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GAS5 promotes glucose metabolism reprogramming and resistance to ferroptosis of endothelial progenitor cells through the miR-495-3p/SIX1 and IGF2BP2/NRF2 dual-regulatory pathways in coronary heart disease

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Abstract

We aimed to explore the potential along with mechanism of lncRNA growth arrest-specific 5 (GAS5) in modulating glucose metabolism and ferroptosis of endothelial progenitor cells (EPCs) in coronary heart disease (CHD). CCK-8, flow cytometry, EdU, colony formation, scratch test as well as transwell assays were implemented to assess cell biological behaviors. Glucose uptake testing, lactic acid production assay, and detection of extracellular acidification rate (EACR) together with oxygen consumption rate (OCR) were used to assess glucose metabolism. Iron, GSH and MDA detection were used to measure ferroptosis. Besides, a series of mechanical experiments were implemented to clarify the modulatory relationship between GAS5 and nuclear factor erythroid 2-related factor 2 (NRF2) as well as sine oculis homeobox 1 (SIX1). We found that GAS5 was down-regulated in CHD patients relative to healthy controls. GAS5 depletion repressed EPCs proliferation, migration along with invasion while elevated cell apoptosis. GAS5 promoted the reprogramming of glucose metabolism and inhibited ferroptosis in EPCs. GAS5 affected glycometabolic reprogramming and ferroptosis resistance through regulating SIX1 and NRF2. On the one hand, GAS5 promoted NRF2 mRNA stability through IGF2BP2. On the other hand, GAS5 regulated the miR-495-3p/SIX1 axis in EPCs. To sum up, GAS5 promotes glucose metabolism reprogramming and resistance to ferroptosis of EPCs through the miR-495-3p/SIX1 and IGF2BP2/NRF2 dual-regulatory pathways in CHD.

Keywords: Coronary heart disease, Endothelial progenitor cells, Ferroptosis, GAS5, Glucose metabolism, NRF2, SIX1.

1. Introduction

Cardiovascular diseases pose a major threat to human health, and coronary heart disease (CHD) is regarded as the primary cause of death among health problems. Based on the China Cardiovascular Health and Disease Report 2021, about 330 million people in China suffer from cardiovascular disease, including about 11.4 million cases of CHD [1]. China is faced with a major public health challenge caused by cardiovascular diseases, whose high incidence, high mortality, and huge medical burden have exerted tremendous pressure on society. A variety of cardiovascular diseases eventually lead to myocardial fibrosis, especially in patients with myocardial infarction, end-stage ischemic myocardial apoptosis, and necrosis, even though interventional or surgical bypass revascularization, restores heart blood supply, but the value is limited, and cannot prevent the occurrence of serious cardiac insufficiency [2]. Therefore, at this stage, it is urgent to actively seek new treatment methods and programs to deal with the problem of myocardial damage and restore its function. Studies have shown that endothelial progenitor cells (EPCs) promote vascular regeneration and regenerative endothelialization after tissue damage by directly participating in mechanisms, such as angiogenesis and secretion of protective cytokines [3]. Therefore, intracoronary transplantation of EPCs has a good clinical application prospect, but there are urgent problems to be solved in the treatment of CHD by EPCs transplantation: the number, function, and aging of EPCs.

It is well known that cardiovascular disease is caused by the interaction of multiple risk factors. Cardiovascular diseases have a significant familial aggregation, and genetic factors play an important role. While individual genetic susceptibility genes have a small effect on disease risk, the number and frequency of susceptibility genes are widespread across the population [4]. In addition, environmental and external factors should not be ignored, such as high blood pressure, smoking, diabetes, obesity, insufficient physical activity, improper diet, alcohol abuse, and air pollution [5]. For a long time, a large number of epidemiolo-

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gical and molecular biological studies have confirmed the key role of iron metabolism disorders as well as oxidative stress in the pathogenesis of cardiovascular diseases [6]. However, a series of recent studies have shown that not only ferroptosis exists in cardiovascular diseases, but also the protective effect of targeted intervention on ferroptosis is exciting [7].

Non-coding RNAs (ncRNAs) belong to molecules that can be transcribed rather than translated into protein products. NcRNAs work as modulatory molecules that mediate many cellular processes, containing chromatin remodeling, transcription, as well as post-transcriptional modification. It is discovered that more than 90% of the human genome contains non-protein-coding RNAs, and nearly 75 percent of these genes code for ncRNAs, so the role of ncRNAs in affecting disease gene expression is much more important and complex than we currently realize [8].

