Long non-coding RNA plasmacytoma variant translocation 1 linked to hypoxia-induced cardiomyocyte injury of H9c2 cells by targeting miR-135a-5p/forkhead box O1 axis

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Abstract

Background: Myocardial infarction occurs due to insufficient (ischemia) blood supply to heart for long time; plasmacytoma variant translocation 1 (PVT1) is a long non-coding RNAs (lncRNAs) involved in the pathogenesis of various diseases, including heart disease; However, few studies have explored its role. The present study evaluated the effects of lncRNA PVT1 on hypoxic rat H9c2 cells.

Methods: Hypoxic injury was examined by measuring cell viability and apoptosis by using cell counting kit-8 activity and flow cytometry assays. Gene expressions after hypoxia were estimated by quantitative real time polymerase chain reaction and the signaling pathway were explored by Western blot analysis. RNA immunoprecipitation and luciferase reporter assays were applied to examine the interactions among genes. Data were analyzed using *t*-test with one-way or two-way analysis of variance.

Results: The lncRNA PVT1 is up-regulated in hypoxia-stressed H9c2 cells and knockdown of PVT1 mitigates hypoxia-induced injury in H9c2 cells. PVT1 acts as a sponge for miR-135a-5p and knockdown of PVT1 attenuated the increased hypoxia-induced injury by up-regulating miR-135a-5p. Forkhead box O1 (*FOXO1*) was identified as a target of miR-135a-5p, and the expression was negatively regulated by miR-135a-5p. The exploration of the underlying mechanism demonstrated that knockdown of *FOXO1* reversed PVT1/miR-135a-5p mediated hypoxia-induced injury in H9c2 cells.

Conclusions: PVT1 plays a crucial role in hypoxia-injured H9c2 cells through sponging miR-135a-5p and then positively regulating *FOXO1*.

Keywords: Acute myocardial infarction; PVT1; miR-135a-5p; *FOXO1*

Introduction

Hypoxia is a state of low oxygen supply to cells, tissues, and organs, which can be characterized by inadequate oxygen transport or the inability of the tissue to take up oxygen.[1] Hypoxia-induced myocardial injury is found to be associated with cardiovascular diseases, such as myocardial ischemia, heart failure, and myocardial infarction (MI), a subtype of cardiovascular disease and one of the leading cause of morbidity and mortality worldwide.[2-4] However, until now there is no effective therapy available for the prevention and treatment of hypoxic heart injury.

Long non-coding RNAs (LncRNAs) are defined as RNA molecules with more than 200 nucleotides and lowly expressed and poorly conserved in DNA sequence, they are found to be functionally associated with multiple pathophysiological processes, such as cancer, neurodegenerative, autoimmune, and cardiovascular diseases by mediating chromatin remodeling, transcriptional and post-transcriptional regulation as well as epigenetics via crosstalk with other RNA species.[4] At the cellular level, dysregulation of lncRNAs can cause cell proliferation, resistance to apoptosis, induction of angiogenesis, promotion of mitosis, and evasion of tumor suppressors.[5,6] lncRNAs play crucial roles as decoys, scaffolds, guide, or enhancer RNAs.[7,8] Recently, it has been shown that lncRNAs are involved in the alleviation of myocardial ischemia/reperfusion (I/R) injury, but very limited reports illustrated their role in cardiovascular diseases. There are several lncRNAs that play a crucial role in MI. For example, up-regulation of zinc finger antisense 1 (*ZFAS1*) was detected in MI patients[9] whereas knockdown of *ZFAS1* can reduce the risk of MI and mitigate ischemic contractile coronary dysfunction in mice[10,11]; HOX transcript antisense intergenic RNA down-regulation limits

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cardiomyocyte apoptosis[12] and relieve oxidative stress-induced H9c2 cell injury.[13] Cardiac autophagy inhibitory factor down-regulation inhibits cardiac autophagy and ameliorating MI.[14] Metastasis-associated lung adenocarcinoma transcript 1 (*MALT1*) up-regulation enhances autophagy and apoptosis of cardiomyocytes in mice after MI[15] and knockdown of *MALT1* can improve cell viability and inhibit cell apoptosis in hypoxia-stressed cardiomyocytes.[16] Growth arrest-specific transcript 5 (*GAS5*) knockdown can attenuate cell apoptosis in hypoxia-stressed cardiomyocytes.[17] Fibroblast growth factor 9-associated factor downregulation inhibits the apoptosis of cardiomyocytes under hypoxia stress.[18] Taurine up-regulated gene 1 up-regulation aggravates hypoxia-induced cell injury[19] and maternally expressed gene 3 knockdown can alleviate hypoxia-induced cell injury in rat.[20] lncRNA H19 attenuated hypoxia induced cardiomyocyte injury by activating the phosphoinositide 3-kinase/protein kinase B and extracellular signal-regulated kinase/p38 MAPKs pathway.[21] Nonetheless, many lncRNAs that may play a role in hypoxia-induced cardiovascular disease remains unexplored.

