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Original Article

Circular RNA MGAT1 regulates cell proliferation and apoptosis in hypoxia-induced cardiomyocytes through miR-34a/YAP1 axis

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Abstract: Congenital heart disease (CHD) has severe morbidity and mortality worldwide. Evidence suggests that circularRNAs (circRNAs) are involved in the pathogenesis of human CHD. However, the regulatory mechanism remains uncertain. This study aimed to explore that mechanism. The levels of circular RNA MGAT1 (circMGAT1) and miR-34a were measured by quantitative polymerase chain reaction (qRT-PCR). Expression of yes-associated protein isoform 1 (YAP1) was assessed by western blot. Caspase-3 activity was evaluated by Caspase 3 Activity Assay Kit. CCK-8 assay was carried out to detect cell proliferation of hypoxia-induced AC16 cells. Cell apoptosis was analyzed by flow cytometry. In addition, dual-luciferase reporter and RNA immunoprecipitation (RIP) assays were performed to verify the relationship between miR-34a and circMGAT1 or YAP1 *in vitro*. The level of circMGAT1 was downregulated, while miR-34a was strikingly increased in CHD tissues and hypoxia-induced AC16 cells. CircMGAT1 was a sponge of miR-34a, and circMGAT1 targeted miR-34a to regulate cell proliferation and apoptosis in hypoxia-induced cardiomyocytes. Dual-luciferase reporter and RIP-assay verified that miR-34a directly targeted YAP1, and the expression of YAP1 was significantly suppressed by miR-34a mimics but was enhanced by miR-34a inhibitor. Interestingly, YAP1 restored the effect of miR-34a on cell proliferation and apoptosis in hypoxia-induced AC16 cells. Besides, circMGAT1 sponged miR-34a to regulate the expression of YAP1. In conclusion, circMGAT1 inhibited cell apoptosis and enhanced cell proliferation by regulating the miR-34a/YAP1 axis, providing a therapy target for the treatment of human CHD.

Keywords: circMGAT1, miR-34a, YAP1, CHD, proliferation, apoptosis

Introduction

Congenital heart disease (CHD) imparts serious morbidity and mortality [1], and it is clinically defined as a structural heart disease since birth [2]. A study indicated that only 30% to 40% of CHD patients will survive 10 years without surgery [3]. Moreover, the mortality rate of CHD treated with surgery remains high in China [4]. The exact changes in pathogenesis of CHD are still not fully understood. Thus, it is urgent to explore the pathogenesis and underlying regulatory mechanisms in CHD progression.

Circular RNAs (circRNAs) are a group of noncoding RNAs that ubiquitously exist in eukaryotic cells, characterized by the covalently closed continuous loops [5, 6]. Evidence indicates that circRNAs function as sponges of microRNAs (miRNAs) and regulate the expression of miRNA-dependent genes, thus contributing to the physiologic/pathologic processes of various diseases [7, 8]. Moreover, the roles of circRNAs have been widely explored in cardiovascular diseases [9]. For instance, circRNA Cdr1as enhanced cell apoptosis to aggravate myocardial infarction in hypoxia-treated cardiomyocytes [10]. Available data indicated that circRNA_010567 negatively regulated miR-141 expression to prevent myocardial fibrosis in mouse cardiac fibroblasts [11]. Has_circ_0075370 (circMGAT1) is a newly discovered circRNA that relates to coronary heart disease progression [12]. However, the functional role of circMGAT1 in CHD is not well elucidated.

MiRNAs are a class of non-coding single-stranded RNA molecules of 22 nucleotides in

length approximately, that regulate multiple human genes [13, 14]. Dysfunction of miRNAs causes a number of diseases, including spinal motor neuron disease [15], Alzheimer's disease [16], liver disease [17] and congenital heart disease [18]. Furthermore, miRNA regulates cardiovascular development [19]. MicroRNA-21 plays an important role in various biologic functions and diseases [20], including cancer and cardiovascular disease. Wang *et al.* proved that microRNA-145 regulated the progression of CHD [21]. MicroRNA-210 was suggested as a novel target gene in the therapy of ischemic heart disease [22]. Moreover, microRNA-34a (miR-34a) modified the cardiac ageing and function [23], suggesting that miR-34a may play a role in the development of CHD.

Yes-associated protein isoform 1 (YAP1), as an important factor in the Hippo pathway, is associated with the process of cardiovascular disease [24, 25]. YAP1 accommodated the formation of endoderm which is required for cardiac precursor cell migration [26]. YAP1 induced cardiomyocyte survival and growth [27]. Moreover, the expression of YAP1 was decreased in ventricular septal defect of the heart [28]. Cell apoptosis of aortic dissection vascular smooth was suppressed by upregulation of YAP1 [29]. Thus, YAP1 may participate in the progression and initiation of CHD, so our research focused on the role of YAP1 in CHD, and the molecular mechanism associated with miR-34a.

Here, the study investigated the roles of circMGAT1, miR-34a, and YAP1 in the development of CHD; their relationship was predicted by starBase v2.0 and DIANA TOOL. Assays were used to explore the functional effects of circMGAT1, miR-34a, and YAP1 on cell proliferation and apoptosis in hypoxia-induced cardiomyocytes.

Materials and methods

Clinical tissue samples and cell culture

CHD tissue samples were isolated from CHD patients (n = 40) who underwent surgery in the Sichuan Academy of Medical Science and normal tissues were obtained from healthy volunteers (n = 15). All the tissue samples were immediately stored at -80°C until used. Clinical samples were procured with informed consent of the patients and the study was approved by the Ethics Committee of the Sichuan Academy of Medical Science.

The study used AC16 cells as *in vitro* cell model, purchased from Be Na collection (Beijing, China). AC16 cells was cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and antibiotics to a final concentration of 100 IU of penicillin and 100 μg/mL streptomycin (Gibco) according to protocol. Then, cells were incubated in a humidified atmosphere with 21% oxygen (normoxia) or hypoxic conditions with only 1% oxygen in the air (hypoxia).