Long non-coding RNAs (LncRNAs) belong to a kind of ncRNAs that is implicated in affecting EPCs progression. They do not have an open reading frame. LncRNAs can modulate gene expression at the epigenetic, transcriptional, as well as post-transcriptional levels. Previously, a novel lncRNA growth arrest-specific 5 (GAS5) was identified to be a tumor repressor in many cancers [9]. Recently, increasing evidence has revealed that GAS5 is also widely involved in the pathological processes of cardiovascular cells, including regulating the apoptosis and inflammatory damage of cardiomyocytes, proliferation, apoptosis, autophagy, and angiogenesis of endothelial cell, and the proliferation, migration, apoptosis as well as differentiation of vascular smooth muscle cells [10, 11]. However, the expression, function, downstream target, and regulatory mechanism of GAS5 in EPCs are not clear.

The purpose of our study was to explore the potential along with mechanism of GAS5 in EPCs, in order to provide new ideas for treating CHD.

2. Materials and methods

2.1. Samples

This study was approved by the Ethics Committee of our hospital. Thirty-four CHD patients as well as 34 healthy volunteers were enrolled in our hospital, and their atherosclerotic peripheral blood was obtained as samples. All the participants signed the informed consent. Peripheral blood was centrifuged, and the supernatant was obtained and stored at -80°C.

2.2. Extraction and culture of EPCs

2 ml peripheral blood was gathered from CHD patients (n=34) and healthy controls (n=34). Through centrifugation, the peripheral blood mononuclear cells were isolated, and then cultured on 6-well plates coated with fibronectin for 24 h before transplanting, and in the endothelial basal medium (Cambrex, USA) supplemented with 30 μg/ml endothelial cell growth supplements (Sigma, USA.), 90 μg/ml heparin (Selleck Chemicals, USA), as well as 1% antibiotics solution at 37 °C. For screening EPCs, the non-adherent cells were removed after 4 days, and the adherent cells were cultured in fresh medium every 3 days. The purity of isolated EPCs was tested by flow cytometry using anti-CD34 as well as anti-VEGFR antibodies.

2.3. Cell transfection

Two specific siRNAs targeting GAS5 and si-NC, NRF2, SIX1 and IGF2BP2 overexpression vector and empty vector (pcDNA3.1), miR-495-3p mimics and NC mimic, as well as miR-495-3p inhibitor and NC inhibitor, were synthesized by GenePharma (Shanghai, China). Cell transfection was implemented utilizing Lipofectamine 2000 (Invitrogen, USA). The samples were gathered after 48 h transfection for further analysis.

2.4. RT-qPCR

EPCs were collected 48 h later for total RNA extraction using miRNeasy Mini Kit of Tiangen Biochemical Company (Beijing, China). Then, total RNA was adopted to synthesize cDNA with the EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Real-time PCR was implemented using SYBR®Premix Ex TaqTM II (TakaRa, China). The comparative threshold cycle (ΔΔCT) method was used for quantification.

2.5. CCK-8

EPCs were inoculated into 96-well culture plates with 5×10^3 cells in each well. Incubated cells were treated with a CCK-8 reagent (Dojindo, Japan) with a volume of 10 μl and absorbance was observed at 450 nm.

2.6. Flow cytometry

EPCs were harvested and stained with Annexin V-FITC/PI (BD Biosciences, USA). Then cells were analyzed by a FACScan flow cytometer (BD Biosciences, USA).

2.7. Colony formation

In this experiment, 500 EPCs were inoculated into a 6-well plate in a 2 ml complete medium that contained 10% FBS. The cells were then cultivated under these conditions for two weeks. The colony was observed every 2 days and the medium was changed. After fixation using 4% polyformaldehyde and staining with 0.1% crystal violet staining, the number of colonies was counted.

2.8. EdU staining

Nucleus staining was performed using Click-it EdU imaging kit (Ribobio, China). Each well was inoculated with 3×10^3 transfected cells into 96-well plates, which were cultured in complete medium for 48 hours, and then cultivated with 50 μM EdU solution for 2 hours. Followed by fixation with 4% paraformaldehyde and treatment with 0.5% Triton X-100, 100 μl of 1 × Apollo staining reaction solution was prepared and incubated with cells in the dark for 30 minutes. Finally, 1 × Hoechst 33342 reaction solution was prepared and cultivated with cells in the dark for 30 min. Nuclei stained by EdU were imaged with fluorescence microscopy (Olympus).

2.9. Wound healing

EPCs were inoculated into 24-well plates (4×10^5 cells per well). After 24 hours, a 200 μl sterile spray gun was used to create scratches in each hole. The cells were washed and then cultured in an incubator. An inverted microscope (Olympus, Japan) was used to detect the migration distance and took images at 0 and 48 h after the scratch test.

