

## Original Article

# Role of miR-24 in myocardial fibrosis induced by angiotensin II

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**Abstract:** Objective: To investigate the role and mechanism of miR-24 in angiotensin II induced myocardial fibrosis, by miR-24 expression and interference recombinant lentiviral transduction in primary cardiac fibroblasts. Method: The primary cardiac fibroblasts were stimulated by angiotensin II to simulate *in vitro* myocardial fibrosis. Cardiac fibroblasts were transduced with previously constructed miR-24 expression interference recombinant lentivirus. Cell proliferation, apoptosis and gene expression levels of AGTR-1,  $\beta$ -arrestin-1, GNAQ and PKC-delta were detected to identify the target of miR-24. PKC-delta expression recombinant lentivirus was constructed to investigate the effect of miR-24 on AGTR1-Gq-PKC signaling pathway. Results: MiR-24 facilitated expression of AGTR-1 and  $\beta$ -arrestin-1 and cell apoptosis, and inhibited cell proliferation and the synthesis of hydroxyproline, exhibiting a negative synergistic effect with PKC-delta. Conclusion: miR-24 negatively regulated PKC-delta by working on AGTR1 and furthermore inhibited myocardial fibrosis induced by angiotensin II.

**Keywords:** miR-24, angiotensin II, myocardial fibrosis

### Introduction

In normal myocardial tissues, collagen fiber synthesis and metabolic imbalance can cause accumulation of collagen fibers and lead to myocardial fibrosis. Such lesions are closely associated with arrhythmia, cardiac dysfunction and sudden cardiac death [1]. Myocardial fibrosis is a complex process, involving multiple regulatory systems including AngII-BK system, ET-NO system, platelet-derived growth factor (PDGF) and thromboxane [2, 3]. Renin-angiotensin system (RAS) is a major regulator of renal and cardiovascular functions, playing an important role in myocardial fibrosis and remodeling process [4]. The physiological function of angiotensin II (AngII) is mediated by its type I receptor (AGTR1) and G protein coupled receptors. In addition, AngII can exert a growth factor-like effect, inducing myocardial cell hypertrophy and stimulating cardiac fibroblast proliferation [5, 6].

Micro RNA (miRNA) is an endogenous non-coding single-stranded small molecule RNA, extensively present in the eukaryotes. miRNA can

inhibit the expression level of target genes through completely or incompletely complementary binding with the 3'-UTR of target miRNA [7]. miRNA participates in various physiological and pathological processes, such as organism development, cell proliferation, differentiation and apoptosis. miR-24, a recently identified miRNA molecule, has been found to be associated with tumor and highly expressed in a variety of malignant tumors (gastric cancer, pancreatic cancer, liver cancer and colorectal cancer, etc.) [8-10]. As a tumor marker, miR-24 participates in signal transduction and apoptosis regulation in tumor cells; however, the role in myocardial fibrosis has not been reported [11-13].

Based on the previously constructed recombinant lentivirus for miR-24 gene expression and interference, this study analyzed primary cardiac fibroblast proliferation, apoptosis and expression of associated factors, screened the targets of miR-24 and analyzed the effect on AGTR1-Gq-PKC signaling pathway, to investigate the role and mechanism of miR-24 in AngII induced myocardial fibrosis.

**Table 1.** Quantitative fluorescence PCR primers

Primers	Sequence (5'-3')
r AGTR1a	F: AACAACTGCCTGAACCCTCTG
r AGTR1a	R: GCGTGCTCATTTTCGTAGACA
r $\beta$ -arrestin-1	F: GATGTCTTGGGTCTGACTTTTCG
r $\beta$ -arrestin-1	R: TCAAAGGTGAAGGGGTAGGC
r actin	F: CCCATCTATGAGGGTTACGC
r actin	R: TTTAATGTCACGCACGATTTC

## Materials and methods

### Cell strains and cell culture

Rat cardiac fibroblasts (CFs) were separated from male SD rat heart. After verified by Vimentin and factor VII staining, CFs were cultured in DMEM complete media containing 1% antibiotics (Penicillin/Streptomycin, Gibco) and 10% FBS (Gibco) at 37°C with 5% CO<sub>2</sub> in an incubator with saturated humidity. 24 h after cell passaging, cell confluence reached about 70%. Serum-free media was used to synchronize cells and after 24 h media was changed. Recombinant lentivirus or drug was added to the designated group and cells were cultured for another 48 h before further detection.