Recently, several reports have suggested the association of lncRNA and microRNA (miRNA) in regulating physiological and pathophysiological processes of cardiovascular disease. For example, miR-17-5p level was significantly up-regulated in I/R-induced cardiac injury in mice and overexpression of miR-17-5p aggravated cardiomyocyte injury with reduced cell viability and enhanced apoptosis.[22] miR-21 plays a critical role in the early phase of MI and has a protective effect on ischemia induced cell apoptosis by targeting Programmed cell death 4.[23] miR-21 and miR-146a synergistically reduced apoptosis in cardiomyocytes under ischemia/hypoxic conditions as compared with either miR-21 or miR-146 alone.[24] Circulating miR-26a-1, miR-146a, miR-199a-1 show higher expression in MI patients and are potential biomarkers for MI.[25] miR-21 negatively regulates the frequency of circulating regulatory T-cells and the expression level of *foxp3*, *TGF-β1*, and *smad7* in patients with coronary heart diseases under *in vitro* and *in vivo* conditions.[26] miR-24 suppresses cardiomyocyte apoptosis, attenuates infract size and reduced cardiac dysfunction by repressing proapoptotic protein Bim.[27] miR-132 negatively regulates the expression of *Rem* in cardiomyocytes during long-term activation of β-adrenergic signalling.[28] miR-101 negatively regulates the expression of *TGF-βR1* which in turn inhibits hypoxia-induced cardiac fibrosis.[29] miR-133a attenuates hypoxia-induced apoptosis by inhibiting *TAGLN2* expression in cardiac myocytes.[30] Lin28/let7 regulatory axis promotes hypoxia mediated apoptosis by inducing the AKT signaling suppressor *PIK3IP1*.[31]

In the present study, we investigated the role of plasmacytoma variant translocation 1 (PVT1) in hypoxia-induced cardiomyocyte injury in H9c2 cells.

Methods

Cell culture and treatment

The rat embryonic ventricular cell line H9c2 was grown in Dulbecco modified Eagle medium (DMEM; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). Cells incubated at 37°C in incubators with an atmosphere containing 95% air and 5% CO_2 (for normoxic condition) were used as control. When the cell confluence reached 80%, the cells were pre-starved for 1 h by using a DMEM (Gibco) which was supplemented with 0.5% FBS (HyClone). To simulate hypoxia injury, the cells were incubated for 12 h in a hypoxic incubator containing 94% N_2, 5% CO_2, and 1% O_2.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA from H9c2 cells was isolated with TRIzol reagent according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). To evaluate the levels of PVT1 and forkhead box O1 (*FOXO1*), the complementary DNA (cDNA) was obtained by using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) and their relative levels were determined with a One Step SYBR® PrimeScript®PLUS RT-RNA PCR Kit (Takara, Kyoto, Japan). With respect to the miR-135a-5p level, a TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II (ThermoFisher, Waltham, MA, USA) were applied according to manufacturer's instructions. The relative expression was assessed by the $2^{-\Delta\Delta Ct}$ cycle threshold method, with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the internal control for PVT1 and *FOXO1*, while U6 was that for miR-135a-5p.

