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ORIGINAL ARTICLE

The protective microRNA-199a-5p-mediated unfolded protein response in hypoxic cardiomyocytes is regulated by STAT3 pathway

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Abstract

The protective effects of downregulated miR-199a-5p on ischemic and hypoxic cardiomyocytes were well recognized, but the underlying mechanism of inhibited miR-199a-5p is not yet clear. The present study explored the relationship between enhanced signal transducer and activator of transcription 3 (STAT3) signaling and lowered production of miR-199a-5p in hypoxic cardiomyocytes. This study firstly found the correlation between elevated interleukin (IL)-6 and IL-11, as well as subsequent STAT3 signaling activation and the downregulation of miR-199a-5p in hypoxic myocardial samples from children with congenital heart disease. Then, using model of hypoxic mice and the intervention of phosphorylated STAT3 (pSTAT3), it was observed that pSTAT3 affected the expression of miR-199a-5p and modulated the expression of its target genes, including endoplasmic reticulum stress (ERS)-related activating transcription factor 6 (ATF6) and 78 kDa glucose-regulated protein (GRP78). Further observation revealed that the pSTAT3 signal in cardiac tissue could affect the expression of pri-miR-199a-2, a precursor of miR-199a-5p. And the chromatin immunoprecipitation (ChIP) assay also confirmed that pSTAT3 could bind to the promoter region of miR-199a-2 gene, which is more significant under hypoxic conditions. In conclusion, the activation of STAT3 signaling in cardiomyocytes during chronic hypoxia leads to downregulation of miR-199a-5p, which promotes the expression of many downstream target genes. This is an important pathway in the adaptive protection mechanism of myocardium during hypoxia.

Keywords Hypoxia · miR-199a-5p · STAT3 · microRNA · Cardiomyocytes · Unfolded protein response

Introduction

Chronic hypoxia of myocardial cells is a common pathophysiological process of many cardiovascular diseases such as coronary heart disease and cyanotic congenital heart disease (CCHD). Under the stimulation of hypoxia, myocardial cells will produce a series of compensatory changes in biochemical and metabolic processes, reduce the consumption of oxygen and energy, and rebuild the structural and functional homeostasis of the cells [12, 13]. The adaptation of the heart to chronic hypoxia is important for the survival and growth of myocardial cells, which is related to the prognosis, treatment, and progression of disease. However, the detailed mechanism remains largely uncharacterized.

In recent years, it has been revealed that microRNAs play important roles in energy metabolism adjustment process of hypoxic or ischemic heart [1]. MicroRNA is a class of endogenous, non-coding single-stranded RNA, which can regulate the expression of many genes by selective targeting 3'-UTR of mRNA, and thus could be involved in many pathological processes. Among them, miR-199a-5p is a highly expressed

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microRNA in the heart. A number of studies observed that miR-199a-5p level will decrease rapidly in hypoxic myocardium, which promotes the expression of its target genes at the post-transcriptional level, including hypoxia inducible factor (HIF), vascular endothelial growth factor (VEGF) and Sirtuin (SIRT), contributing to the protective effects for hypoxic-ischemic myocardium by adjusting myocardial oxygen metabolism and cardiac angiogenesis [15, 24]. Previous studies have also found that miR-199a-5p can negatively regulate unfolded protein response (UPR)-related genes including 78 kDa glucose-regulated protein (GRP78), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) in various cells [3, 11]. Our recent study already demonstrated that moderate hypoxia can reduce miR-199a-5p in cardiomyocytes, and subsequently upregulate the expression of its target genes, UPR-related ATF6 and GRP78 [30]. The enhanced UPR alleviated endoplasmic reticulum stress (ERS) and avoid the occurrence of cellular apoptosis. Although many studies have reported that the downregulation of miR-199a-5p under hypoxia can exert protective effect on heart by increasing expression of a wide range of genes, the question of which mechanism causes the decrease of miR-199a-5p in hypoxic cardiac cells is not clear at present.