### Detection of AGTR1 and $\beta$ -arrestin-1 mRNA level

CFs were digested, centrifuged and collected. Total RNA was extracted using TRIzol reagent (Qiagen). Reverse transcription and SYBR green I quantitative fluorescence PCR (Eppendorf) were performed to detect the mRNA level of AGTR1 and  $\beta$ -arrestin-1. Primer sequences are listed in **Table 1**. PCR conditions were as follows: 94°C 4 min, 94°C 20 sec, 60°C 30 sec, 72°C 30 sec, 35 cycles. Each sample was repeated in triplicates.

### Detection of protein level of AGTR1, $\beta$ -arrestin-1, GNAQ and PKC-delta

CFs were digested, centrifuged and washed with PBS once. 0.1 ml RIPA lysis buffer was added to 10<sup>6</sup> cells. Cells were vortexed, resuspended and placed on ice for 5 min. Cell lysis was centrifuged at 12000 g, 4°C for 5 min. The supernatant was used for SDS-PAGE and Western blot. The film was scanned and the intensity of target bands was analyzed using UVP Image Analysis System Labworks 4.6 software.

### Detection of cell apoptosis by flow cytometry

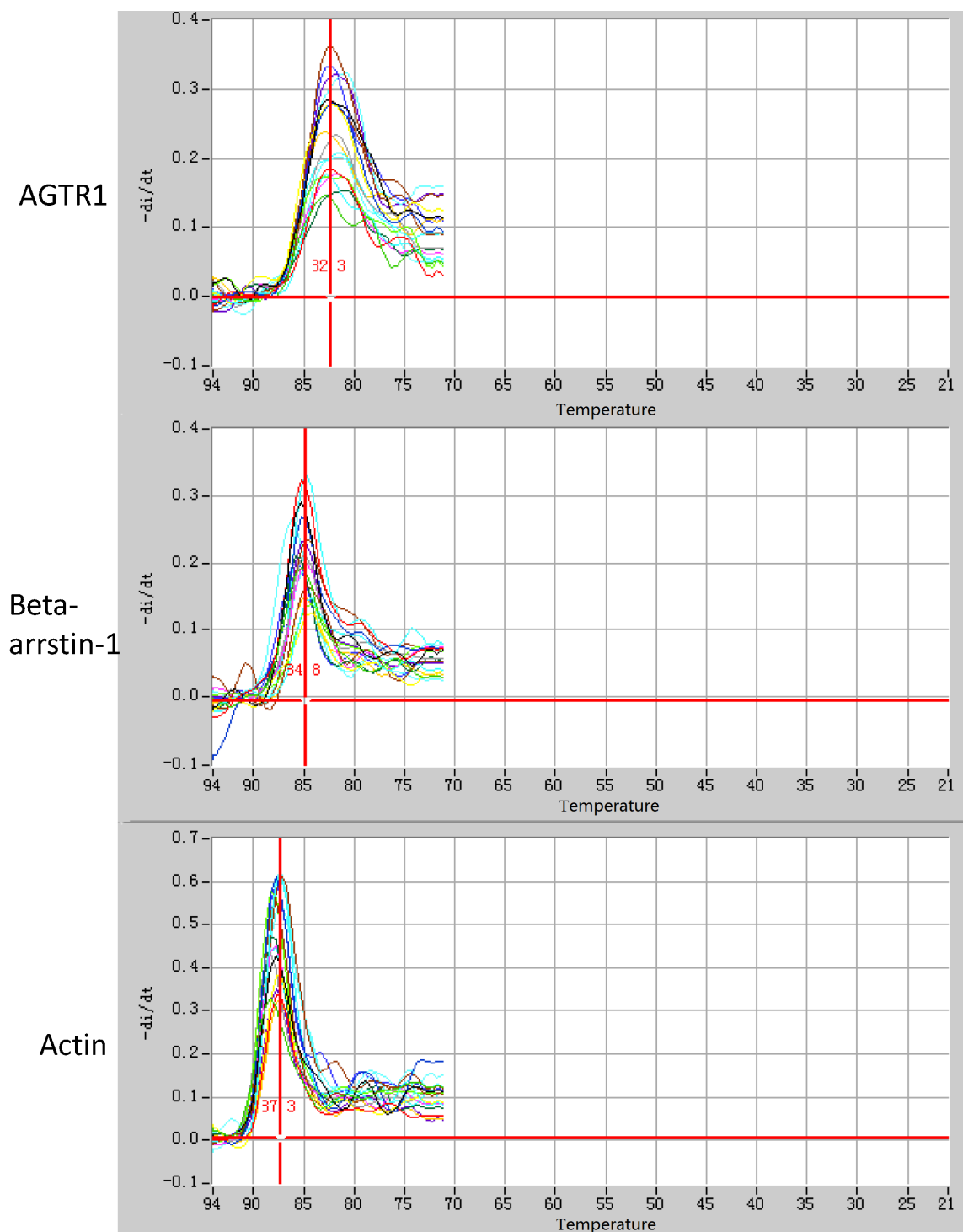
CFs were digested and washed with PBS twice. 1~5×10<sup>4</sup> cells were collected, added into 200  $\mu$ l Annexin V-FITC binding buffer (BD) and incubated at room temperature for 10 min without light. The supernatant was discarded after centrifugation. 190  $\mu$ l Annexin V-FITC binding buffer was added to resuspend cells. Cells were mixed with 10  $\mu$ l propidium iodide and placed on ice without light for flow cytometry detection.

