscle actin α-SMA, cell proliferation marker protein EDU and then reduced the CF cell proliferation in the acute myocardium injure model. Further research discovered that miR 126 mimics could inhibited expression levels of EGFL7, AKT, pAKT, ERK, Perk mRNA compared with CFs group. Epidermal Growth Factor like domain 7 (Egfl7) also known as Vascular Endothelial-statin (VE-statin) codes for a gene mostly expressed in endothelial cells [23]. Silencing (knockdown) of the egfl7 gene in the zebrafish inhibits vascular tubulogenesis and embryos have little or no blood circulation [24]. The mice which did not express egfl7 had various vascular defects and the observed phenotypes were later attributed to the concomitant inactivation of the miR-126 locus [25]. Over-expression of egfl7 specifically in endothelial cells in mice induces embryonic lethality with head haemorrhages, cardiac defects and head and yolk sac vasculature defects [26]. Therefore, there was a potential therapy method for acute myocardial injure though activation of miRNA 126 to down regular expression of gene egfl7 and to further inhibit expression of EGF-like domain-containing protein and to reduce myocardial cell proliferation and to alleviate acute myocardial injure.

In conclusion, miR-126 inhibits the proliferation and differentiation of myocardial fibroblasts, possibly, through increasing the expression of EGFL7 activating AKT/ERK signaling pathway.

Disclosure of conflict of interest

None.

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