In the case of myocardial ischemia and hypoxia, the accumulation of reactive oxygen metabolites and toxic free radicals causes immune cells to produce inflammatory and anti-inflammatory reaction through pathway such as complement activation and NF-κB signal, generating large amounts of cytokines, including interleukin (IL)-1β, IL-6, TNFα, Monocyte chemoattractant protein (MCP) 1, etc. [14, 21]. In addition to the damage to myocardial cells caused by inflammatory factors, some cytokines have a protective effect on myocardial cells, such as IL-6 and IL-11, which can exert anti-inflammatory and antioxidant effects, reduce myocardial hypertrophy and myocardial fibrosis, and prevent myocardial cell apoptosis. The protective effects of these cytokines usually depend on the activation of signal transducer and activator of transcription 3 (STAT3) signal [17, 22]. In clinical application, the exogenous injection of these cytokines and the activation of STAT3 signaling have shown good prospect for myocardial protection [23]. Our past studies have found that activation of IL-6/STAT3 pathway contributed to myocardial adaptation to chronic hypoxia in cyanotic congenital heart disease [6]. Recent studies have found that STAT3 signaling can regulate the expression of some microRNAs such as miR-17 and miR-146 [2, 25]. A previous study has revealed STAT3-mediated regulation of miR-199a-5p in cardiomyocytes under normal physiological condition, as well as a link between reduced STAT3 and increased miR-199a-5p in pathophysiological process of heart failure [8]. We would like to know, in myocardial cells under chronic hypoxia condition, whether cytokines such as IL-6 and IL-11 could affect the expression of miR-199a-5p through activation of STAT3 signaling, thereby affecting its downstream target genes and promoting myocardial cells adapt to hypoxia.

In the present study, we firstly observed the relation between the STAT3 signal activation and the downregulation of miR-199a-5p in hypoxic myocardial samples from cyanotic congenital heart disease (CCHD). Then, animal model of hypoxic mice and intervention on STAT3 signaling were used to study whether and how STAT3 affect the expression of miR-199a-5p under hypoxic pathophysiological process. Taken UPR/ERS-related genes as an example, the expression of miR-199a-5p-target genes were also observed. These studies will enrich our knowledge of myocardium protective adaptation to hypoxia.

Methods

Subjects

The participants in this study include 17 patients diagnosed with cyanotic congenital heart disease (Tetralogy of Fallot; O_2 saturation 81.6 ± 7.5%; aged 2.9–16 years) and 15 cases of acyanotic congenital heart disease (ACHD, includes atrial and ventricular septal defects and pulmonary arteriovenous malformation; O_2 saturation 96.2 ± 3.5%; aged 2.6–17 years), who were all from department of cardiovascular surgery of our hospital. The research program was approved by the hospital's ethics committee, and the signed informed consent forms were obtained from parents of the children. After cardiopulmonary bypass during the operation process, the myocardial tissue samples in right ventricular outflow tract were removed and stored in liquid nitrogen for subsequent study.

Mice treatment

BALB/c male mice, 8–10 weeks old, were purchased from experimental animal center of Third Military Medical University. The mice lived continuously in normal atmosphere (oxygen pressure 21.15 kPa, 159 mmHg), or in a low-pressure hypoxic environment (oxygen pressure 11.01 kPa, 82.8 mmHg, the oxygen concentration is equivalent to half of the normal atmospheric environment) for 7 days and 21 days, part of mice were administered every other day with STAT3 specific inhibitor S3I-201 (SelleckChemicals, Houston, TX, USA) by intraperitoneal injection at the dose of 5 mg/kg, or treated with miR-199a-5p antagomir (Genepharma, Shanghai, China) simultaneously at the dose of 20 mg/kg. The control group was administered with equal volume of saline. All animal handling was consistent with the standards of experimental animal ethics.

Detection of protein expression

The proteins were collected from myocardial tissues using the corresponding nuclear and cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Jiangsu, China), and protein concentrations were quantified by BCA method. Then, each protein sample was taken 20 g for SDS-PAGE gel electrophoresis. After being transferred to PVDF blotting membrane, nonspecific binding was minimized by the addition of dried skimmed milk. The primary antibodies include anti-GRP78 rabbit polyclonal antibody (BD Pharmingen, SanJose, CA, USA), anti-ATF6, anti-CHOP rabbit polyclonal antibodies (Santa Cruz Biotechnology, CA, USA), anti-pSTAT3(Tyr705) anti-STAT3 antibodies (Cell Signaling, Beverly, MA, USA), and anti-GAPDH antibody (Sigma), and the secondary antibody was horseradish peroxidase conjugated goat anti-rabbit IgG (Beyotime Biotechnology). The protein bands were detected by the method of ECL, and the densities were analyzed using ImageJ 1.45 s software (NIH, Bethesda, MD, USA).

Detection of cytokines

The plasma isolated from venous blood of patients were stored at − 70 °C. The enzyme linked immunosorbent assay (ELISA) method was applied for the detection of plasma IL-6 and IL-11 level, and the detection kit were purchased from R&D company (Minneapolis, MN, USA) and RayBiotech (Norcross, GA, USA), respectively. The detection step was in strict accordance with the manufacturers' instruction.