### Preparation of PKC-delta recombinant lentivirus

The synthesized PKC-delta gene was cloned into a shuttle plasmid pLV5 for ligation. E.coli competent cells were transformed with the plasmid. Positive clones were selected, sequenced and named pLV5/PKC-delta. Plasmids were extracted using high purity and endotoxin-free plasmid midi-extraction kit (NucleoBond Xtra Midi Plus, Macherey-Nagel) and mixed with packaging plasmid PG-p1-VSVG, PG-P2-REV and PG-P3-RRE (Tronolab), respectively, in a certain ratio to transduce 293T cells. 293T cells were cultured for 72 h and the culture supernatant was collected. The supernatant was centrifuged, filtered, and precipitated in PEG8000. The pellete was resuspended and ultracentrifuged using CsCl and dialyzed to purify virus. 293T cells were seeded in a 96-well plate at a concentration of 1×10<sup>5</sup>/well and cultured in an incubator. After 24 h, the purified virus was diluted in ten-fold DMEM and 100  $\mu$ l virus dilution was added to each well (10<sup>-2</sup>-10<sup>-6</sup>). Control group was also set up. Cells were cultured for 24-72 h in the incubator and observed under inverted light microscope to count positive cells. The virus transducing unit (TU/ml) was calculated with dilution factor.

### Detection of CFs proliferation by MTT assay after recombinant lentiviral transduction

CFs were seeded in a 96-cell plate at a density of 1×10<sup>4</sup>/well and after 24 h, serum-free media was used to synchronize cells for 24 h. Following media change, cells were treated by groups and cultured for another 48 h. 10  $\mu$ l 5 mg/ml MTT solution (Sigma) was added into each well and cells were cultured for 4 h. The media was discarded and 100  $\mu$ l DMSO was added in each well. The plate was shaken at a low rate for 10 min at room temperature. The absorbance at 490 nm wavelength was detected using a spectrophotometer. DMSO blank control was included in the experiment.