Transient transfection

Oligonucleotides were small interfering RNA (siRNA) against circMGAT1 (si-circMGAT1) and the control (si-NC), miR-34a mimics (miR-34a) and the negative control (miR-NC), miR-34a inhibitor (anti-miR-34a) and inhibitor negative control (anti-NC), siRNA against YAP1 (si-YAP1) and siRNA negative control (si-NC). Special vectors: pcDNA3.1-circMGAT1 overexpression vector (circMGAT1) and the control (pcDNA), pcDNA3.1-YAP1 overexpression vector (YAP1) and pcDNA3.1 empty vector (pcDNA), luciferase reporters of circMGAT1-WT, circMGAT1-MUT, YAP1-WT or YAP1-MUT were synthetized by Genepharma (Shanghai, China). Subsequently, above oligonucleotides or vectors were transfected into AC16 cells with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, respectively. After transfection for relative times in hypoxic conditions, all the cells were harvested for subsequent assays.

Quantitative polymerase chain reaction (qRT-PCR)

QRT-PCR was carried out to measure the level of miR-34a, and YAP1 in CHD tissues and hypoxia-induced cardiomyocytes. First, the total RNA were extracted from tissues and AC16 cells by Trizol (Thermo Fisher Scientific, Rockford, IL, USA) and stored at -80°C immediately prior to use. Then cDNA was synthesized by HiScript II Q Select RT SuperMix for qPCR (Vazyme, Nanjing, China) according to protocol. Subsequently, AceQ Universal SYBR qPCR Master Mix (Vazyme) was used to mix the reaction solution for qRT-PCR on the basis of manu-

facturer's protocol. Finally, solubility curve were received by ABI 7900 Real-time PCR System (Applied Biosystems, Rockford, IL, USA). The relative expression was calculated using $2^{-\Delta\Delta Ct}$ method. U6 and GAPDH were used as internal controls. The special primers synthetized in Genepharma as below: circMGAT1: (forward, 5'-AAGAGAGAAGCTGCACTTTGG-3', reverse: 5'-CAGGCCTTAGAAGCGGCAT-3'); miR-34a: (forward, 5'-GTGCAGGGTCCGAGGT-3', reverse: 5'-CATGGCAGTGTCTTAGCTGGTT-3'); YAP1: (forward: 5'-GGCAGGGGAGAGTGATACAGA-3', reverse: 5'-GAAGCCAATTCTCACGAAGGG-3'); GAPDH (forward: 5'-ACTTTGTGAAGCTCATTTCCTGGTA-3', reverse, 5'-GTGGTTTGAGGGCTCTTACTCCTT-3'); U6: (forward, 5'-CTTCGGCAGCACATATACT-3', reverse, 5'-AAAATATGGAACGCTTCACG-3').

RNase R digestion

Total RNA isolated from AC16 cells were incubated with 6 units of RNase R (Epicenter Biotechnologies, Shanghai, China). Then, the total RNA was incubated with RNase R for 15 min at 37°C. Finally, the expression of circMGAT1 was determined using qRT-PCR.

Circularization detection of circMGAT1

Random primers could amplify all RNA, while oligo $(dT)_{18}$ primers could only amplify RNA containing poly (A) tails. CircMGAT1 and MGAT1 were amplified with random primers or oligo $(dT)_{18}$ primers, followed by qRT-PCR to confirm whether circMGAT1 contained poly (A) tails.

Western blot

The total protein was harvested from AC16 cells using lysis buffer (Thermo Fisher Scientific), and concentration of the lysate was quantitative by BCA Protein Assay Kit (Sangon Biotech, Shanghai, China). Subsequently, protein tubes were placed in boiling water for 10 minutes, and SDS-polyacrylamide gel electrophoresis was used to separated quantitative protein. All the proteins isolated were transfected onto 0.45 μm polyvinylidene fluoride (PVDF, Millipore, Bedford, MA, USA) membranes which were blocked with 5% (w/v) bovine serum albumin (BSA, Solarbio, Beijing, China) for 2 hours at room temperature subsequently. Then membranes were incubated with primary antibody overnight at 4°C. Residual solution was washed off with Tris Buffered saline Tween (TBST) (Sangon Biotech) for 3 times (5 min each). Membranes were covered by secondary antibody in a table concentrator for 40 min at room temperature. Afterwards, PVDF membranes were washed with TBST for 4 times (6 min each). Finally, special proteins on membranes were visualized by SuperSignal West Femto (Thermo Fisher Scientific) and analyzed by Image Lab software (Bio-Rad, Hercules, CA, USA). The primary antibodies of YAP1 (catalog no. ab52770, 1:5000) and b-actin (catalog no. ab115777, 1:200) were purchased from Abcam (Cambridge, MA, USA).

Cell Counting Kit-8 (CCK-8) assay

Cell proliferation was analyzed by CCK-8 (MedChemExpress, NJ, USA). After transfection with corresponding vectors into hypoxia-induced AC16 cells, WST-8 reagent (MedChemExpress) was added into AC16 cells according to manufacturer's instruction and cardiomyocytes were incubated for another 2 h at hypoxia condition. Subsequently, iMark™ Microplate Absorbance Reader (Bio-Rad) was employed to detect the absorbance at 450 nm.

Caspase 3 activity analysis

In the assay, Caspase 3 Activity Assay Kit was used to detect caspase 3 activity. First, cells were digested with trypsin and washed in phosphate buffer saline (PBS, Hyclone). Lysate was added at a ratio of 100 μL to 2 million cells for 15 min on ice and then centrifuged at 16,000-20,000 g for 15 min. Subsequently, upper solution was reacted with acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) for 1.5 h at 37°C according to producer's manual. Finally, sample's A405 was evaluated by iMark™ Microplate Absorbance Reader (Bio-Rad). The standard curve was made synchronously in the experiment.