Detection of miRNA and mRNA by fluorescent quantitative PCR

The total RNA from samples of patients and mice were extracted using the RNAiso kit (TaKaRa, Dalian, China). Detection of human and mouse microRNAs were performed by the All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia, Guangzhou, China), using U6 as internal control. The primers for human and mouse miR-199a-5p, miR-199a-3p and U6 were also purchased from GeneCopoeia company. For detection of mRNA, the Takara PrimeScript RT application kit was used for reverse transcription step, the expression of pri-miR-199a-1 and pri-miR-199a-2 were measured using fluorescence quantitative PCR method, and the Power SYBR Green PCR Master the Mix Kit was purchased from Applied Biosystems company. The primer sequences were as follows: mmu-miR-199a-1: forward 5'-CTGGCCTGTACCATGGCC-3′ and reverse 5'-CAGACTACTGTACATGTCCC-3′ mmu-miR-199a-2: forward 5'-GACAGGCTCTCCCCAGCC-3′ and reverse 5'-GACAGGCTCTCCCCAGTC-3′. The relative expression of miRNA and mRNA were calculated by $2^{-\triangle\triangle}$Ct method.

Immunohistochemistry staining

The formalin-fixed, paraffin-embedded myocardial tissues were sectioned. After procedures of dewaxing, hydration, and heat-induced epitope retrieval, the tissue slides were incubated in 3% H_2O_2 to remove endogenous excessive nutrient enzymes, followed by a 15-min blocking step with 1% skim milk. The 1:200 rabbit polyclonal anti-GRP78/Bip (Santa cruz, CA, USA) was added, incubated for 1 h at 37 °C, and stayed overnight at 4 °C. Next, sections were washed with PBS and incubated with biotin-labeled secondary antibody (Zhongshan Goldenbridge Company, Beijing, China) for 1 h. The expression of GRP78 was visualized by DAB coloration, and the nucleus was stained with hematoxylin.

Chromatin immunoprecipitation

The SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads; no. 9005; Cell signaling Technology) was used for chromatin separation and immunoprecipitation. The 25 mg myocardial tissue from experimental mouse was cut and placed in PBS containing protease inhibitor. After cross-linking in 1.5% formaldehyde solution for 20 min at room temperature, glycine solution was added to terminate the reaction. The nucleus was extracted according to the kit instructions, and the chromatins were broken into small fragments using the micrococcus nuclease. The DNA protein complex was captured by rabbit anti-p-STAT3 through overnight incubation, using anti-histone 3 antibody as positive control group or normal rabbit IgG as negative control. After adding the ChIP-grade protein G beads and resting at 4 °C for about 2 h, the DNA was recovered by chromatin rinsing and precipitation. The fluorescent quantitative PCR detection was carried out using the SYBR Green PCR Master Mix-PLUS Kit (TOYOBO, Osaka, Japan) and specific primers designed in the miR-199a-2 promoter region (− 640: 0). Forward (F), AAA GGG GGG AGC CCC AAC TTA TCT G; reverse (R), TTC CTG CAC CAGGGG CTT GT.

Statistical analysis

For data with a normal distribution, the mean values of multiple groups were compared using one-way analysis of variance (ANOVA) and the least significant different (LSD) tests. For data that did not conform to normal distribution, multiple groups were compared using the nonparametric rank sum test. The correlations were assessed by Pearson (for normally distributed data) and Spearman (for non-normally distributed data) methods. The significance level was $P < 0.05$, and $P < 0.01$ was considered highly significant. All the statistical analyses were performed using the SPSS13.0 software.

Results

The different levels of IL-6 and IL-11, as well as STAT3 and miR-199a-5p in myocardial tissue of CCHD and ACHD

In children with CCHD, the chronic hypoxia induced significant increase of IL-6 and IL-11 levels in peripheral blood compared to the acyanotic group (Fig. 1a, b). Correspondingly, as the downstream signaling of IL-6 and IL-11, the expression ratio of phosphorylated STAT3 (p-Tyr-705-STAT3) in nuclear protein and cytoplasmic STAT3 protein in hypoxic myocardial tissue of CCHD was significantly higher than that in ACHD group (Fig. 1c). At the same time, myocardial miR-199a-5p levels of cyanotic group were significantly lower than those of acyanotic group. However, as a complementary sequence of miR-199a-5p, the expression of miR-199a-3p in both CCHD and ACHD groups were significantly lower than that of miR-199a-5p, and there was no significant difference in the levels of miR-199a-3p between the two groups (Fig. 1d). Correlation analysis showed a significant negative correlation between miR-199a-5p expression and the two cytokines, IL-6 and IL-11, in myocardial tissue of CCHD children (Fig. 1e, f), and miR-199a-3p is not associated with these two cytokines.