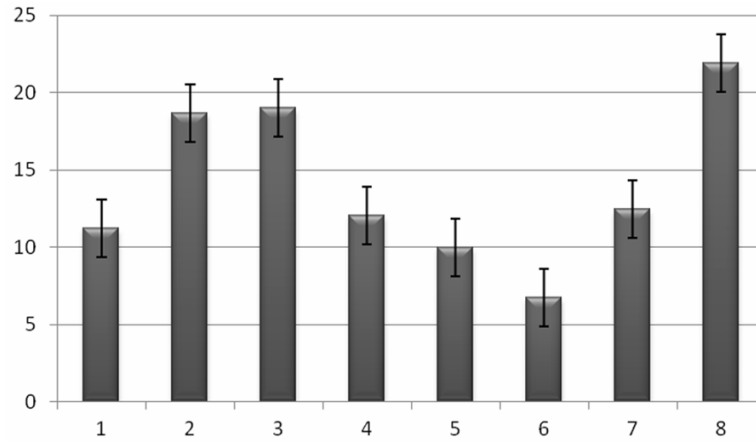


**Figure 1.** Quantitative PCR melting curve.

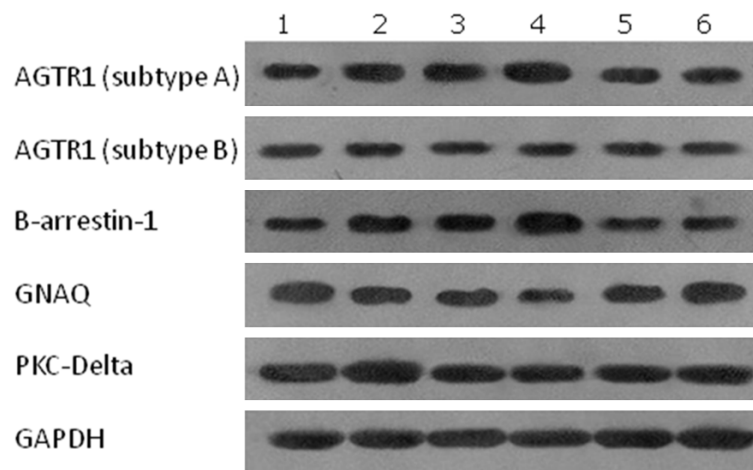
#### Detection of hydroxyproline level in CFs

The hydroxyproline level in CFs was detected using a double antibody sandwich ELISA kit. CFs were treated following aforementioned

methods. Cell culture supernatant was collected. Cells were digested, resuspended with 100  $\mu$ l PBS and subjected to repeated freeze-thaw cycles. Cells were centrifuged. The supernatant was collected and mixed with culture super-



**Figure 2.** mRNA expression of AGTR1 and  $\beta$ -arrestin-1 (1-8 present Control Group; AngII group; AngII + empty vector group; AngII + MiR-24 expression group; AngII + empty vector + Rottlerin group; AngII + MiR-24 expression + Rottlerin group; AngII + MiR-24 expression + empty vector group; AngII + MiR-24 expression + PKC delta expression group, respectively).



**Figure 3.** The protein detection level of AGTR1 (subtype A), AGTR1 (subtype B),  $\beta$ -arrestin-1, GNAQ and PKC-delta. (1-6 present control group, AngII group, AngII + empty vector group, AngII + MiR-24 expression group, AngII + MiR-24 interference and AngII + AngII inhibitor group, respectively).

nantant. ELISA was performed following instruction of the kit and the hydroxyproline level in each sample was calculated using standard curve.

#### Statistical analysis

Triplicates were set up for each experiment. Data were analyzed using SPSS 10.0 software. The mean values between two samples were compared using t-test.  $P < 0.05$  was considered significant difference.