2.10. Transwell

Cell invasion assay was performed by means of Transwell chambers (Costar, USA) with matrigel (BD

Biosciences, USA). 1×10^5 cells were placed onto the upper insert. The lower chamber was filled with 800 µl medium containing 20% FBS. After cultivation for 48 h, cells on the lower surface were fixed with ethanol as well as stained with 0.2% crystal violet. The number of invaded cells was calculated.

2.11. Glucose uptake testing

Cells were collected and inoculated into 96-well cell culture plates with 1×10^4 cells per well, and then cultured overnight at 37°C. Then, cells were deprived of sugar for 2 hours. Afterwards, 10 µl 2-deoxy-d-glucose (2-DG) was injected into each well and incubated for 20 min. After cultivating with 100 ml Krebs-HEPES, 10 µl 2-DG was injected into each well. The cells were then gathered. The glucose intake was measured at 412 nm wavelength by OD method.

2.12. Lactic acid production assay

The L-lactic acid test kit (colorimetry) was used to assess the amount of lactic acid produced. The transfected cells were first inoculated into a 96-well cell culture plate and then cultivated overnight at 37°C. After 2 hours of starvation, the supernatant was collected and the lactic acid yield was measured. Lactate production levels were measured at 450 nm.

2.13. Iron detection

Cells were seeded in a 10 cm^2 plate, followed by treatment with Erastin, RSL3, or DMSO for 12 h. After washing, and homogenized on ice, and then centrifuged at 4°C to remove insoluble material. The supernatant was gathered and iron reducer was added to each sample before mixing, and incubating for 30 min. After that, 100 µL of the iron probe was added to each sample, mixing and incubating the reaction for 1 h. The absorbance at 593 nm was measured with a microplate reader.

2.14. GSH detection

Cells were planted in 10 cm plates, followed by treatment with Erastin, RSL3, or DMSO for 12 h. After washing, cells were lysed, and GSH levels were measured by spectrophotometry at 412 nm.

2.15. MDA detection

Standard diluent of MDA was prepared and tested with optimal dilution of cell lysate. Then, thiobarbituric acid solution was added to standard sample as well as test sample and incubated at 95°C for 1 h. The sample was then cooled to room temperature. Then, 200 µL of each mixture was placed into a 96-well microplate for immediate colorimetric determination at 532 nm. MDA concentration was calculated.

2.16. Detection of extracellular acidification rate (EACR) and oxygen consumption rate (OCR)

Cells were inoculated on 96-well cell culture plates containing 10% FBS and incubated overnight at 37°C. After baseline concentration was measured, glucose, oligomycin, as well as 2-DG were added to each well successively for ECAR measurement. At the same time, oligomycin, FCCP, antimycin A and rotenone were injected successively for OCR detection.

2.17. Subcellular fractionation analysis

A PARIS™ kit (Invitrogen, USA) was implemented for subcellular fractionation analysis. RT-qPCR was implemented to analyze cytoplasmic and nuclear RNA extracts, and GAPDH and U6 were adopted to be the reference genes.

2.18. Luciferase reporter assay

GAS5 and SIX1 3'UTR fragments including wild-type (Wt) and mutation (Mut) binding site with miR-495-3p were inserted into pmirGLO vector (Promega, USA) to construct GAS5-Wt, GAS5-Mut, SIX1 3'UTR-Wt, as well as SIX1 3'UTR-Mut. Then, cells were co-transfected with luciferase reporter vectors together with miR-495-3p mimics/mimics NC, followed by examining the luciferase activity by a dual luciferase reporter kit (Promega, USA).

2.19. RNA immunoprecipitation (RIP) assay

A Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) was implemented to determine the relationship between GAS5/IGFBP2 and NRF2. Briefly, cells were resuspended in RIPA buffer, NRF2 antibody or IgG with Dynabeads protein G (Invitrogen, USA) and were added into cells for cultivation overnight at 4°C. After washing, samples were purified and analyzed by RT-qPCR.

2.20. RNA pull-down assay

In short, 1×10^7 cells were lysed on ice with polysome extraction buffer. The supernatant was gathered and cultivated with 2 µg biotin-labeled probes to generate an RNA-protein complex. The incubating pre-treated Streptavidin Magnetic beads were added to the reaction mix. After washing, the precipitated RNA-protein mixture was subjected to RT-qPCR analysis.