Flow cytometry assay

Cell apoptosis was identified by Annexin V-fluorescein isothiocyanate (Annexin V-FITC) Apoptosis Detection Kit (Sigma, St. Louis, MO, USA). Cells were washed with PBS (Hyclone) at first, Afterwards, 100 μL binding buffer were used to resuspend cells, and then Annexin V and Propidium Iodide solution (PI) were both added into AC16 cells according to the protocol, and AC16 cells were incubated for 10 min

in the dark place. In the end point, apoptosis signals were received by flnt cytometer (BD Biosciences, San Jose, CA, USA).

Dual-luciferase reporter assay

The binding sites of miR-34a and circMGAT1 or YAP1 were predicted by starBase v2.0 or DIANA TOOL. Special vectors contained common fragments with miR-34a and wild type (circMGAT1-WT or YAP1-WT) or mutant (circMGAT1-MUT or YAP1-MUT) of circMGAT1 or YAP1 were synthesized by Genepharma. Subsequently, AC16 cells were co-transfected with pRL-CMV renilla vector (Promega, Madison, WI, USA) and circMGAT1-WT, circMGAT1-MUT, YAP1-WT, or YAP1-MUT and then cultured for 48 hours. The luciferase activity was measured with dual-luciferase assay kit (Promega) and THERMO Varioskan Flash (Thermo Fisher Scientific) was performed to analyze fluorescence intensity.

RNA immunoprecipitation (RIP) assay

RIP-assay was carried out to verify the relationship between miR-34a and circMGAT1 or YAP1 by Imprint® RNA immunoprecipitation kit (Sigma). First, AC16 cells were transfected with miR-34a mimics or paired control, and cultured for 72 hours. Then, RIP-buffer was aimed to lyse the total cells according to manufacturer's instruction. Lysis solution was incubated with magnetic beads covered with Argonaute-2 (AGO2, Abcam) or IgG (Abcam) antibodies. In the end point, RNA was washed and harvested, and the enrichment of circMGAT1 or YAP1 was determined by qRT-PCR.

Statistical analysis

The data were exhibited as the mean ± standard deviation (SD) by three independent assays, and analyzed by Student's t-test for two group comparisons and one-way analysis of variance for multiple group comparisons. $P < 0.05$ was considered significant.

Results

Downregulation of circMGAT1 and overexpression of miR-34a in CHD tissues and hypoxia-induced cardiomyocytes

In order to investigate the role of circMGAT1 and miR-34a in CHD, the level of circMGAT1 and miR-34a was detected by qRT-PCR. CircMGAT1 was lowly expressed in CHD tissues compared with adjacent normal tissues (**Figure 1A**). Moreover, after treatment with hypoxic condition for 24 h, 48 h or 72 h, the level of circMGAT1 was decreased in AC16 cells (**Figure 1B**). Our data showed that circMGAT1 was resistant to RNase R, while linear RNA MGAT1 could be digested by RNase R (**Figure 1C**). Meanwhile, circMGAT1 was markedly reduced in AC16 cells when using the oligo $(dT)_{18}$ primers (**Figure 1D**). Furthermore, miR-34a expression was significantly promoted in CHD tissues and hypoxia-induced AC16 cells (**Figure 1E** and **1F**). These assays suggested that circMGAT1 and miR-34a may play important roles in human CHD development.

CircMGAT1 sponged miR-34a

As shown in **Figure 2A**, starBase v2.0 predicted that miR-34a contained the binding sites of circMGAT1. Then, dual-luciferase reporter assay and RIP assay were used to verify the relationship between circMGAT1 and miR-34a. Our results indicated that luciferase activity was very inhibited in circMGAT1-WT group, while there was no striking difference in circMGAT1-MUT group (**Figure 2B**). Moreover, RIP assay revealed that circMGAT1 and miR-34a were significantly enriched in anti-Ago2 compared with that in anti-IgG (**Figure 2C**). To further investigate the interrelation between circMGAT1 and miR-34a, AC16 cells were transfected with pcDNA, circMGAT1, si-NC, or si-circMGAT1 and incubated for 48 hours. QRT-PCR results showed that the expression of miR-34a was effectively suppressed by circMGAT1, but distinctly enhanced by si-circMGAT1 *in vitro* (**Figure 2D**). In conclusion, the assay suggested that circMGAT1 was a sponge of miR-34a and the expression of miR-34a was regulated by circMGAT1 *in vitro*.

CircMGAT1 promoted cell proliferation and inhibited cell apoptosis by targeting miR-34a in hypoxia-induced cardiomyocytes

We explored the biological role of circMGAT1 and miR-34a in the progression of CHD. As described in **Figure 3A** and **3B**, the level of miR-34a was notably suppressed by circMGAT1 overexpression or significantly enhanced by circMGAT1 deletion, while these effects were partially blocked by miR-34a mimic or inhibitor in

