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Original Article

Down-regulation of microRNA-184 contributes to the development of cyanotic congenital heart diseases

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Abstract: Purpose: We aimed to investigate the roles of miR-184 in adaptation of hypoxic cardiomyocytes, as well as to elucidate the possible mechanisms of miR-184 in the development of cyanotic congenital heart diseases (CHD). Materials and methods: We conducted quantitative real-time polymerase chain reaction (qRT-PCR) to determine the expression of miR-184 in patients with cyanotic cardiac defects. The embryonic rat ventricular myocardial H9c2 cells were transfected with miR-184 inhibitor and negative scramble RNA. Mock group was untreated by anything. We then used MTT assay and in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) to determine whether inhibition of miR-184 in vitro affect cell proliferation and apoptosis under hypoxic conditions. Besides, the expression levels of caspase-3 and caspase-9 in hypoxic H9c2 cells were determined by western blot. Results: MiR-184 was significantly down-regulated in CHD patients with cyanotic cardiac defects. In addition, miR-184 was successfully inhibited in hypoxic H9c2 cells. Moreover, inhibition of miR-184 markedly decreased cell viability and obviously induced apoptosis under hypoxic conditions in vitro. Besides, the expression levels of caspase-3 and caspase-9 in hypoxic H9c2 were significantly increased after miR-184 inhibition. Conclusions: Our findings indicate that inhibition of microRNA-184 may contribute to the development of cyanotic CHD via decreasing proliferation and inducing apoptosis of cardiomyocytes. Moreover, miR-184 inhibition may promote hypoxia-induced apoptosis via activation of caspase-3 and caspase-9. Congenital down-regulation of miR-184 may be a mechanism leading to CHD development.

Keywords: Congenital heart diseases, microRNA-184, chronic hypoxia, caspase-3, caspase-9

Introduction

Congenital heart diseases (CHD) is the most common congenital defect that can occur from genetic variations, environmental exposures, and other factors [1, 2], especially cyanotic CHD. Chronic hypoxia is commonly observed in patients with cyanotic CHD and is considered to be the basic pathophysiological process of CHD, which can affect mitochondrial oxidative metabolism and subsequently heart remodeling [3]. Therefore, a better understanding of the underlying mechanism of the protective adaptation of cardiomyocytes to chronic hypoxia will help to develop novel molecular therapies for reducing myocardial damage during cardiac surgery.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs (approximately 18-25 nucleotides), which can regulate gene expression by binding to the 3' untranslated region (3' UTR) of target mRNA transcripts and subsequently affect their biological functions [4, 5]. Mounting evidence has demonstrated that miRNAs play a pivotal role in cardiac protection and development [6]. Several miRNAs are also reported to promote cardiac regeneration and may serve as potential therapeutic targets for patients with congenital and acquired cardiovascular diseases [7]. Recently, microRNA-184 (miR-184) is found to be involved in a wide range of biological and pathological processes, such as cell proliferation, apoptosis and oncogenesis [8, 9]. Furthermore, the potential roles of microRNA-184 (miR-184) in cardiac development and diseases have aroused more the more attention. Dickinson et al. discovered that plasma miR-184 was dysregulated in response to hypertension-induced heart failure [10]. Dysre-

gulation of miR-184 is also shown to be implicated in the process of cardiomyocyte hypertrophy [11, 12]. Besides, researchers have shown that miR-184 can be oxidatively modified by ROS that is generated under hypoxia conditions, and subsequently, oxidative modification of miR-184 can target Bcl-xL and Bcl-w to induce the initiation of apoptosis of rat heart cell line H9c2 [13], implying the important role of miR-184 in hypoxia-induced cardiomyocytes apoptosis. Despite these, the functions and potential mechanisms of miR-184 in CHD are less well-studied.