2.21. Western blot

Cells were lysed in lysis buffer, and the protein concentration was verified with a BCA assay (Thermo Fisher, USA). Afterwards, proteins could be resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad, USA). After being blocked with 5% skim milk, the membranes were incubated with the primary antibodies at 4°C overnight. After washing, the membranes were incubated with secondary antibody incubation for 1.5 h. Immunodetection was obtained by using the chemiluminescence reagent (Thermo Fisher, USA).

2.22. Statistical analysis

Each experiment was implemented at least 3 times. All data were analyzed using the SPSS 22.0 software. The data are exhibited as mean ± SD. Student's t-test or one-way analysis of variance (ANOVA) was used for comparison. P value < 0.05 was meant statistically significant.

3. Results

3.1. GAS5 depletion inhibits EPCs proliferation, migration along with invasion

To explore GAS5 expression in CHD, RT-qPCR analysis was adopted to examine GAS5 expression in CHD patients. As shown in Fig. 1A, GAS5 was down-regulated in CHD patients relative to healthy controls. Subsequently, the potential of GAS5 in the growth of EPCs was investigated. The EPCs were transfected with either si-NC or si-

GAS5#1/2. RT-qPCR outcomes revealed that si-GAS5#1 was the most effective si-RNA for GAS5 gene knockout. Therefore, si-GAS5#1 was selected for follow-up assays (Fig. 1B). The experimental results of CCK-8 revealed that when the expression of GAS5 was inhibited in EPCs, the proliferation ability of EPCs was significantly reduced (Fig. 1C). EPCs apoptosis was elevated after GAS5 knockdown (Fig. 1D). The colony formation capacity of GAS5 knockdown cells was significantly weakened (Fig. 1E). The EdU proliferation experiment of EPCs displayed that the EPCs proliferation was significantly lessened after GAS5 knockout (Fig. 1F). Additionally, it was manifested that, knockdown GAS5 expression repressed EPCs migration and invasion (Fig. 1G-1H).

3.2. GAS5 promotes the reprogramming of glucose metabolism and inhibits ferroptosis in EPCs

To clarify the possible mechanism of action of GAS5, the localization of GAS5 cells was analyzed. lncALTAS database (https://lncatlas.crg.eu/) showed that GAS5 was mainly presented in the cytoplasm (Fig. 2A). Meanwhile, our experimental results also showed GAS5 was mainly located in the cytoplasm (Fig. 2B), reflecting that GAS5 may be involved in post-transcriptional regulation, which provided a direction for our future research on the core mechanism of GAS5. In addition, KEGG pathway analysis (https://www.genome.jp/kegg/pathway.html) showed that GAS5 was mainly involved in nuclear epigenetic regulation, metabolic regulation and iron metabolism regulation (Fig. 2C), which provided a direction for us to further study the core mechanism of GAS5.

It was speculated that GAS5 may have a certain role in modulating glycolysis. The outcomes showed that glucose uptake, lactic acid and ATP production decreased after GAS5 knockout (Fig. 2D-2F). After GAS5 knockout, the ECAR of the total glycolytic flux was decreased, while the mitochondrial oxidative respiratory index OCR was increased (Fig. 2G-2H). These results confirmed that GAS5 promoted the reprogramming of glucose metabolism in EPCs. Ferroptosis is an important factor leading to EPCs death. Next, we investigated the effects of ferroptosis activator Erastin and RSL3 on EPCs activity. We transfected EPCs with si-NC or si-GAS5#1, followed by treatment with Erastin or RSL-3, the results showed that GAS5 gene knockout increased Erastin or RSL-3 mediated cell death in EPCs compared to control. More importantly, this effect was offset after treatment of ferroptosis inhibitor Fersint-1 countered this effect (Fig. 2I). Subsequently, we analyzed the effects of GAS5 on iron accumulation, GSH and MDA production during ferroptosis. We first analyzed the effect of GAS5 on intracellular iron and Fe^{2+} concentrations using iron kits. The data showed that in Erastin or RSL-3-treated EPCs, iron and Fe^{2+} levels were elevated after GAS5 gene knockout (Fig. 2J). In addition, GAS5 silencing lessened the production of GSH content in Erastin or RSL3-treated EPCs (Fig. 2K). GAS5 silence also increased mitochondrial MDA levels in EPCs (Fig. 2L).