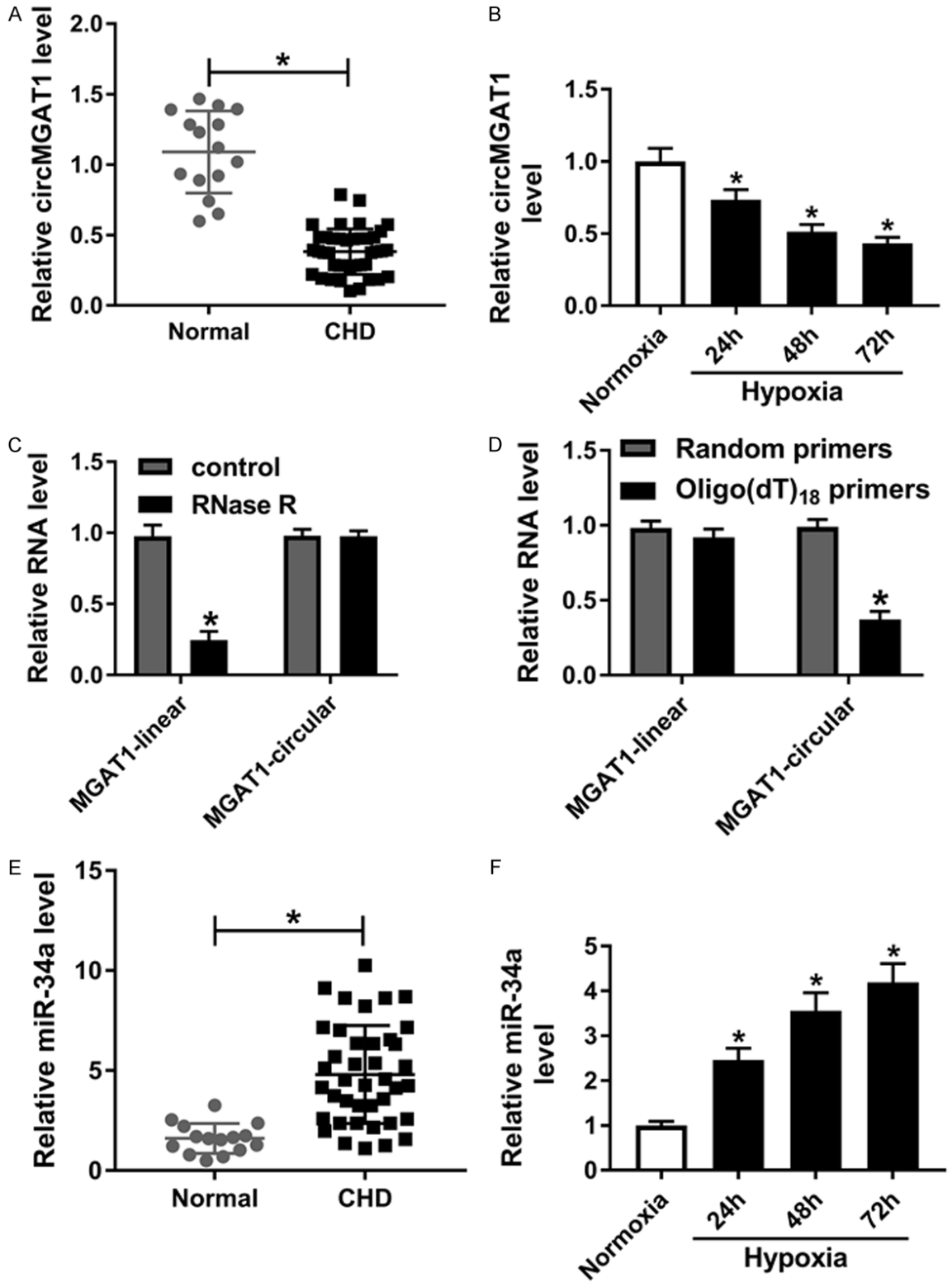

Figure 1. Downregulation of circMGAT1 and overexpression of miR-34a in CHD tissues and hypoxia-induced cardiomyocytes. A. The level of circMGAT1 was measured by qRT-PCR in CHD tissue samples. B. QRT-PCR assay was performed to detect circMGAT1 level in hypoxia-induced for 24 h, 48 h or 72 h AC16 cells compared with normoxic control group. C. The relative expression levels of circMGAT1 and MGAT1 in AC16 cells were detected by qRT-PCR after treatment with RNase R. D. The level of circMGAT1 and MGAT1 in AC16 cells were assessed by qRT-PCR after

being normalized with random primers and oligo $(dT)_{18}$ primers. E. The expression of miR-34a was measured by qRT-PCR in CHD tissue samples. F. The level of miR-34a was determined by qRT-PCR in hypoxia-induced for 24 h, 48 h or 72 h AC16 cells compared with a normoxic control group. *$P < 0.05$.

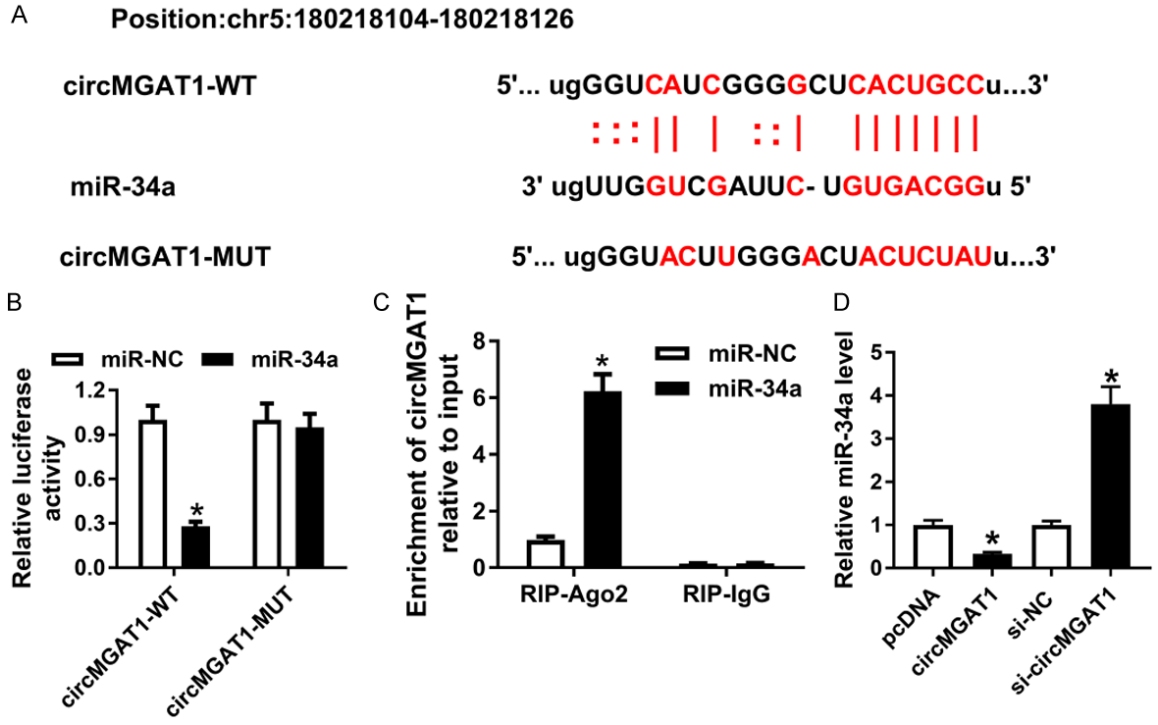

Figure 2. MiR-34a is a target gene of circMGAT1. A. The binding sites of miR-34a and YAP1 were predicted by starBase v2.0. B. The luciferase activity in AC16 cells co-transfected with miR-NC, miR-34a, circMGAT1-WT, or circMGAT1-MUT was detected by dual-luciferase reporter assay. C. The relationship between circMGAT1 and miR-34a was confirmed by RIP-assay. D. The expression of miR-34a was evaluated by qRT-PCR. *$P < 0.05$.