## Results

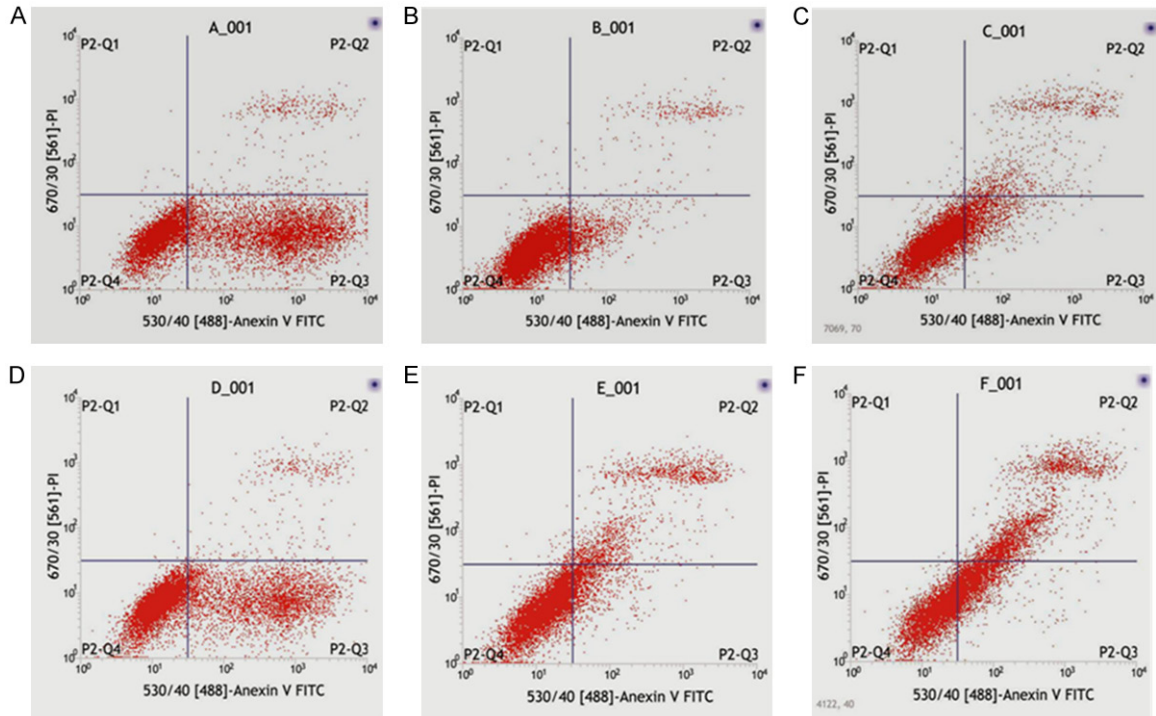
### miR-24 promoting AGTR1 and $\beta$ -arrestin-1 expression

The melting curve of quantitative fluorescence PCR demonstrated a good specificity of all primers of AGTR1,  $\beta$ -arrestin-1 and actin gene (**Figure 1**). The standard curve showed that all three PCR systems had an amplification efficiency higher than 95%. The relative quantitative result showed that AngII significantly promoted gene transcription of AGTR1 and  $\beta$ -arrestin-1 in CFs ( $P < 0.05$ ), while AngII inhibitor exhibited a significant effect only on AGTR1 gene transcription. The recombinant lentivirus control failed to exhibit an effect on AngII induced AGTR1 and  $\beta$ -arrestin-1 gene transcription in CFs ( $P > 0.05$ ). Under the effect of miR-24 expression and interference recombinant lentivirus, gene transcription of AGTR1 and  $\beta$ -arrestin-1 were partially promoted and inhibited, respectively (**Figure 2**).