Cell transfection

For knockdown of PVT1 and *FOXO1*, the small interfering RNA sequences specific for PVT1 (siPVT1) and *FOXO1* (siFOXO1), synthesized by GenScript Company (Nanjing, China), were sub-cloned into the BLOCK-iT™ U6 RNAi Entry Vector (ThermoFisher) to generate PVT1-shRNA (shPVT1) and FOXO1-shRNA (shFOXO1), both using the empty vector as control (shControl). For overexpression of PVT1 and *FOXO1*, the corresponding cDNA for PVT1 or *FOXO1* were amplified and ligated into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to obtain pcDNA3.1/PVT1 and pcDNA3.1/FOXO1 with the empty vector (Vector) as control. Lipofectamine 3000 reagent (Life Technologies) was used for cell transfection. The miR-135a-5p mimics and inhibitor were provided by Life Corporation with the scramble miRNAs as control (NC-miRNAs). Subsequently, the transfection of the above sequences or plasmids into the H9c2 cells were carried out by the use of Lipofectamine 3000 reagent. Primers or relevant sequences are listed in Supplementary Table 1, http://links.lww.com/CM9/A354.

Cell viability assay

To assess cell viability, 1×10^5 cells were seeded in triplicate in 60 mm dishes. At 48 h after the corresponding transfection, the cells were treated under hypoxia for additional 12 h. Afterward, the cells were washed with phosphate buffered saline (PBS) and cell viability was evaluated as previously described.[32]

Flow cytometry analysis

Cell apoptosis analysis was processed as described previously.[33] Briefly, PBS-washed cells were stained successively by propidium iodide (PI) and fluorescein isothiocynate (FITC)-conjugated annexin V (FITC-annexin V) (Sigma Aldrich, Shanghai, China) following the manufacturer's guide. Then, the apoptotic cells were sorted using a FACScan (Beckman Coulter, Brea, CA, USA) and analyzed with FlowJo software V9 (Tree Star Inc., Ashland, OR, USA).

Luciferase reporter assay

The wild-type PVT1 (PVT1-WT) and wild-type FOXO1 (FOXO1-WT) were formed, respectively, by cloning the full-length PVT1 and *FOXO1* including a fragment of the 3′-untranslated region (with the putative miR-135a-5p binding sites) into the pmirGLO vector (Promega, Madison, WI, USA). Similarly, the PVT1-MT and mutant *FOXO1* (FOXO1-MT) were obtained by cloning the sequences with the predicted binding sites mutated. Afterward, H9c2 cells were transfected appropriately with the above vectors together with miR-135a-5p mimics or NC-miRNA, 48 h after the transfection, the luciferase activity was tested using the Dual Luciferase Reporter Assay System (Promega).

RNA immunoprecipitation (RIP) assay

RIP assays were performed to validate the interaction between PVT1 and miR-135a-5p by the use of a Magna RNA-binding protein immunoprecipitation kit (Millipore, Burlington, MA, USA) based on the manufacturer's guide. Briefly, the cells were incubated with protein G agarose beads containing Argonaut 2 (Ago2; ab186733; Abcam, Cambridge, MA, USA) or immunoglobulin G (IgG) antibody (ab172730; Abcam) after lysis. The normal rabbit IgG served as a negative control. Afterward, RNAs precipitated by Ago2 or IgG were detected by qRT-PCR.

Western blot analysis

For western blot analysis, the total protein was extracted by radioimmunoprecipitation assay buffer lysis buffer (Beyotime, Shanghai, China) with the concentration measured using a Pierce™ bicinchoninic acid Protein Assay Kit (ThermoFisher). Thereafter, proteins were separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by electrophoretic transfer into polyvinylidene difluoride membranes. After being blocked using non-fat milk, the membranes were incubated at 4°C with anti-rabbit Bax (1:1000 dilution, ab32503, Abcam), Bcl-2 (1:1000 dilution, ab32124, Abcam), FOXO1 (1:1000 dilution, ab39670, Abcam) or GAPDH (1:5000 dilution, ab8245, Abcam) antibodies overnight, rinsed with Tris buffered saline containing Tween 20 and further incubated with secondary anti-rabbit antibody (1:5000 dilution, A0208, Beyotime) at room temperature for 1 h. Finally, the blots were observed by using the enhanced chemiluminescence solution (ThermoFisher) with the signals analyzed by Image Lab™ Software 5.2 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The results from at least three experiment were displayed as mean ± standard error with the statistical analyses processed using Graphpad 6.0 software (GraphPad Software, San Diego, CA, USA). The calculation of differences between the two groups was estimated by Student's *t* test. Differences with a P value < 0.05 were indicated to be of statistical significance, which was shown as asterisk.