hypoxia-induced AC16 cells, respectively. Cell proliferation was measured by CCK-8 assay. Results demonstrated that miR-34a upregulation could attenuate circMGAT1 overexpression-mediated promotion effect on cell proliferation in hypoxia-induced AC16 cells (**Figure 3C**), whereas the inhibitory effect of circMGAT1 deletion on cell proliferation was abolished by miR-34a inhibitor (**Figure 3D**). Moreover, the results suggested that circMGAT1 overexpression repressed cell apoptosis, which was harbored by miR-34a mimic in hypoxia-induced AC16 cells (**Figure 3E**). CircMGAT1 downregulation-induced cell apoptosis was hindered by miR-34a inhibitor (**Figure 3F**). Additionally, Caspase 3 Activity Assay Kit was used to measure the expression of caspase-3, a marker of cell apoptosis. The findings were consistent with the cell apoptosis results (**Figure 3G** and **3H**). In brief, circMGAT1 regulated cell proliferation and apoptosis in hypoxia-induced AC16 cells by sponging miR-34a.

YAP1 was a direct target gene of miR-34a

The study aimed to clarify the regulatory mechanism of miR-34a in the development of CHD. The binding sites between miR-34a and YAP1 have been predicted by DIANA TOOL and shown in **Figure 4A**. In order to verify the relationship between them, common fragments of miR-34a and wildtype (YAP1-WT) or mutant (YAP1-MUT) YAP1 were constructed. Afterwards, one of them and miR-34a mimics were co-transfected into AC16 cells, luciferase activity was very decreased in YAP1-WT group, while luciferase activity in YAP1-MUT group had no striking difference (**Figure 4B**). In the meanwhile, the targeted relationship between miR-34a and YAP1 was also verified by RIP assay (**Figure 4C**). To further investigate the interrelation between miR-34a and YAP1, AC16 cells were transfected with miR-NC, miR-34a, anti-miR-NC or anti-miR-34a and incubated for 48 hours, the total protein was gathered and western blot results

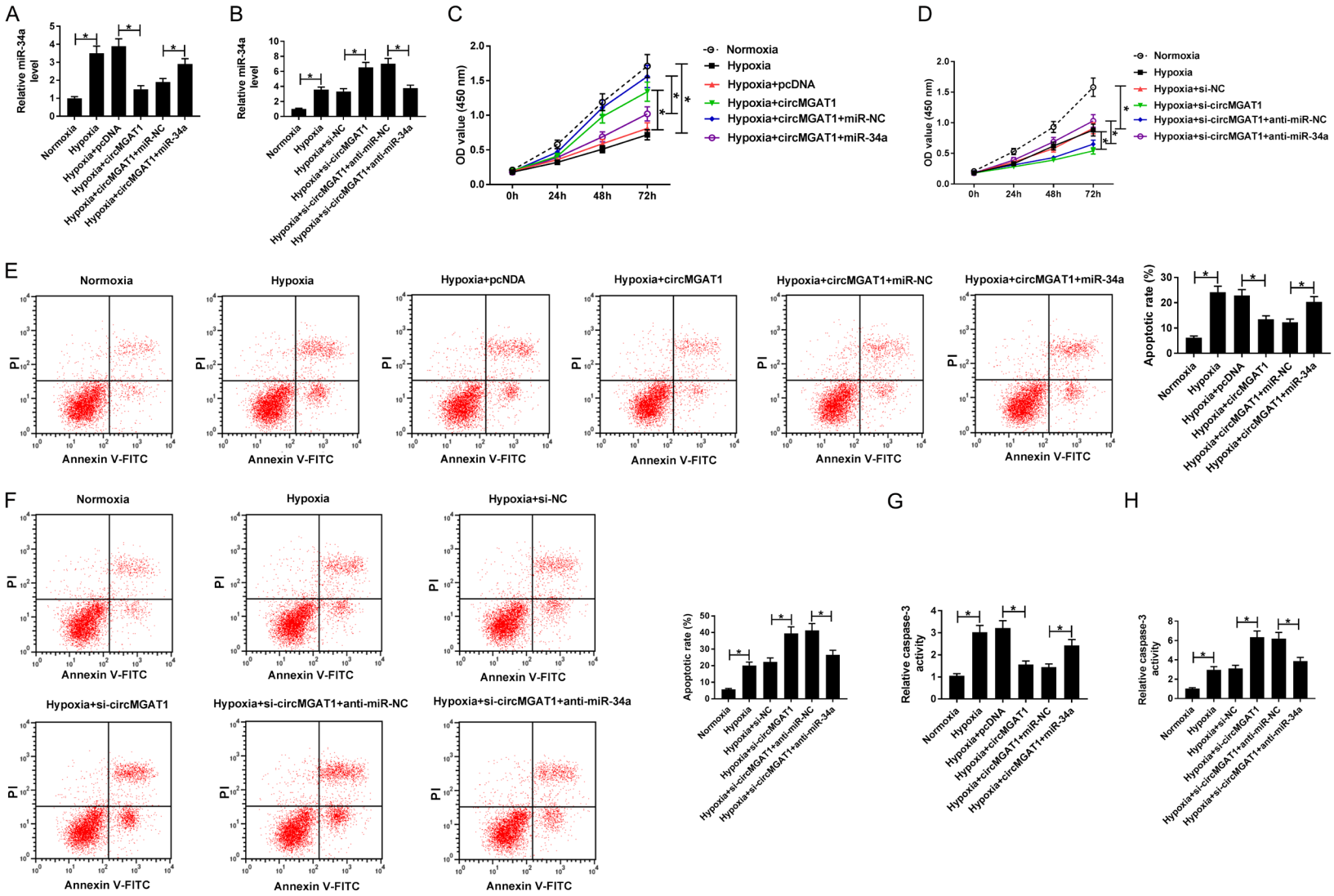

Figure 3. CircMGAT1 promoted cell proliferation and inhibited cell apoptosis by targeting miR-34a in hypoxia-induced cardiomyocytes. A, B. The expression of miR-34a was determined by qRT-PCR. C, D. Cell proliferation was measured by CCK-8 assay. E, F. Cell apoptosis was detected by flow cytometry. G, H. Caspase 3 activity was detected by Caspase 3 Activity Assay Kit. *$P < 0.05$.