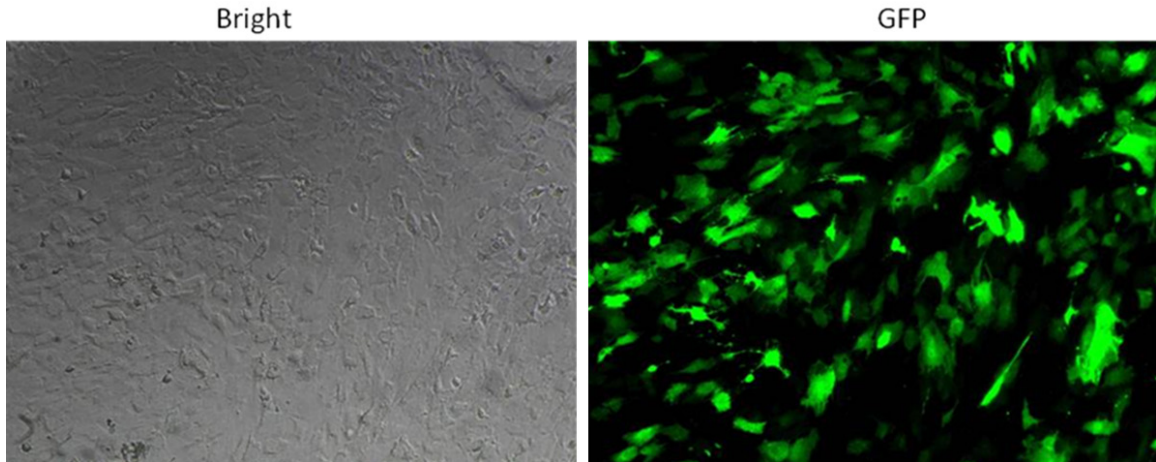
Western blot and intensity analysis (**Figure 3**) showed that under the effect of miR-24 expression and interference recombinant lentivirus, AGTR1 (A subtype) and  $\beta$ -arrestin-1 protein expression levels were promoted and inhibited, compared with recombinant lentivirus control group. Protein expression level of AGTR1 (B subtype), GNAQ and PKC-delta was barely influenced by miR-24 ( $P > 0.05$ ).

### MiR-24 promoting cell apoptosis

Flow cytometry results (**Figure 4**) showed that the cell survival rate in control, AngII, AngII + empty vector, AngII + MiR-24 expression, AngII + MiR-24 interference and AngII + AngII inhibi-



**Figure 4.** CFs apoptosis detection results. (1-6 present control group, AngII group, AngII + empty vector group, AngII + MiR-24 expression group, AngII + MiR-24 interference and AngII + AngII inhibitor group, respectively).



**Figure 5.** Lenti-PKC-delta infection CFs.

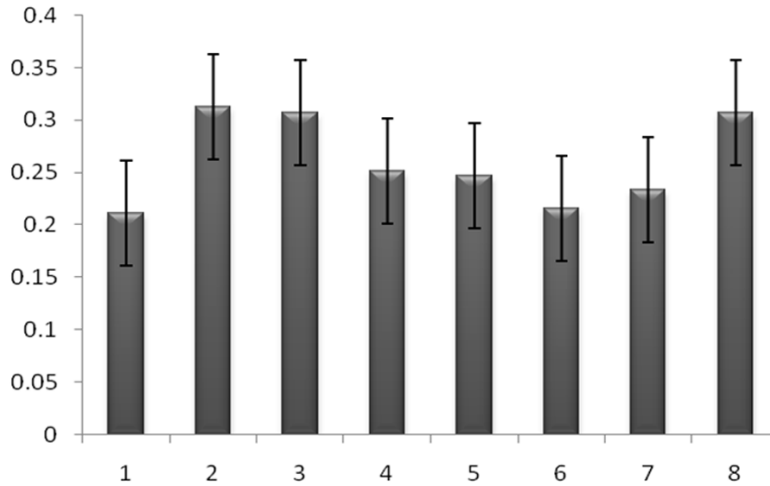
tor groups was  $(46.82 \pm 0.10)\%$ ,  $(87.47 \pm 0.03)\%$ ,  $(76.59 \pm 0.03)\%$ ,  $(61.33 \pm 0.03)\%$ ,  $(72.37 \pm 0.37)\%$  and  $(46.75 \pm 0.09)\%$ , respectively. AngII significantly facilitated CFs survival ( $P < 0.05$ ) while AngIIinhibitorcompletelyoffsetthis effect. Recombinant lentivirus control had a certain effect on CFs survival following AngII stimulation ( $P < 0.05$ ). Compared with recombinant lentivirus control, CFs survival rate was reduced

under the effect of miR-24 expression recombinant lentivirus ( $P < 0.05$ ), while miR-24 interference recombinant lentivirus failed to exert a significant effect on CFs survival ( $P > 0.05$ ).