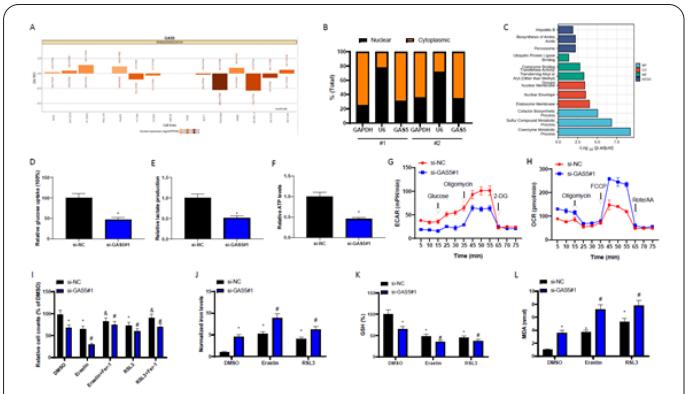

Fig. 2. GAS5 promotes the reprogramming of glucose metabolism and inhibits ferroptosis in EPCs. (A) lncALTAS database showed that GAS5 was mainly located in the cytoplasm. (B) Subcellular fractionation assay examined the location of GAS5. (C) KEGG pathway analysis showed the regulatory function of GAS5. (D-E) Relative glucose uptake and lactate production in EPCs after GAS5 knockdown. (F) ATP levels in EPCs after GAS5 knockdown. (G-H) ECAR and OCR in EPCs after GAS5 knockdown. (I) Cell death in si-GAS5#1 transfected EPCs after treatment with Erastin, Erastin+Fersint-1, RSL3 and RSL3+Fersint-1. (J) Iron levels in si-GAS5#1 transfected EPCs after treatment with Erastin and RSL3. (K-L) GSH and MDA in si-GAS5#1 transfected EPCs after treatment with Erastin and RSL3. $^{*}P<0.05$, $^{\#}P<0.05$, $^{\&}P<0.05$.

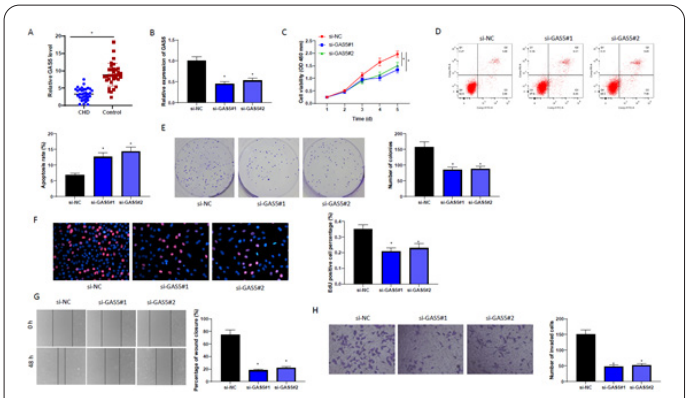

Fig. 1. Knockdown of GAS5 inhibits EPCs proliferation, migration and invasion. (A) GAS5 expression in CHD patients via RT-qPCR. (B) RT-qPCR examined transfection efficiency of GAS5 knockdown in EPCs. (C) CCK-8 examined EPCs viability after GAS5 knockdown. (D) Flow cytometry analysis examined EPCs apoptosis after GAS5 knockdown. (E-F) Colony formation and EdU assays examined EPCs proliferation after GAS5 knockdown. (G-H) Scratch test and transwell assays assessed EPCs migration and invasion after GAS5 knockdown. $^{*}P<0.05$.

3.3. GAS5 affects glycometabolic reprogramming and ferroptosis resistance through regulating SIX1 and NRF2

We first knocked down GAS5 to observe the influence on the expression of these transcription factors. The results showed that only SIX1 and NRF2 were affected by GAS5 depletion (Fig. 3A). Afterwards, we explored whether the functionality of GAS5 depended on SIX1 or NRF2. Further functional studies showed that overexpression of either SIX1 or NRF2 reversed the decrease in EPCs proliferation induced by GAS5 silencing (Fig. 3B). It was also worth mentioning that overexpression of SIX1 could reverse the reduced metabolic reprogramming ability caused by GAS5 silencing (Fig. 3C-3D), while overexpression of NRF2 could reverse the increase in ferroptosis (Fig. 3E-3F) caused by GAS5 silencing. These results indicated that GAS5 affected glycometabolic reprogramming and ferroptosis resistance through regulating SIX1 and NRF2.