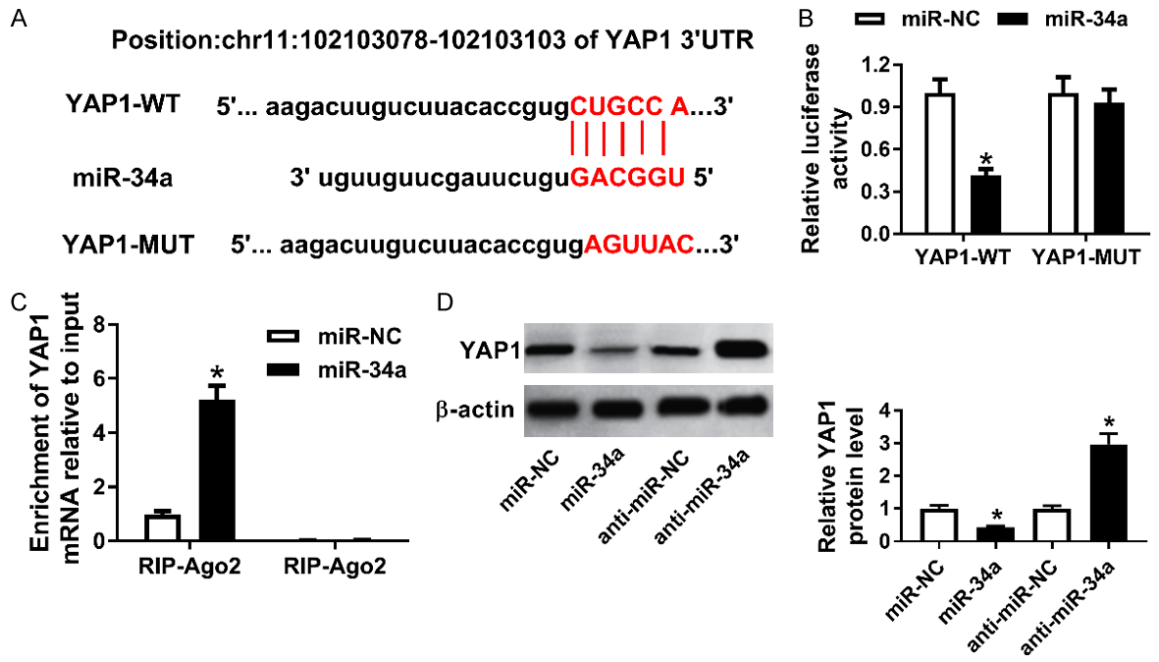

Figure 4. YAP1 is a direct target gene of miR-34a. A. The binding sites of miR-34a and YAP1 were predicted by DIANA TOOL. B. YAP1-WT or YAP1-MUT was co-transfected with renilla vector into AC16 cells and luciferase activity was measured by dual-luciferase reporter assay. C. RIP-assay was carried out to verify the relationship between miR-34a and YAP1. D. The expression of YAP1 was evaluated by western blot after transfected with miR-NC, miR-34a, anti-miR-NC, or anti-miR-34a into AC16 cells. *$P < 0.05$.

showed that the expression of YAP1 was effectively retarded by miR-34a, but distinctly boosted by anti-miR-34a *in vitro* (**Figure 4D**). In conclusion, the assay suggested that miR-34a directly targeted YAP1 and the expression of YAP1 was regulated by miR-34a *in vitro*.

YAP1 reversed the effects of miR-34a on cell proliferation and apoptosis in vitro

The experiment focused on the molecular mechanism of miR-34a and YAP1 in CDH. The level of YAP1 in hypoxia-induced AC16 cells was detected by western blot. Results suggested that the expression of YAP1 was decreased in AC16 cells at hypoxic condition for 24 h, 48 h or 72 h (**Figure 5A**). Afterwards, miR-NC, miR-34a, miR-34a + pcDNA or miR-34a + YAP1 were transfected into AC16 cells respectively, and cultured for 72 h at hypoxia condition. Expression of YAP1 was strikingly inhibited by miR-34a, while the inhibitory effect was restored by overexpression of YAP1 at hypoxia condition (**Figure 5B**). Then, anti-miR-NC, anti-miR-34a, anti-miR-34a + si-NC or anti-miR-34a + si-YAP1 were transfected into hypoxia-induced AC16 cells for 72 hours. Results revealed that downregulation of miR-34a obviously increased the level of YAP1, while knockdown of YAP1 reversed the promoted effect in AC16 cells at hypoxia condition (**Figure 5C**). Cell proliferation was measured by CCK-8. Overexpression of YAP1 rescued the repressive effect induced by miR-34a on cell proliferation (**Figure 5D**), and the promotion effect of miR-34a inhibitor on cell proliferation was regained by low-expression of YAP1 (**Figure 5E**). The results proved that miR-34a mimics enhanced cell apoptosis while the stimulation was recovered by the upregulation of YAP1 (**Figure 5F**). Additionally, miR-34a inhibitor inhibited cell apoptosis, while the inhibitory effect was rescued by si-YAP1 (**Figure 5G**). Furthermore, the activity of caspase-3 also verified the inhibitory or promoted effect of miR-34a and YAP1 in flow cytometry assay on cell apoptosis *in vitro* (**Figure 5H** and **5I**). Thus, miR-34a inhibited cell proliferation and boosted cell apoptosis by regulating YAP1 expression in hypoxia-induced AC16 cells.