Results

Hypoxia-stress induces the expression of the lncRNA PVT1 in H9c2 cells

Previously, H9c2 cells were used to simulate myocardial infarction *in vitro*.[32,33] Present investigation used H9c2 cells to explore PVT1 function in MI. H9c2 cells were cultured in the hypoxic condition to investigate their viability and apoptosis. It has been shown that hypoxia dramatically inhibited cell viability and significantly induced H9c2 cell apoptosis as compared to the untreated control cells [Figures 1A and 1B]. Furthermore, we analyzed the protein levels of both the B-cell lymphoma 2 (Bcl-2) and Bcl-2 Associated X-protein (Bax), as they were regularly used as an indicator of anti-apoptotic and pro-apoptotic conditions, respectively.[32,34,35] The hypoxic treatment substantially increased the accumulation of Bax but significantly reduced the level of Bcl-2 in H9c2 cells compared with the untreated control [Figure 1C]. These results indicated that hypoxia stress induced H9c2 cell injury and this was consistent with the previous reports.[32,36] Additionally, to determine whether PVT1 played a role in hypoxia-induced H9c2 cell injury, qRT-PCR was performed to detect the expression of PVT1 in response to hypoxic stress. Our results showed that mRNA level of PVT1 was significantly increased under hypoxic condition as compared to the control, suggesting a crucial role of PVT1 in hypoxia-mediated apoptotic cell death of H9c2 [Figure 1D].

Knockdown of PVT1 alleviates hypoxia-induced H9c2 cell apoptosis

To further explore the role of PVT1 in hypoxia-induced H9c2 cell injury, we knockdown PVT1 in H9c2 cells by transfecting it with shPVT1 or shControl, used as control. The PVT1 mRNA level was found to be profoundly decreased as compared to the control [Figure 2A]. Afterward, we examined the viability and apoptosis of these cells transfected with shPVT1 or shControl to explore the functional role of PVT1 in H9c2 cells under hypoxia stress. The knockdown of PVT1 alleviated the decreased viability and suppressed the apoptosis of hypoxia-stressed H9c2 cells [Figure 2B and 2C]. Further, protein levels of *Bax* and *Bcl-2* in hypoxia stressed H9c2 cells were determined after these cells were transfected with shPVT or shControl. The significantly lower level of Bax and higher level of Bcl-2 were observed in PVT1 knockdown hypoxia-stressed H9c2 cells as compared to the control [Figure 2D], suggesting that knockdown PVT1 alleviated hypoxia-induced H9c2 cells injury, which validating the point that PVT1 acts as a positive regulator in hypoxia-induced H9c2 cells injury.