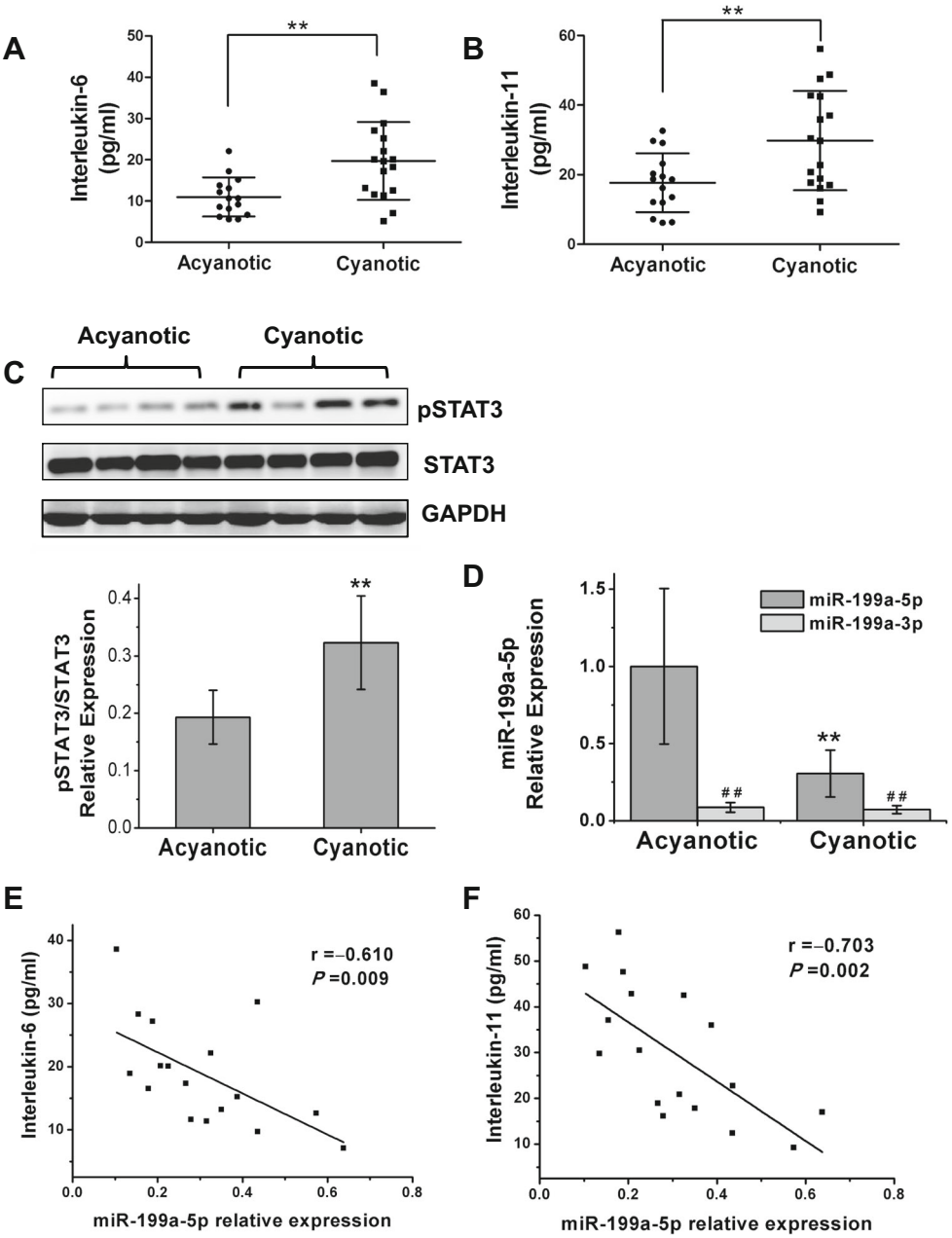

Fig. 1 Effects of chronic hypoxia on IL-6/IL-11 production, STAT3 activation, and miR-199a-5p levels. **a**, **b** The ELISA results of IL-6 and IL-11 in plasma for cyanotic and acyanotic groups. Each sample was measured two times and the average value was taken. The lines represent median and interquartile range. **c** The phosphorylation of STAT3 in myocardial tissue specimens was detected by Western-blot. The above are the representative bands of each group. The relative expression of pSTAT3/STAT3 is shown below (acyanotic group $n = 18$; cyanotic group $n = 16$). **d** The level of miR-199a-5p and miR-199a-3p in myocardial tissue specimens of cyanotic and acyanotic groups. **e**, **f** The analysis of the correlation between the level of miR-199a-5p in myocardial tissue and plasma IL-6/IL-11 contents in cyanotic patients. **Compared with the acyanotic group, $P < 0.01$; ## Compared with the miR-199a-5p level of each group, $P < 0.01$