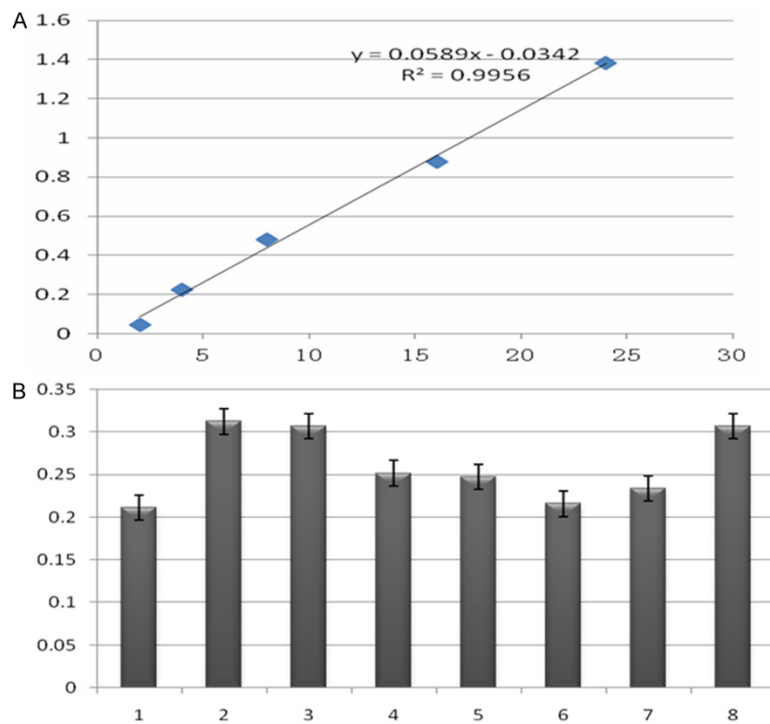
#### CFs transduction with Lenti-PKC-delta

The titer of Lenti-PKC-delta was  $5 \times 10^8$  TU/ml after packaging and purification. The negative control of recombinant lentivirus without exog-





**Figure 6.** MTT Assay Result of CFs following Recombinant Lentiviral Transduction (1-8 present Control Group; AngII group; AngII + empty vector group; AngII + MiR-24 expression group; AngII + empty vector + Rottlerin group; AngII + MiR-24 expression + Rottlerin group; AngII + MiR-24 expression + empty vector group; AngII + MiR-24 expression + PKC delta expression group, respectively).



**Figure 7.** A: ELISA standard curve; B: Hydroxyproline levels.

enous genes was prepared using the same method by Weisiteng Biotechnology Center, with a titer of  $1 \times 10^8$  TU/ml. The two types of recombinant lentivirus were used to transduce CFs at a dose of MOI=15. After 96 h, the trans-

duction rate of Lenti-PKC-delta reached above 80%, as shown in **Figure 5**.

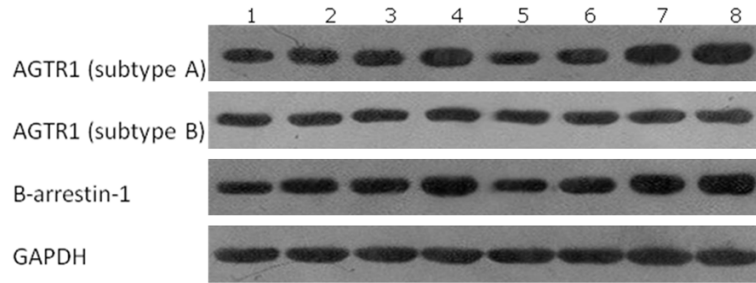
#### *CFs proliferation following recombinant lentiviral transduction*

MTT results at 48 h following recombinant lentiviral transduction and drug treatment are shown in **Figure 6**. AngII significantly promoted CFs proliferation. Recombinant lentivirus control had no effect on CFs proliferation following AngII stimulation. Under the effect of miR-24 expression recombinant lentivirus, cell proliferation was partially inhibited, and the effect was comparable with that of PKC delta inhibitor Rottlerin. MiR-24 expression exhibited a positive synergistic effect with Rottlerin, while the effects of miR-24 expression and PKC delta expression offset each other.