In the present study, we first investigated whether miR-184 was aberrantly expressed in patients with cyanotic cardiac defects. We then used MTT assay and in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) to determine whether inhibition of miR-184 *in vitro* affect cell proliferation and apoptosis under hypoxic conditions. Finally, the expression levels of caspase-3 and caspase-9 in hypoxic H9c2 cells was determined by western bolt analysis. The objective of our study was to determine the roles of miR-184 in the protective adaptation of cardiomyocytes to chronic hypoxia, as well as to elucidate the possible mechanisms of miR-184 in cyanotic CHD development. Our findings will help to devise possible approaches for the treatment of cyanotic CHD in a clinical application.

Materials and methods

Collection of myocardial samples

A total of 20 patients who underwent cardiac operations were enrolled in our study, including 10 CHD patients with cyanotic cardiac defects and 10 controls with acyanotic cardiac defects. Cardiac operations after standardized anesthesia were performed as previously described [14]. After cardiac arrest, the myocardial samples were taken from the right ventricular outflow tract immediately. Then all samples were snap frozen in liquid nitrogen and stored at -80°C for subsequent experiments. All procedures were approved by the local ethical committee and confirmed with the principles outlined in the Declaration of Helsinki. All patients were informed consent before participation.

Cell culture and plasmid transfection

The embryonic rat ventricular myocardial H9c2 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) at 37°C in an atmosphere with a gaseous mixture of 94% N_2, 5% CO_2 and 1% O_2. H9c2 cells were seeded in 60 mm plates to culture until reaching 50-60% confluence. Then cells were transfected with miR-184 inhibitor and negative scramble RNA by Lipofectamine2000 (Invitrogen Inc., Carlsbad, CA, USA) following the manufacturer's protocols. The sequences of miR-184 inhibitor and negative scramble RNA were designed and synthesized by Invitrogen as follows: miR-184 inhibitor, 5'-ACCCUUAUCAGUUCUCCGUCCA-3'; and negative scramble RNA, 5'-UUCUCCGAACGUGUCACGUTTACGUGACACGUUCGGAGAATT-3'. Mock group was untreated by anything.

RNA extraction and real-time PCR

Total RNA was extracted from using Trizol reagent (Invitrogen Inc., Carlsbad, CA, USA) according to the instruction of manufacturer. The quality of total RNA was assessed by spectrophotometer (260/280 ratio: 1.8-2.0). The miR-184 expression level was then determined with All-in-One™miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA) following the manufacturer's instructions. In brief, cDNA was firstly synthesized from 100 ng RNA in a 25 μl reaction system at 37°C for 60 min, followed by 85°C for 5 min. The PCR conditions for quantification were as follows: 10 min at 95°C, 40 cycles of 10 s at 95°C, 20 s at 62°C, and 30 s at 72°C. The endogenous U6 snRNA was used as an internal control. For miRNAs. The relative expression of miR-184 was calculated using the $2^{-\Delta\Delta CT}$ method as previously described [15]. Each sample was tested in triplicate.

Cell viability assay

Cell viability was assessed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT: Dojindo, Kumamoto, Japan) assay. In brief, H9c2 cells (2.5×10^4 cells/mL) were plated in 24-well plates and grown overnight at 37°C in an atmosphere with a gaseous mixture of 94% N_2, 5% CO_2 and 1% O_2. Afterwards, 50 μL of 5 mg/mL of MTT was added into each well at 72 h and 7 d after transfection and incubated for another 4 h. Formazan crystals were then dissolved in 1 mL of DMSO and the absorbance was measured at 570 nm using an Infinite M200 (Tecan, Mannedorf, Switzerland).

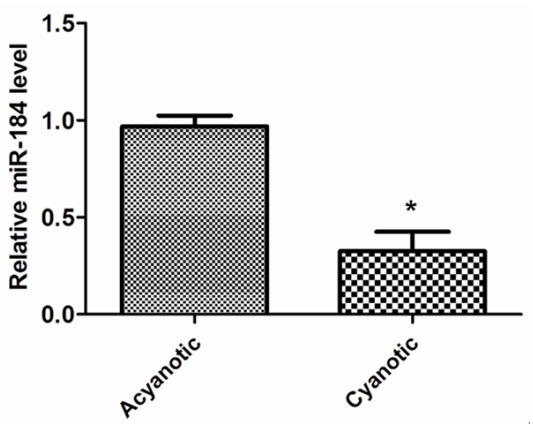

Figure 1. RT-PCR analysis displayed the expression of miR-184 in myocardial samples from patients with cyanotic cardiac defects and controls with acyanotic cardiac defects, respectively. Error bars indicate means ± SD and *indicates significant difference compared with control group (P < 0.05).