STAT3 signaling affects miR-199a-5p production by regulating the expression of pri-miR-199a-2

In order to ascertain whether the activation of STAT3 has a causal relationship with the decrease of miR-199a-5p level, we examined miR-199a-5p levels in myocardial tissue of mice when STAT3 activation was inhibited during 7-day and 21-day hypoxic condition. It was observed that hypoxia induced a significant decrease in the level of miR-199a-5p in the myocardial tissue of mice, and the inhibition of STAT3 phosphorylation by S3I-201 at the same time could significantly prevent the decrease of miR-199a-5p (Fig. 2a).

MiR-199a-5p comes with two different original microRNAs, namely, pri-miR-199a-1 and pri-miR-199a-2. Further PCR experiments showed that hypoxia and activation of STAT3 mainly affected the pri-miR-199a-2 expression, but there was no significant effect on the expression of pri-miR-199a-1 (Fig. 2b, c).

Then, ChIP assay was performed to determine whether STAT3 signal can directly regulate the gene expression of pri-miR-199a-2. We found that pSTAT3 could combine with miR-199a-2 promoter region, and under hypoxia, the binding of pSTAT3 and miR-199a-2 promoter region was further strengthened (Fig. 2d).

STAT3 intervention affects expression of miR-199a-5p target gene

The previous studies of our group have found that the expression of two proteins related to endoplasmic reticulum stress, ATF6 and GRP78, increased in myocardial tissue of children with CCHD. ATF6 and GRP78 were also the target genes of