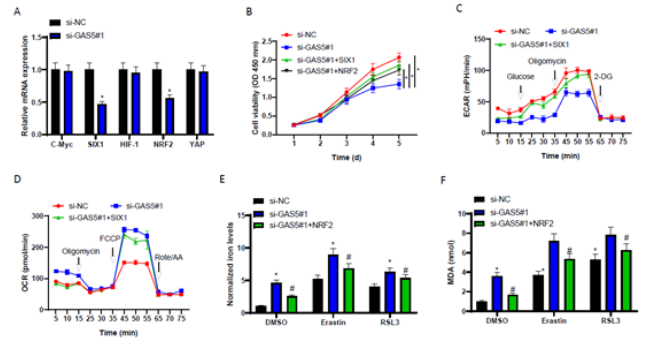

Fig. 3. GAS5 affects glycometabolic reprogramming and ferroptosis resistance through regulating SIX1 and NRF2. (A) Expression of C-myc, SIX1, HIF-1, NRF2 and YAP in si-GAS5#1 transfected EPCs by RT-qPCR. (B) CCK-8 examined EPCs viability in different groups. (C-D) ECAR and OCR of EPCs in different groups. (E-F) GSH and MDA of EPCs in different groups. *P<0.05, #P<0.05.

3.4. GAS5 promotes NRF2 mRNA stability through IGF2BP2

To explore the modulatory relationship between GAS5 and NRF2, RIP together with RNA pull-down assays were performed. It was unveiled that NRF2 mRNA was specifically enriched in MS2-GAS5 or GAS5 groups (Fig. 4A-4B). NRF2 protein expression declined in EPCs (Fig. 4C), suggesting that regulation of NRF2 by GAS5 happened at the transcriptional or post-transcriptional level. However, luciferase reporter experiments manifested that GAS5 had no regulatory effect on NRF2 transcription (Fig. 4D). Our study explored the effect of down-regulated GAS5 gene expression on NRF2 mRNA stability by conducting mRNA stability experiments. It was revealed that the down-regulation of GAS5 gene decreased the stability of NRF2 mRNA, implying that GAS5 promoted NRF2 expression via regulating the stability of NRF2 mRNA (Fig. 4E). We observed the relationship between GAS5 and common mRNA stabilizers by RIP experiment, and the results confirmed that GAS5 interacted with IGF2BP2, an important mRNA stabilizer (Fig. 4F). Subsequently, we also confirmed the direct interaction between IGF2BP2 and NRF2 mRNA by RIP experiments, and the interaction could be weakened by knocking down GAS5 expression (Fig. 4G). In addition, mRNA stability experiments showed that knocking down GAS5 expression promoted the degradation of NRF2 mRNA, while IGF2BP2 overexpression reversed this process (Fig. 4H).

3.5. GAS5 regulates the miR-495-3p/SIX1 axis in EPCs

As displayed in Fig. 5A-5B, miR-495-3p expression was negatively correlated with GAS5 and SIX1 expression. We then analyzed the localization of GAS5 in EPCs, and it was discovered that GAS5 was majorly present in the cytoplasm of EPCs (Fig. 5C), indicating that GAS5 may be a miRNA sponge. Then, according to bioinformatics analysis, it was found that miR-495-3P may bind to GAS5 (Fig. 5D). To verify this interaction, luciferase reporter experiments unveiled that miR-495-3p weakened luciferase activity of wild-type GAS5, whereas had no significant effect on GAS5 mutant activity (Fig. 5E). In addition, RT-qPCR analysis showed that GAS5 gene knockout significantly promoted miR-495-3p expression in EPCs (Fig. 5F). Similarly, miR-495-3p was found to bind to SIX1 (Fig. 5G). Luciferase reporter experiments manifested that miR-495-3p repressed luciferase intensity of wild-type SIX1 3'-UTR, whereas had no significant effect on SIX1 3'-UTR mutant activity (Fig. 5H). More importantly, SIX1 mRNA was elevated in the miR-495-3p inhibitor group (Fig. 5I).

4. Discussion

Cardiovascular disease is a severe threat to human life and health, its pathogenesis of is complicated, and the current treatment can only alleviate the symptoms, but cannot solve the problem fundamentally. Seeking new pathogenesis and new drug intervention targets is a critical breakthrough in preventing and treating cardiovascular diseases.

Oxidative stress and iron metabolism disorder are common factors implicated in the development of cardiovascular diseases [12]. Especially since the birth of the concept

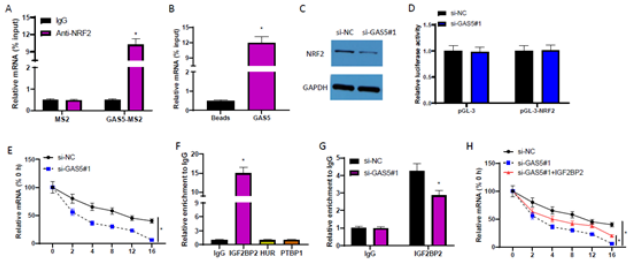

Fig. 4. GAS5 promotes NRF2 stability through IGF2BP2. (A-B) RIP assay and RNA pull-down assay assessed the combination of NRF2 and GAS5 in EPCs. (C) Western blot assessed NRF2 protein level in EPCs after GAS5 silence. (D) Luciferase reporter assay assessed the transcription activity of NRF2 in EPCs after GAS5 silence. (E) GAS5 mRNA stability detection in EPCs after GAS5 silence. (F) RIP assay examined the combination of NRF2 and RNA-binding proteins. (G) RIP assay examined the combination between NRF2 and IGF2BP2 in EPCs after GAS5 silence. *P<0.05.