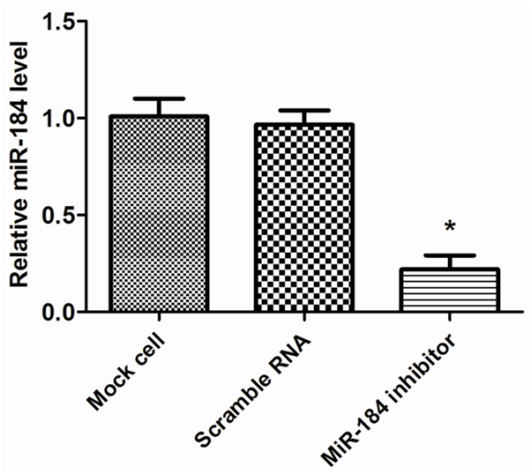

Figure 2. RT-PCR analysis displayed the expression of miR-184 in hypoxic H9c2 cells after transfection. Error bars indicate means ± SD and *indicates significant difference compared with mock group (P < 0.05).

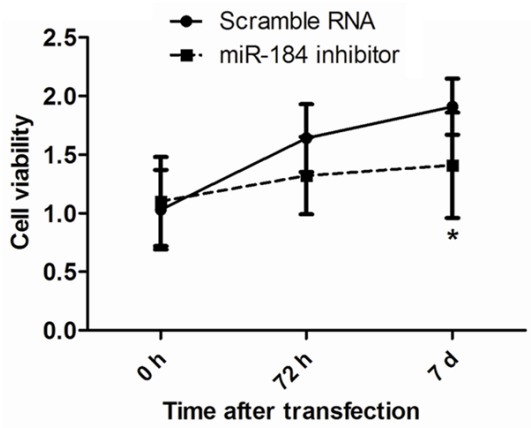

Figure 3. MTT assay showed the cell viability in an experimental period of 7 d after transfection under hypoxic conditions. Error bars indicate means ± SD and *indicates significant difference compared with mock group (P < 0.05).

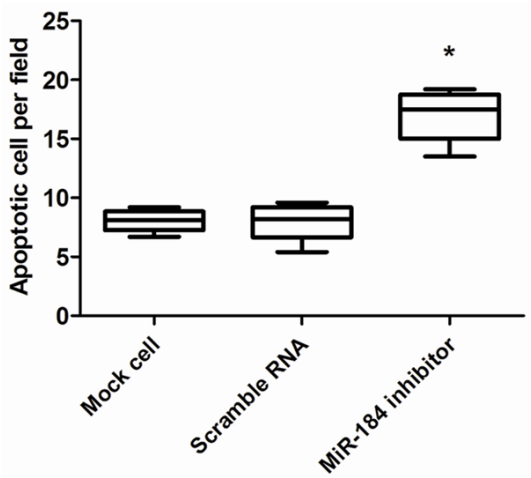

Figure 4. TUNEL staining displayed the number of apoptotic cells after transfection under hypoxic conditions. Error bars indicate means ± SD and *indicates significant difference compared with mock group (P < 0.05).

Each experiment was performed in triple. All collected were finally analyzed using i-Control version 1.0 software (Tecan).

TUNEL assay

To detect DNA fragmentation, in situ TUNEL method was performed using in situ apoptosis detection kit (APOPTAG, Qbiogene, Carlsbad, CA). The principle of this assay is that the terminal deoxynucleotidyl transferase can label the 3'-OH ends of DNA resulting from cell apoptosis with biotinylated nucleotides, and subsequently, these DNA fragments are detected by immunoperoxidase staining. All steps were performed in accordance with the manufacturer's instructions. Cells with stained nuclei were counted in 10 randomly selected fields with an upright microscope (Olympus BX51). The counting was carried out by two independent investigators.