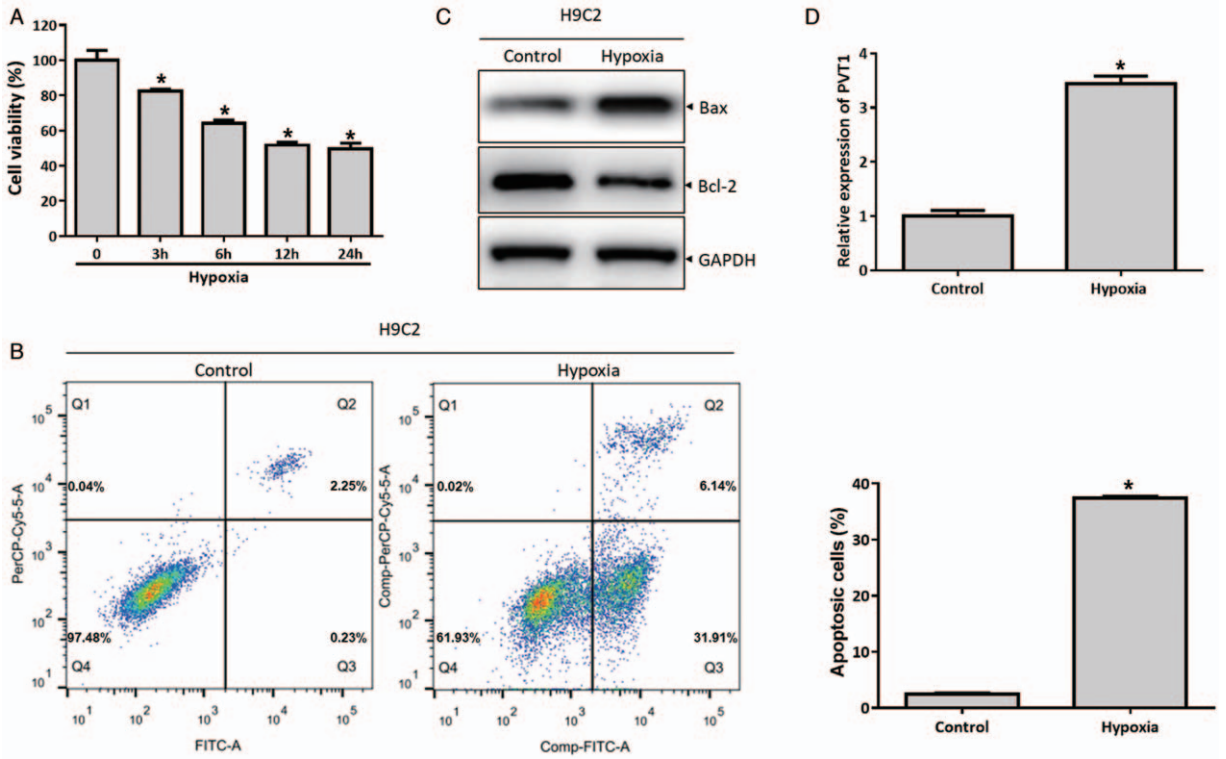

Figure 1: Hypoxia stress induces the expression of PVT1 in H9c2 cells. (A) Viability of H9c2 cells after hypoxia treatment for 3, 6, 12, and 24 h. (B). Apoptosis of H9c2 cells after hypoxia treatment for 12 h. (C). Western blot analysis of Bax and Bcl2 in H9c2 cells after 12 h of hypoxia treatment. (D). qRT-PCR detection of PVT1 mRNA level under normoxic condition (control) or 12 h hypoxia condition. Data shown are the means ± SE. $^{*}P < 0.05$. Bax: Bcl-2 Associated X-protein; Bcl-2: B-cell lymphoma 2; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; H9C2: H9C2 cells; PVT1: Plasmacytoma variant translocation 1; SE: Standard error.