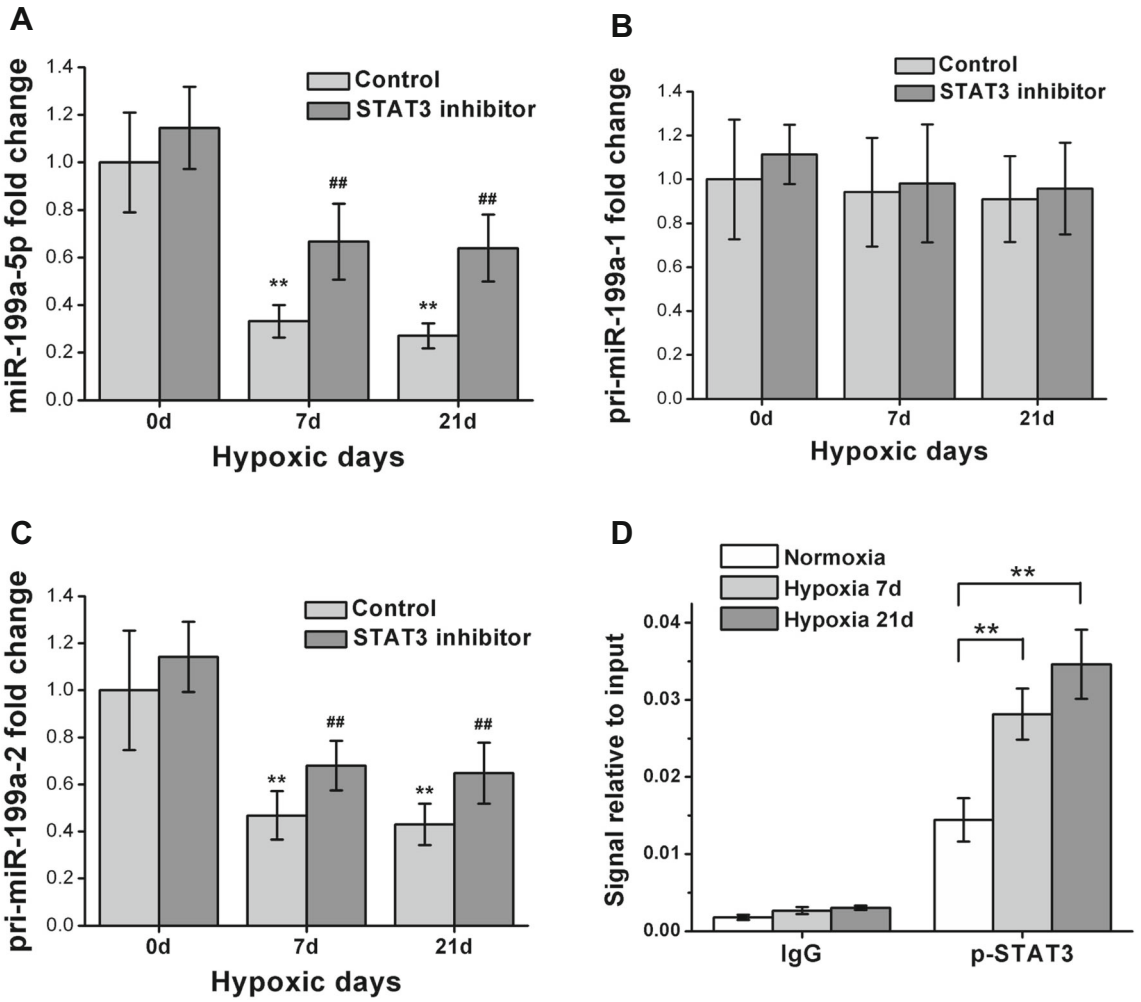

Fig. 2 The effect of STAT3 activation on the expression of miR-199a gene. **a** Effects of hypoxia and inhibition of STAT3 phosphorylation on the expression of miR-199a-5p in myocardial tissue ($n = 6$). **b**, **c** Effects of hypoxic condition and STAT3 intervention on the expression of pri-miR-199a-1 and pri-miR-199a-2 (primary miRNA of miR-199a-5p) in the myocardium of mice ($n = 6$). **d** ChIP analysis of p-STAT3 binding to miR-199a-2 promoter region in myocardium of mice under normoxic and hypoxic environments. ** Compared with the normoxic group, $P < 0.01$. ## Compared with hypoxic un-intervention group, $P < 0.01$

miR-199a-5p. We speculate that STAT3 activation during hypoxia should promote the expression of ATF6 and GRP78 by inhibition of miR-199a-5p. We test this hypothesis by mice experiments. As shown in Fig. 3a and b, during living at hypoxic environment, when the intervention of STAT3 signal pathway hampered the decrease of miR-199a-5p, the expression of ATF6 (both p90 and p50) and GRP78 were also significantly inhibited at the same time. In addition, the expression of CHOP, a myocardial ERS-related apoptosis protein also increased. If the mice were injected with miR-199a-5p antagonist to inhibit its level when interfering STAT3 signal, the increasing expression of ATF6 and GRP78 during hypoxia reappeared, as well as inhibited expression of the apoptosis protein CHOP. The immunohistochemical analysis of the expression of GRP78 in cardiac specimens of these mice also clearly reflects the similar results (Fig. 3c).

To verify the above phenomena in myocardial specimens of patients with CCHD, we analyzed the correlations between pSTAT3/STAT3 ratio, miR-199a-5p levels, and ATF6 and GRP78 protein expressions. As shown in Fig. 3d, significant correlations between these indicators were all observed in CCHD patients, especially the close correlation between