Western blot analysis

Cells were harvested 72 h after transfection. Proteins were then extracted from the whole-

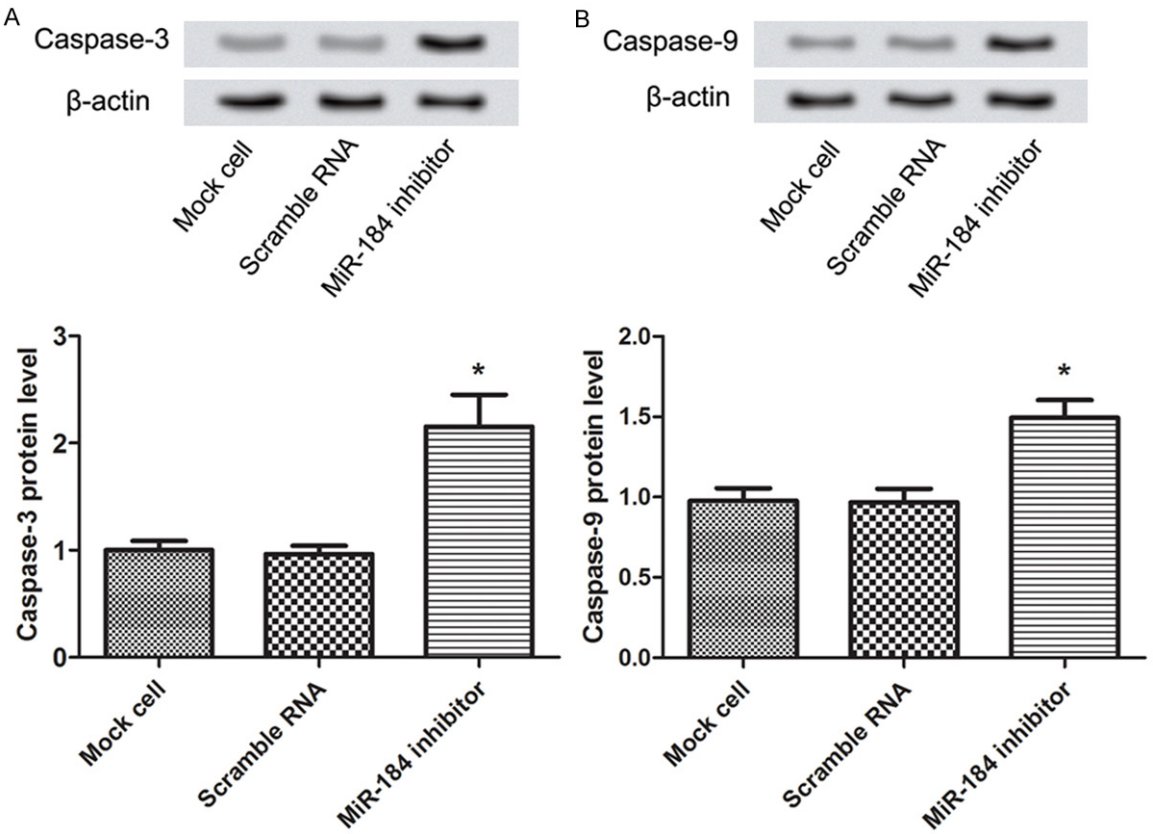

Figure 5. Western bolt analysis showed the expression levels of caspase-3 and caspase-9 in hypoxic H9c2 cells after transfection. Error bars indicate means ± SD and *indicates significant difference compared with mock group ($P < 0.05$).