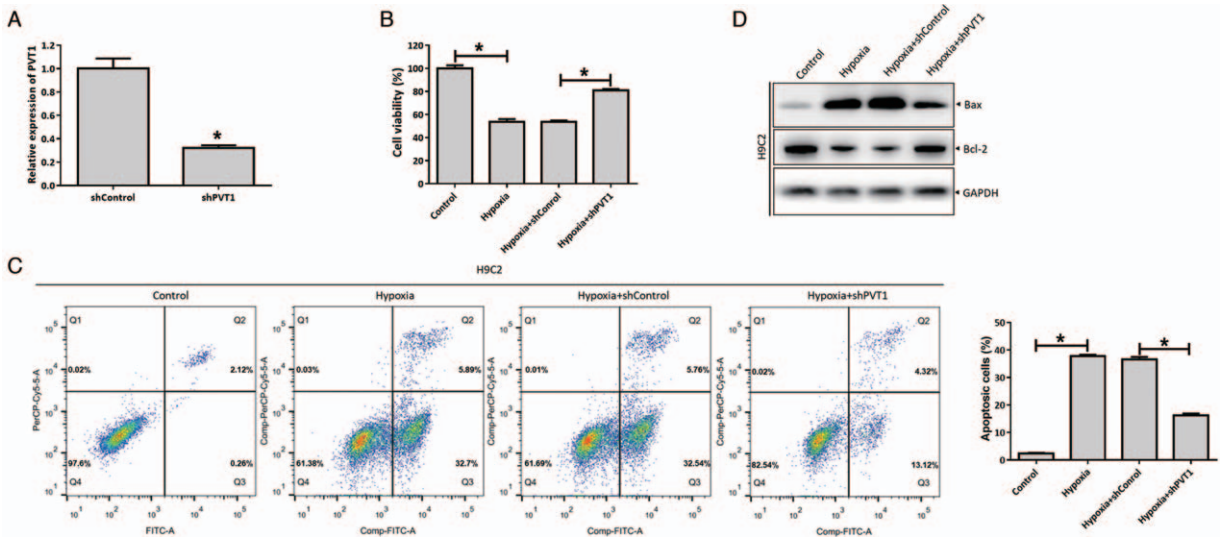

Figure 2: PVT1 aggravated hypoxia-injured H9c2 cell injury. (A). Relative expression level of PVT1 in H9c2 cells after transfected with shControl or shPVT1 for 48 h. (B). Cell viability of H9c2 cells after transfected with shControl or shPVT1 for 48 h and following 12 h hypoxia condition. (C). Cell apoptosis rate of H9c2 cells after transfected with shControl or shPVT1 for 48 h and following 12 h hypoxia stress treatment. (D). Bax and Bcl-2 protein detection in H9c2 cells after transfected with shControl or shPVT1 for 48 h and following 12 h hypoxia stress treatment. Data are shown as the means ± SE. $^{*}P < 0.05$. Bax: Bcl-2 Associated X-protein; Bcl-2: B-cell lymphoma 2; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; H9C2: H9C2 cells; PVT1: Plasmacytoma variant translocation 1; SE: Standard error; shControl: Empty vector (BLOCK-iT™ U6 RNAi Entry Vector); shPVT1: Plasmids with siRNA sequences specific for PVT1 subcloned into the BLOCK-iT™ U6 RNAi Entry Vector.

PVT1 functions as a sponge of miR-135a-5p

It is well known that lncRNAs can act as competing endogenous RNA (ceRNA) to regulate target gene expression and, thus, function in various physiological processes.[37,38] As one of the lncRNAs, PVT1 plays a crucial role in regulating the development of several cancers by directly interacting with miRNAs, such as miRNA-186 and miR-143.[17,39] Therefore, to explore the underlying mechanistic link between PVT1 and hypoxia-induced H9c2

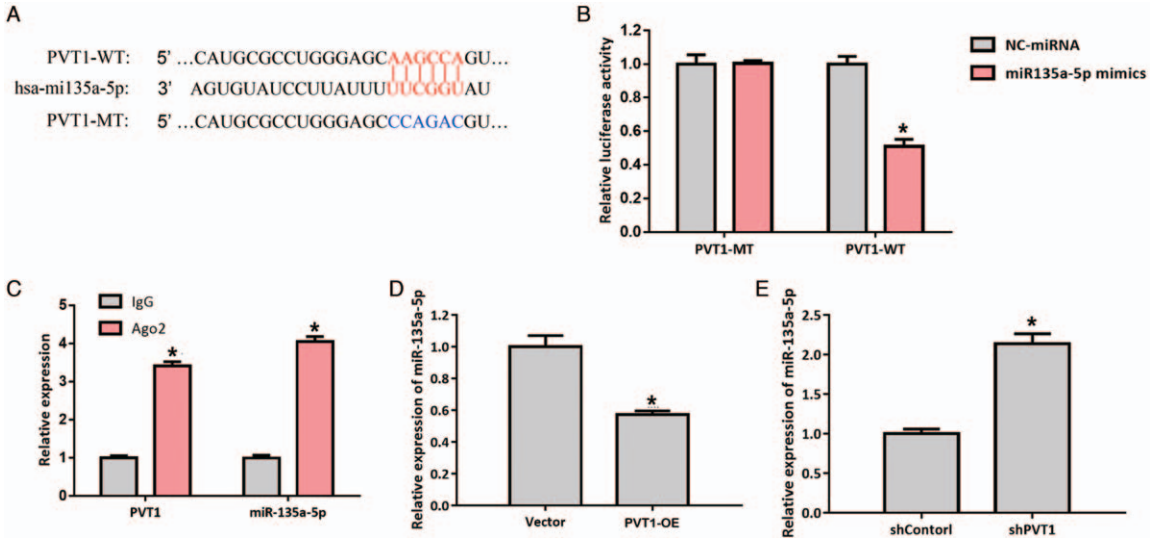

Figure 3: PVT1 sponged miR-135a-5p in H9c2 cells. (A) Schematic representation of the predicted target site for miR-135a-5p in PVT1. (B) Relative luciferase activities of luciferase reporters bearing wild-type or mutant PVT1 following transfection with the indicated miR-135a-5p mimics or NC-miRNA in H9c2 cells. (C) The enrichment of PVT1 or miR-135a-5p against Anti-Ago2 or IgG in RIP assay. (D, E) Relative expression level of miR-135a-5p in H9c2 cells transfected with PVT1-OE (D) or shPVT1 (E) for 48 h. Data shown are the means ± SE, $^{*}P < 0.05$. Ago2: Protein G agarose beads containing Argonaut 2; IgG: Protein G agarose beads containing immunoglobulin G; miR135a-5p: MicroRNA-135a-5p; NC-miRNA: Negative control for miR-135a-5p; PVT1: Plasmacytoma variant translocation 1; PVT1-OE: Plasmids with full length cDNA of PVT1 ligated into pcDNA3.1; PVT1-WT: Wildtype 3′-UTR sequence of PVT1; PVT1-MT: Mutated 3′-UTR sequence of PVT1; SE: Standard error; Vector: Empty vector (pcDNA3.1).