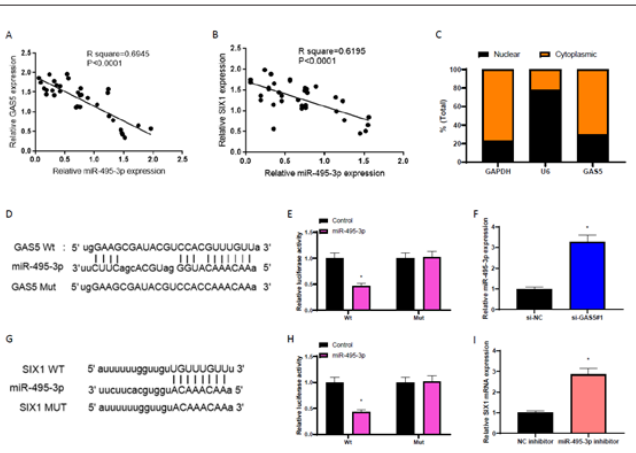

Fig. 5. GAS5 regulates the miR-495-3p/SIX1 axis in EPCs. (A-B) Correlation between miR-495-3p expression and GAS5 and SIX1. (C) Subcellular fractionation assay examined the location of GAS5. (D) Binding sites between miR-495-3p and GAS5. (E) Luciferase reporter assay assessed the luciferase activity of GAS5-Wt and GAS5-Mut in EPCs after miR-495-3p overexpression. (F) RT-qPCR examined miR-495-3p expression in EPCs after GAS5 silence. (G) Binding sites between miR-495-3p and SIX1 3'UTR. (E) Luciferase reporter assay assessed the luciferase activity of SIX1 3'UTR-Wt and SIX1 3'UTR-Mut in EPCs after miR-495-3p overexpression. (F) RT-qPCR examined SIX1 mRNA expression in EPCs after miR-495-3p inhibition. *P<0.05.

of "ferroptosis" in 2012, numerous experimental evidences and clinical studies have suggested that ferroptosis is a new mechanism of cardiovascular diseases, which can mediate pathophysiological changes of cardiovascular diseases through multiple angles, such as accelerating the structural reconstruction of myocardium and blood vessels, affecting systolic and diastolic functions, and leading to energy metabolism disorders [13]. It is exciting to note that interventions targeting ferroptosis as a therapeutic target can significantly improve cardiovascular outcomes [14]. At the same time, more scientific studies are expected to elucidate novel molecular mechanisms of ferroptosis, achieve regulation of lesions at the cellular level and organelle level, and minimize damage to surrounding tissues [15].

LncRNAs belong to a class of RNAs that cannot code for proteins but with important biological roles. LncRNAs can modulate gene expression through different mechanisms, containing recruitment of chromatin regulators as well as transcription factors [16]. Recently, numerous studies have used in vitro cell lines and in vivo simulation systems to reveal the function lncRNAs in EPCs in different individuals [17]. For example, lncRNA WTAPP1 accelerates EPCs migration and angiogenesis via MMP1 via the miR-3120 and Akt/PI3K/ autophagy pathways [18]. Another study has reported that lncRNA TUG1 works as a ceRNA of miR-6321 [19].

GAS5, as an ncRNA, has shown relevance in a variety of diseases [20]. We used RT-qPCR to measure GAS5 expression in patients with CHD. It was discovered that in the CHD group, GAS5 expression was significantly downregulated, reflecting that GAS5 may have a certain modulatory role in the pathogenesis of CHD. Subsequently, the function of GAS5 in the growth of EPCs was explored. The findings suggested that GAS5 depletion hindered the proliferation, migration, as well as invasion of EPCs while elevating cell apoptosis. Consistently, Yao et al. also pointed out that GAS5 silence hindered EPC proliferation while elevated senescence [21].