CircMGAT1 regulated YAP1 expression by sponging miR-34a

Next, we investigated the regulatory mechanism of circMGAT1, miR-34a and YAP1. As

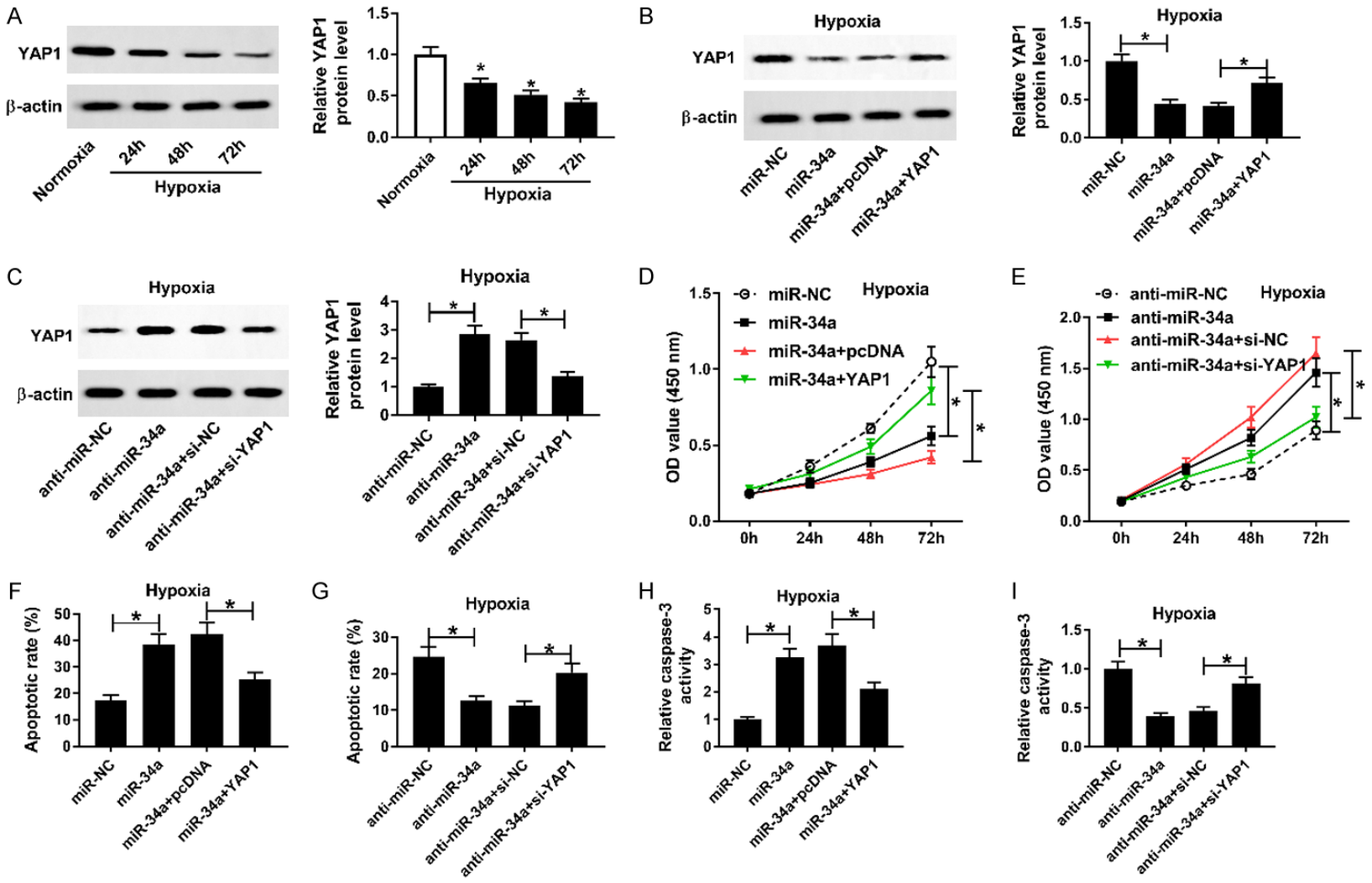

Figure 5. YAP1 reversed the effects of miR-34a on cell proliferation and apoptosis in vitro. A. AC16 cells were cultured for 24 h, 48 h or 72 h at hypoxic condition, cells were collected and YAP1 expression was detected by western blot. B. MiR-NC, miR-34a, miR-34a + pcDNA or miR-34a + YAP1 were transfected into hypoxia-induced AC16 cells and the level of YAP1 was analyzed by western blot. C. Hypoxia-induced cardiomyocytes were transfected with anti-miR-NC, anti-miR-34a, anti-miR-34a + si-NC or anti-miR-34a + si-YAP1 and the expression of YAP1 was identified by western blot. D, E. CCK-8 assay was used to detect cell proliferation after transfected with relative vectors, respectively. F, G. Cell apoptosis was determined by flow cytometry. H, I. After the above treatment in hypoxia-induced AC16 cells, the activity of caspase-3 was examined by Caspase 3 Activity Assay Kit. *$P < 0.05$.