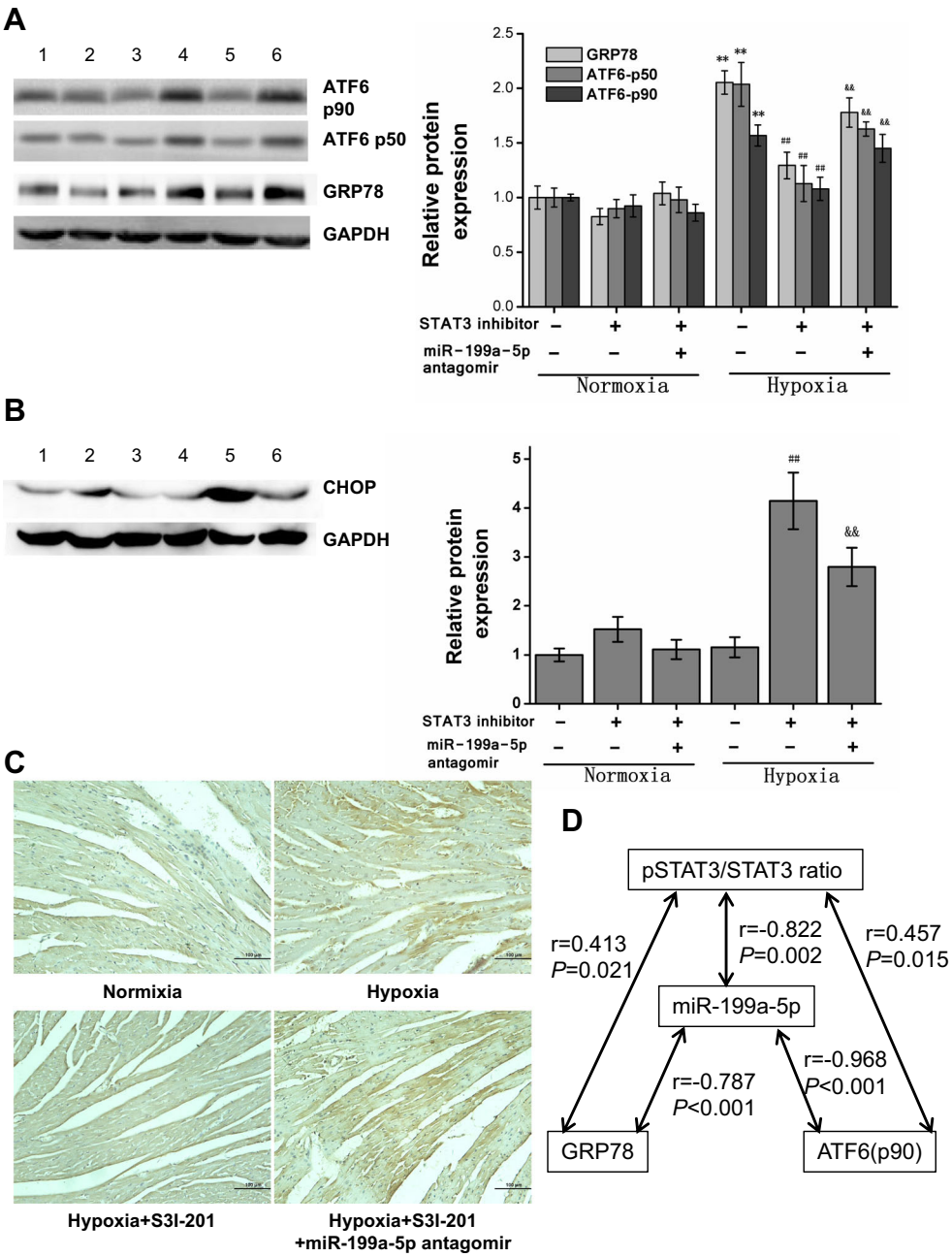

Fig. 3 Effect of inhibiting STAT3 activation on expression of miR-199a-5p target genes related to endoplasmic reticulum stress. **a** The protein expression of miR-199a-5p target genes ATF6 and GRP78 in the myocardium of hypoxic mice when inhibiting STAT3 activation ($n = 6$). Western band lane 1–3 (normoxic mice), lane 4–6 (hypoxia mice) were representative of non-interventional, only inhibiting STAT3 activation by S3I-201 and inhibiting both STAT3 signaling and miR-199a-5p level, respectively. **b** Effect of STAT3 intervention on expression of apoptosis-related protein CHOP in myocardium of hypoxic mice($n = 6$). **c** Representative sections of immunohistochemical staining for GRP78 expression (depicted in brown) in heart specimen slices of these experimental mice. ** Compared with the normoxic group, $P < 0.01$. $^{\#\#}$ Compared with hypoxic un-intervention group, $P < 0.01$. $^{\&\&}$ Compared with hypoxic STAT3-inhabited mice, $P < 0.01$. **d** The correlation analysis of pSTAT3/STAT3 ratio and miR-199a-5p, ATF6, and GRP78 protein levels in myocardial tissues of CCHD patients. Spearman's or Pearson's correlation coefficient (r) and P values are labeled

pSTAT3/STAT3 ratio and miR-199a-5p levels, as well as the strong association between miR-199a-5p and its two target genes (ATF6 and GRP78).

Discussion

Although previous studies have found that both IL-6/IL-11-STAT3 signaling and miR-199a-5p have protective effects on the hypoxic-ischemic heart by regulation of various genes, this study firstly found the relationship between these two approaches. Using two research models including clinical samples of cyanotic congenital heart disease and hypoxic mice, we demonstrated that activation of STAT3 signaling by IL-6/IL-11 downregulated miR-199a-5p levels in myocardial cells, which is involved in the adaptive protective UPR mechanism during cardiac chronic hypoxia.