#### *Changes of CFs hydroxyproline level following recombinant lentiviral transduction*

ELISA standard curve was constructed by the OD value of standard, as shown in **Figure 7A**. The hydroxyproline concentration of samples was calculated using interpolation method and the result is shown in **Figure 7B**. AngII significantly elevated the level of hydroxyproline in CFs. Recombinant lentivirus control had no significant effect on the level of hydroxyproline in CFs following AngII stimulation. Under

the effect of miR-24 expression recombinant lentivirus, hydroxyproline level was partially inhibited, and PKC delta inhibitor Rottlerin exhibited the same effect. MiR-24 expression exhibited a positive synergistic effect with



**Figure 8.** The protein detection level of AGTR1 (subtype A), AGTR1 (subtype B),  $\beta$ -arrestin-1, GNAQ and PKC- $\delta$ . (1-8 present Control Group; AngII group; AngII + empty vector group; AngII + MiR-24 expression group; AngII + empty vector + Rottlerin group; AngII + MiR-24 expression + Rottlerin group; AngII + MiR-24 expression + empty vector group; AngII + MiR-24 expression + PKC  $\delta$  expression group, respectively).

Rottlerin, while the effects of miR-24 expression and PKC  $\delta$  expression offset each other.

#### *Change of AGTR1 and $\beta$ -arrestin-1 protein level in CFs after recombinant lentiviral transduction*

Western blot and intensity analysis (**Figure 8**) showed that AngII significantly promoted AGTR1 (A subtype) and  $\beta$ -arrestin-1 expression in CFs, while recombinant lentivirus control had no significant effect on the two protein expression in CFs after AngII stimulation. Under the effect of miR-24 expression recombinant lentivirus, the expression levels of both proteins were elevated. PKC  $\delta$  inhibitor Rottlerin reduced the levels of the two proteins. MiR-24 expression exhibited a positive synergistic effect with PKC  $\delta$  and a negative synergistic effect with Rottlerin. The change of protein expression level of AGTR1 (B subtype) was relatively small ( $P > 0.05$ ).

#### **Discussion**

Based on the recent progress of miRNAs in the research field of biosynthesis, mechanism of action and disease association, great breakthrough has been made in the association between miRNAs and tumor occurrence and development [14, 15]. The increase or reduction of miRNAs expression level can lead to changes in genetic information, thereby resulting in certain pathophysiological status. PKC is an important intracellular mediator of signal transduction. The catalytic activity of PKC is activated only when binding with membrane so that PKC can participate in intracellular and

extracellular signal transduction and regulation of cell metabolism, differentiation and proliferation. It has been demonstrated that PKC participates in the process of myocardial remodeling [16]. AngII can bind with AGTR1 and activate PKC- $\delta$ , promoting fibroblast proliferation, adherence and migration [17, 18].

The preliminary study showed that miR-24 was able to inhibit AngII induced CFs proliferation and hydroxyproline syn-

thesis. Further studies found that miR-24 facilitated cell apoptosis and also promoted expression of AGTR1 (A subtype) and  $\beta$ -arrestin-1, while exhibiting subtle influence on AGTR1 (B subtype), GNAQ and PKC- $\delta$ . To investigate the effect of miR-24 on AGTR1-Gq-PKC signaling pathway, recombinant lentivirus expressing PKC- $\delta$  was constructed. Working synergistically with recombinant lentivirus expressing miR-24, PKC- $\delta$  antagonized the inhibitory effect of miR-24 on cell proliferation and hydroxyproline synthesis, which was comparable with the effect of PKC- $\delta$  inhibitor Rottlerin. The results showed that AngII induced myocardial fibrosis was blocked by the inhibitory effect of miR-24 on PKC- $\delta$ .

Regulation of PKC is an effective means of intervention in the treatment of human heart failure. Recent clinical trials showed that systemic application of PKC inhibitors exhibited good tolerability, but drugs with tissue specificity of *in vivo* distribution are still waiting to be developed. The role and mechanism of miR-24 in angiotensin II induced myocardial fibrosis enrich our understanding on myocardial fibrosis and provide more possibilities for research of treatment target, drug development and even gene therapy.

#### **Disclosure of conflict of interest**

None.

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