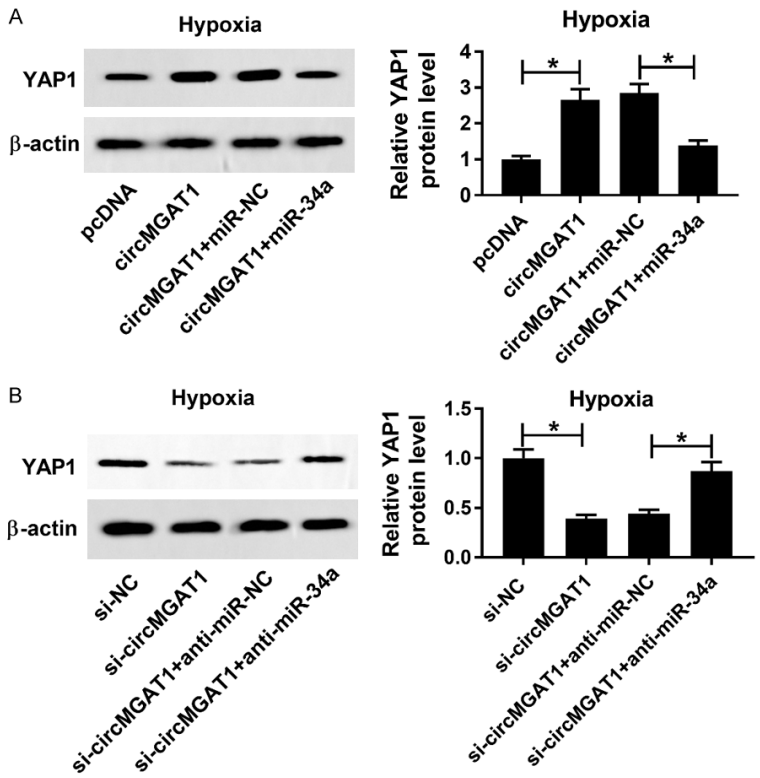

Figure 6. CircMGAT1 regulated YAP1 expression by sponging miR-34a. A. Hypoxia-induced AC16 cells were transfected with pcDNA, circMGAT1, circMGAT1 + miR-NC, or circMGAT1 + miR-34a for 72 hours, the protein expression of YAP1 was determined by western blot. B. Hypoxia-induced AC16 cells were transfected with si-NC, si-circMGAT1, si-circMGAT1 + anti-miR-NC, or si-circMGAT1 + anti-miR-34a for 72 hours, the protein expression of YAP1 was determined by western blot. *$P < 0.05$.

shown in **Figure 6A**, we found that YAP1 protein expression was increased by circMGAT1, which was partially reversed by miR-34a. Moreover, the decrease of YAP1 protein expression was attenuated by miR-34a inhibitor in hypoxia-induced AC16 cells (**Figure 6B**). These data demonstrated that circMGAT1 regulated YAP1 expression by targeting miR-34a.

Discussion

CHD is the most frequent major congenital anomaly, while the birth prevalence of CHD varies among studies worldwide [30]. 8 per 1000 live births is usually accepted as the best approximation in the world [31]. Over the past decades, reports have shown pathogenesis and initiation of CHD, but risk factors for adverse outcome are not defined completely. Therefore, the aim of this study was to investigate the partial regulatory mechanism of the progression of CHD.

Numerous circRNAs have been discovered to act as vital regulators in human disease [32, 33]. However, the role and underlying mechanism of circRNAs in various human diseases, such as CHD, remain poorly understood. In particular, a previous study discovered that circRNA HRCR played a vital role in cardiac hypertrophy [34]. Hsa_circ_0007623 was demonstrated to be a new biomarker for acute myocardial ischemia prognosis and treatment [35]. In this study, our data illustrated that the expression of circMGAT1 was downregulated in CHD tissues and hypoxia-induced cardiomyocytes, consistent with the result of the previous study [12].

miRNAs are associated with cancer and cardiovascular diseases [36]. The expression of miRNA was altered in Alzheimer's disease [37], and the level of miRNAs was associated with heart function [38]. MiRNAs can function as treatment targets in the therapy of ischemic heart disease [39]. Duan *et al*. discovered the functional role of microRNA-1 and its dysregulation in heart diseases [40]; MicroRNA-21 was upregulated and participated in the process of cardiovascular diseases [41]; MicroRNA-197 and microRNA-223 were involved in cardiovascular death of symptomatic coronary artery disease [42]. miRNA is closely related to the pathogenesis of heart failure. In addition, miR-34a was upregulated in human atherosclerotic [43], and it functioned as a potential biomarker of coronary artery disease [44]. MiR-34a induced cardiomyocyte apoptosis after myocardial infarction [45]. In the present study, the results exhibited that miR-34a was notably increased in CHD tissues and hypoxia-induced cardiomyocytes. Furthermore, circMGAT1 sponged miR-34a. Other studies showed that circRNAs served as miRNA sponges to exert their biologic function [46]. Our findings demonstrated that circMGAT1 targeted miR-34a to elevate cell proliferation and sup-

press cell apoptosis in hypoxia-induced cardiomyocytes *in vitro*. All the evidence proved that circMGAT1 and miR-34a participated in the processes of human CHD.

YAP1, as a classical factor of Hippo signaling pathway, has been discovered to be involved in the progression and initiation of cancer [47, 48]. Hippo pathway was also cited in development and regeneration in heart [49, 50]. Recent research showed that YAP1 was also related to human heart diseases [51], and hypoxia-induced cardiomyocyte apoptosis was regulated by YAP1 [52]. In our research, YAP1 was verified as a target gene of miR-34a and negatively regulated by miR-34a *in vitro*. Moreover, YAP1 reversed the effect of miR-34a on cardiomyocyte proliferation and apoptosis. Besides, circMGAT1 can regulate the expression of YAP1 by sponging miR-34a.

In conclusion, circMGAT1, miR-34a, and YAP1 are involved in the pathogenesis and process in human CHD, and circMGAT1 suppressed cardiomyocyte apoptosis and contributed to proliferation in hypoxia-induced AC16 cells.

Conclusion

The expression of circMGAT1 was dramatically decreased, while the level of miR-34a was significantly increased in CHD tissue samples and hypoxia-induced cardiomyocytes. Moreover, miR-34a was a target of circMGAT1 and circMGAT1 regulated cell growth and apoptosis by regulating miR-34a in hypoxia-induced AC16 cells. Furthermore, YAP1 was directly targeted by miR-34a and strikingly repressed by miR-34a mimics. YAP1 reversed the effects of miR-34a on cell proliferation and apoptosis in hypoxia-induced AC16 cells. The expression of YAP1 was regulated by circMGAT1 and miR-34a. In brief, circMGAT1 modified cell proliferation and apoptosis by the miR-34a/YAP1 axis in hypoxia-induced cardiomyocytes.

Disclosure of conflict of interest

None.

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