In the absence of oxygen, the cellular mitochondrial respiration steady-state was destroyed, and the free radicals production was increased, which leads to oxidative damage and activation of inflammatory response [5]. Previous studies have confirmed that the systemic inflammatory response caused by chronic intermittent hypoxia did not cause obvious injury in the heart. On the contrary, it was observed that compensatory anti-inflammatory response obviously protect cells from damage in the hypoxic heart [9]. The protective effect of cytokines such as IL-6 and IL-11 on the heart has been well recognized, and its main mechanism is activating JAK2-STAT3 signal. Previous studies have confirmed that STAT3 activation is beneficial to myocardial cells against a variety of physiological and pathological stress conditions, STAT3 gene knockout mice are more prone to heart failure compared to wild-type mice under various stress conditions [16]. Studies have also shown that the pharmacological mechanisms of some potential cardio-protective drugs, such as rapamycin, are associated with STAT3 activation [4]. For the first time, the present study suggested that the protective effect of STAT3 pathway during hypoxic stress condition might be realized by means of miR-199a-5p regulation in pathophysiological process of hypoxia, which was a further extension of STAT3's role in regulating miR-199a-5p discovered by Haghikia et al. One of the important target genes of miR-199a-5p is HIF1α. As a transcription factor, HIF1α can upregulate the expression of several cytokines, such as vascular endothelial growth factor (endothelial cell, growth factor, VEGF) and erythropoietin (erythropoietin, EPO), to play protective effect on hypoxic and ischemic myocardium by increasing angiogenesis and improving tissue oxygen supply.

The precursors of miR-199a-5p, pri-miR-199a are encoded by the miR-199a-1 and miR-199a-2 genes [7]. The miR-199a-1 is located on human chromosome 19 (chromosome 9 for mice), and the miR-199a-2 gene is located on chromosome 1. In this study, we found that hypoxia reduced the level of miR-199a-5p in myocardial cells mainly by inhibiting the expression of miR-199a-2. Furtherly, we confirmed the binding capacity of STAT3 to miR-199a-2 promoter by ChIP assay. MiR-199a-2 and miR-214 are located in the intron of the reverse strand of DNM3 gene (Dynamin-3 opposite strand, DNM3os) (3′-5′). It is reported that Twist-1 can increase the level of miR-199a and miR-214 by promoting the transcription of DNM3os in multiple tissues and organs during embryonic period [19]. Both Twist-1 and STAT3 are important transcription factors. They may cooperatively maintain the normal level of miR-199a-5p, and also played a regulatory role on miR-199a-5p production in stress such as hypoxia.

Studies of our team and others detected the increasing of UPR effector molecules ATF6 and GRP78 in hypoxic or infarcted myocardial tissue [26]. At present, it has not been reported that the main signal pathways of hypoxic sensing, such as hypoxia inducible factor (HIF) 1/2 alpha and beta can directly regulate the expression of ATF6 and GRP78. Our preliminary promoter analyses using JASPAR database (http://jaspar.genereg.net) [18] and LASAGNA-Search 2.0 (http://biogrid-lasagna.engr.uconn.edu/lasagna_search/) [20] also showed that there is no binding site with high probability for HIF (including HIF-1α, HIF-2α or their β subunits) in the promoter regions of human or murine ATF6 and GRP78. Our observation on hypoxic mice with the intervention of STAT3 and miR-199a-5p confirmed that hypoxia regulate the expression of ATF6 and GRP78 through a pathway not directly depending on HIF signaling, but involving STAT3 and miR-199a-5p pathway, to prevent apoptosis induced by ERS. It has been reported that some drugs, such as Elatoside C, can enhance the unfolded protein response of myocardial cells to hypoxia and reoxygenation, so as to avoid the apoptosis induced by ERS and thus to protect the heart [28]. This paper reported that the pharmacological mechanism of Elatoside C is related to STAT3 activation, but did not explain how downstream of the STAT3 signaling acted on ERS- or UPR-related molecules. The results of this study solved this problem and revealed that the regulation of STAT3 on UPR is mediated by miR-199a-5p.

As an important transcription factor, except for miR-199a-5p, it is not excluded that the activation of STAT3 signal during hypoxia can positively or negatively regulate the expression of other microRNA or coding genes. In addition, there are a large number of target genes regulated by miR-199a-5p. In this study, we only focused on the changes of ATF6 and GRP78, the two endoplasmic reticulum stress related genes. It was also reported in recent literature that ERS could activate inflammation [10, 21, 29]. Then, it is possible that ERS-induced inflammation and subsequent STAT3 signaling adversely affects ERS intensity by miR-199a-5p-mediated UPR process, which forms a negative feedback of excessive ERS and avoids damaging of cells. In the future, we can make a further study on the above problems.

In summary, our results demonstrated that chronic hypoxia could downregulate miR-199a-5p levels through activating STAT3 pathway. Decreasing miR-199a-5p promotes the expression of GRP78 and ATF6, the two major ER stress sensors and UPR effectors, thus benefiting UPR process and the protection of cardiocytes against ERS-related apoptosis. This is a novel adaptation mechanism of cardiac myocytes to chronic hypoxia. We propose that STAT3 and miR-199a-5p could be valuable diagnostic and therapeutic targets for patients of CCHD and other hypoxic-ischemic heart disease [27].

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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