The survival and proliferation of proliferating cells need not only ATP but also biological macromolecules such as nucleic acids, fatty acids, proteins, as well as membrane phospholipids. Glycolysis, as an important metabolic pathway, can provide the raw materials and intermediates required for the synthesis of these biological macromolecules, so as to meet the energy and material requirements of cell proliferation [22]. LncRNA can mediate the reprogramming of glucose metabolism through various pathways. Liu et al. reported that lncRNA NBR2 regulated AMPK activity through glucose starvation induction [23]. YY1/GAS5 complex promotes cerebral ischemia/reperfusion injury via enhancing neuronal glycolysis [24]. Therefore, the potential of GAS5 in glucose metabolism is undoubtedly of great importance. In our study, it was discovered that glucose uptake, lactic acid, and ATP production decreased after GAS5 knockout. At the same time, GAS5 knockout decreased the ECAR of total glycolytic flux, while increased the mitochondrial oxidative respiratory index OCR. These results confirmed that GAS5 promoted the reprogramming of glucose metabolism in EPCs.

Ferroptosis belongs to a mode of programmed cell death and is featured by reactive oxygen species (ROS) along with high levels of intracellular iron. Excessive ROS can cause peroxidation as well as disintegration of lipid membranes, leading to cell death. The modulation of ferroptosis is mainly dependent on the neutral reaction between GSH and ROS-7 [25]. A large number of animal and cell experiments have shown that antioxidant therapy and inhibiting iron death can effectively reduce ischemic or hypoxic injury of heart or myocardial cells [26]. It has been documented that GAS5 has a crucial role in programmed cell death in heart failure [27]. In our study, it was suggested that GAS5 silence reduced the GSH content in Erastin or RSL3-treated EPCs, and increased mitochondrial MDA levels. These findings indicated that GAS5 knockdown could induce the ferroptosis of EPCs, that is, GAS5 promoted the ferroptosis resistance of EPCs.

Nuclear factor erythroid 2-related factor 2 (NRF2) belongs to a transcription factor encoded by the NFE2L2 gene and belongs to the basic leucine zipper group. NRF2 was initially thought to be a key regulator of cellular REDOX balance [28]. With the deepening of research, the role of NRF2 is not limited to REDOX balance, but also possesses an important role in protease and cell proliferation, carbohydrate metabolism, lipid metabolism, iron metabolism, mitochondrial function, DNA repair and drug/heterobiological metabolism, and the above functions of NRF2 are closely related to cell survival, disease prevention, and treatment [29]. Since NRF2 is an important transcription factor induced by oxidative stress, many target genes of NRF2 mediate the initiation of lipid peroxidation and ferroptosis in cells [30]. As a transcription factor, sine oculis homeobox 1 (SIX1) is closely associated with the progression of cardiovascular diseases. SIX1 is also documented to be involved in regulating glucose metabolism and ferroptosis [31, 32]. Similarly, our study also proved that GAS5 affected glycometabolic reprogramming and ferroptosis resistance through regulating NRF2 and SIX1.

RNA binding proteins can mediate the post-transcriptional modulation of lncRNA. As a famous RNA binding protein, IGF2BP2 can mediate post-transcriptional modulation of gene expression associated with cell glucose metabolism as well as ferroptosis [33]. Accumulating studies have suggested that IGF2BP2 belongs to an N6-methyladenosine (m6A) reader that takes part in disease progression through cooperating with lncRNAs [34]. In our study, we discovered IGF2BP2 could interact with GAS5, promoting NRF2 mRNA stability, promoting NRF2 expression. In addition, lncRNAs can serve to be miRNA sponges to repress the modulatory impact of miRNAs on their target genes. The lncRNA-miRNA interaction also has crucial roles in many biological processes [35]. Likewise, our study proved that GAS5 regulated the miR-495-3p/SIX1 axis in EPCs.

5. Conclusion

In conclusion, our study demonstrates that promotes glucose metabolism reprogramming and resistance to ferroptosis of endothelial progenitor cells through the miR-495-3p/SIX1 and IGF2BP2/NRF2 dual-regulatory pathways in CHD, which provide a novel sight for CHD therapy.

Conflict of Interests

The authors declare no competing interests.

Consent for publications

The author read and approved the final manuscript for

publication.

Ethics approval and consent to participate
We have received approval from the Ethics Committee of Jinhua Municipal General Hospital and Zhujiang Hospital of Southern Medical University.

Informed Consent
We have received informed consent from the Ethics Committee of Jinhua Municipal General Hospital and Zhujiang Hospital of Southern Medical University.

Availability of data and material
If you have any additional questions about the study's original contributions, please contact the corresponding author.

Authors' contributions
LY contributed to the study conception and design. Experimental operation, data collection and analysis were performed by ZM, XW, TB, ZQ and JZ. The first draft of the manuscript was written by ZM. All authors commented on previous versions of the manuscript.

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