cell lysis of the H9c2 cells and protein concentrations were determined by a bicinchoninic acid assay (Biotech, USA). Equal amount of protein (30 μg/lane) were separated by 10% SDS-PAGE electrophoresis for western blot analysis. The protein blots were then transferred to polyvinylidene difluoride membranes (Millipore, USA) using a semi-dry electroblot apparatus (Bio-Rad, USA). The membranes were blocked in 5% skim milk for 1 h at room temperature, followed by appropriate primary antibodies overnight at 4°C. Caspase-3 antibody (1:1000), Caspase-9 antibody (1:1000) and β-actin antibody (#12620; 1:1000) were all purchased from Cell Signal Technology. After washing, secondary antibody conjugated with horseradish peroxidase (1:2000, Santa Cruz Biotechnology) was added to incubate the membrane at room temperature for 1 h. The membranes were washed and then visualized by a Chemiluminescence Detection Kit (P90719, Millipore, Billerica, MA, USA). The expression level of these proteins were normalized to the β-actin and analyzed with a FluorChem M System (ProteinSimple, San Jose, CA, USA).

Statistics analyses

All the measurement data were are presented as the mean ± SD and were tested for the normal distribution using one-sample K-S test. The statistical analyses were then analyzed by student t-test or one-way ANOVA in SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Further between-group-comparison was then performed by post-hoc Tukey test. A value of $P < 0.05$ represents statistically significant.

Results

MiR-184 was down-regulated in CHD patients with cyanotic cardiac defects

Compared with control patients with acyanotic cardiac defects, we found the expression of miR-184 was significantly down-regulated in myocardial samples from CHD patients with

cyanotic cardiac defects using RT-PCR analysis ($P < 0.05$, **Figure 1**).

The expression of miR-184 was successfully inhibited in H9c2 cells

To explore the roles of miR-184 in hypoxic cardiomyocytes, hypoxic H9c2 cells were transfected with miR-184 inhibitor and negative scramble RNA, respectively. The results showed that the expression of miR-184 in cells transfected with miR-184 inhibitor was markedly lower than that in mock cells or cells transfected with scramble RNA ($P < 0.05$, **Figure 2**), indicating the expression of miR-184 was successfully inhibited in hypoxic H9c2 cells by miR-184 inhibitor.

Inhibition of miR-184 decreased cell viability under hypoxic conditions

MTT assay showed the cell viability in an experimental period of 7 d after transfection under hypoxic conditions. As shown in **Figure 3**, the cell viability of hypoxic H9c2 cells transfected with miR-184 inhibitor was significantly lower than scramble RNA transfected group ($P < 0.05$), indicating that inhibition of miR-184 expression obviously decreased cell viability under hypoxic conditions.

Inhibition of miR-184 induced cell apoptosis under hypoxic conditions

TUNEL staining displayed apoptotic cells in different group. By counting the average apoptotic cells in 10 randomly selected fields with a microscope, we found that the number of apoptotic cells in miR-184 inhibitor transfected group significantly increased compared with scramble RNA transfected group or mock group ($P < 0.05$) while there were no significant differences between scramble RNA transfected group and mock group ($P > 0.05$) (**Figure 4**), indicating that inhibition of miR-184 induced cell apoptosis under hypoxic conditions.

Analysis of the expression levels of caspase-3 and caspase-9 under hypoxic conditions

To further investigate the regulatory mechanism of miR-184 in hypoxic cardiomyocytes, we determined the expression levels of caspase-3 and caspase-9 by western bolt analysis. As shown in **Figure 5**, similar results were obtained that the expression levels of caspase-3 and caspase-9 in hypoxic H9c2 cells transfected with miR-184 inhibitor were significantly increased compared with that in scramble RNA transfected cells or mock cells ($P < 0.05$).

Discussion

Chronic hypoxia is a common pathophysiological process of cyanotic CHD. However, the protective mechanisms against hypoxia-induced damage are largely unknown. In the present study, we discovered that miR-184 was down-regulated in patients with cyanotic CHD. Moreover, *in vitro* inhibition of miR-184 markedly decreased cell viability and induced apoptosis under hypoxic conditions. Besides, the expression levels of caspase-3 and caspase-9 in hypoxic H9c2 were significantly increased after miR-184 inhibition. Therefore, inhibition of miR-138 is likely to be a potential mechanism for cardioprotection against chronic hypoxia, and the roles of miR-138 in the development of cyanotic CHD merit further discussion.

In the cardiovascular field, miRNAs are known to play important roles in the process of myocardial adaptation to stress [16]. MiR-210 is shown to exert cytoprotective effects in hypoxic cardiomyocytes via Akt-and p53-dependent pathways [17]. Inhibition of miR-15 can also protect against cardiac ischemic injury and thus enhance cardiac function in response to myocardial infarction [18]. In addition, miRNAs have been observed to be widely involved in cardiac apoptosis program [19]. Down-regulation of miR-199a can induce apoptosis of cardiomyocytes via inhibiting hypoxia-inducible factor (HIF)-1a and its stabilization of p53 [20]. Up-regulation of miR-26a promotes reactive oxygen species (ROS)-mediated apoptosis of hypoxic rat neonatal cardiomyocytes [21]. A previous study also have confirmed that oxidative modification of miR-184 by ROS can target Bcl-xL and Bcl-w, thereby inducing the initiation of apoptosis of rat heart cell line H9c2 [13]. Besides, the role of miR-184 has been studied in a variety of cancer cells. Gao et al. demonstrated that miR-184 silencing inhibited cellular proliferation and induced cell apoptosis in human hepatocellular carcinoma [22]. It has also been suggested that miR-184 inhibits glioma progression via inhibiting cell proliferation and inducing cell apoptosis [23]. In the present study, miR-184 was down-regulated in cardiomyocytes from patients with cyanotic CHD and

in vitro inhibition of miR-184 markedly decreased cell proliferation and induced apoptosis of H9c2 cells under hypoxic conditions. Although the role of miR-184 in cardiac apoptosis is not clear, we speculate that down-regulation of miR-184 may decrease proliferation and induce apoptosis of hypoxic cardiomyocytes, and congenital down-regulation of miR-184 may be a mechanism leading to the development of cyanotic CHD.

To further investigate the regulatory mechanism of miR-184 in hypoxic cardiomyocytes, the expression levels of key apoptotic proteins, such as caspase-3 and caspase-9 in hypoxic H9c2 cells were determined by western blot. Caspase-3 is an executioner caspase known to be involved in the final execution phase of apoptosis [24]. It has been reported that overexpression of miR-378 can attenuate ischemia-induced apoptosis in cardiomyocytes via by inhibiting the expression of caspase-3 [25]. Moreover, caspase-3 has been considered as a key downstream target that executes the apoptotic cascade after hypoxia [26]. Kim et al. suggested that Acetylcholine might inhibit hypoxia-induced apoptosis in mouse embryonic stem cells through regulation of caspase-3 [27]. In addition, caspase-9 is also considered to be a critical regulator of mitochondria-mediated apoptosis. Overexpression of HS-1 associated protein-1 (HAX-1) is observed to protect cardiac myocytes from apoptosis through inhibition of caspase-9 [28]. Yuan et al. confirmed that sanguinarine could inhibit Angiotensin II-induced H9c2 cardiac cells apoptosis through decreasing the expression and activity of caspase-3 and caspase-9 proteins [29]. Taken together, caspase-3 and caspase-9 may be key downstream molecules involved in hypoxia-induced apoptosis. In our study, the expression levels of caspase-3 and caspase-9 in hypoxic H9c2 were significantly increased after miR-184 inhibition. Although the directly functional link between miR184 and caspase-3 or caspase-9 has not been investigated, our results also provide new clues that miR-184 inhibition may promote hypoxia-induced apoptosis via activation of caspase-3 and caspase-9.

In conclusion, our findings indicate that inhibition of microRNA-184 may contribute to CHD development through decreasing proliferation and inducing apoptosis of cardiomyocytes. Moreover, miR-184 inhibition may promote hypoxia-induced via activation of caspase-3 and caspase-9. Congenital down-regulation of miR-184 may be a mechanism leading to CHD development.

Disclosure of conflict of interest

None.

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