cell injury, we screened the possible miRNAs that might interact with PVT1 by using LncBase Predicted v.2 in DIANA tools (http://www.carolina.imis.athena-innovation.gr), and found miR-135a-5p was a possible target of PVT1 [Figure 3A]. To verify the association between PVT1 and miR-135a-5p, the luciferase reporter assay and RIP assay were performed. In luciferase reporter assay, only the luciferase activity of WT PVT1 rather than the mutant PVT1 was found to be specifically decreased by miR-135a-5p mimics but not by NC-miRNA [Figure 3B]. Moreover, the RIP assay exhibited that PVT1 and miR-135a-5p in H9c2 cells were both enriched in Ago2 IP, indicating PVT1 could directly interact with miR-135a-5p in H9c2 cells [Figure 3C]. We further explored the link of PVT1 on the expression of miR-135a-5p by evaluating the expression of miR-135a-5p while overexpressing or silencing PVT1 in H9c2 cells [Figures 3D and 3E]. The expression of miR-135a-5p was significantly reduced in H9c2 cells with PVT1 overexpression whereas predominantly increased in PVT1-silenced H9c2 cells compared with control. These results showed that PVT1 directly interacted with miR-135a-5p and negatively regulated the expression of miR-135a-5p in H9c2 cells.

PVT1 aggravates the hypoxia-induced H9c2 injury by sponging miR-135a-5p

To explore whether miR-135a-5p was involved in the hypoxia-induced H9c2 cell injury, we first performed qRT-PCR to detect the expression of miR-135a-5p in H9c2 cells and found its expression was significantly reduced in hypoxic stressed H9c2 cells as compared with the untreated control [Figure 4A], corroborating that miR-135a-5p might play a significant role in hypoxia-induced H9c2 injury. To further determine the role of miR-135a-5p in hypoxia-induced H9c2 injury, the mimics of miR-135a-5p and NC-miRNA (control) were transfected into H9c2 cells. The miR-135a-5p mimics, instead of NC-miRNA, caused an obvious increase in the expression of miR-135a-5p [Figure 4B]. Then, the effects of overexpressed miR-135a-5p was examined on cell viability and apoptosis of hypoxia-stressed H9c2 cells. miR-135a-5p overexpression rescued the significant reduction of cell viability and increased the cell apoptosis under hypoxia stress [Figures 4C and 4D]. Furthermore, Western blot analysis showed that transfecting of miR-135a-5p mimics predominantly attenuated the accumulation of Bax whereas significantly enhanced the content of Bcl-2 compared to that of NC-miRNA in hypoxia-stressed H9c2 cells, indicating that miR-135a-5p negatively regulated hypoxia-induced H9c2 cell injury.

PVT1 could directly interact with miR-135a-5p and negatively regulate its expression [Figure 3], thus, it might be possible that PVT1 regulates hypoxia-induced H9c2 injury via sponging miR-135a-5p. PVT1 was co-transfected into hypoxia treated H9c2 cells after overexpressing miR-135a-5p. Then, the effect of PVT1 overexpression on cell viability and apoptosis of hypoxia-induced H9c2 cells was analyzed. Intriguingly, PVT1 overexpression attenuated the dramatic increase of cell viability and an obvious decrease of cell apoptosis as induced by miR-135a-5p overexpression [Figures 4C and 4D]. Similarly, PVT1 overexpression reversed the accumulation of apoptosis-related proteins triggered by the overexpression of miR-135a-5p in hypoxia stressed H9c2 cells [Figure 4E]. Collectively, these investigations demonstrated that PVT1 was apparently involved in the hypoxia-induced H9C2 cell injury through sponging miR-135a-5p.

miR-135a-5p negatively regulates hypoxia-induced H9c2 cell injury by targeting FOXO1

To found whether PVT1 was involved as a ceRNA in hypoxia-induced injury, the downstream targets of miR-

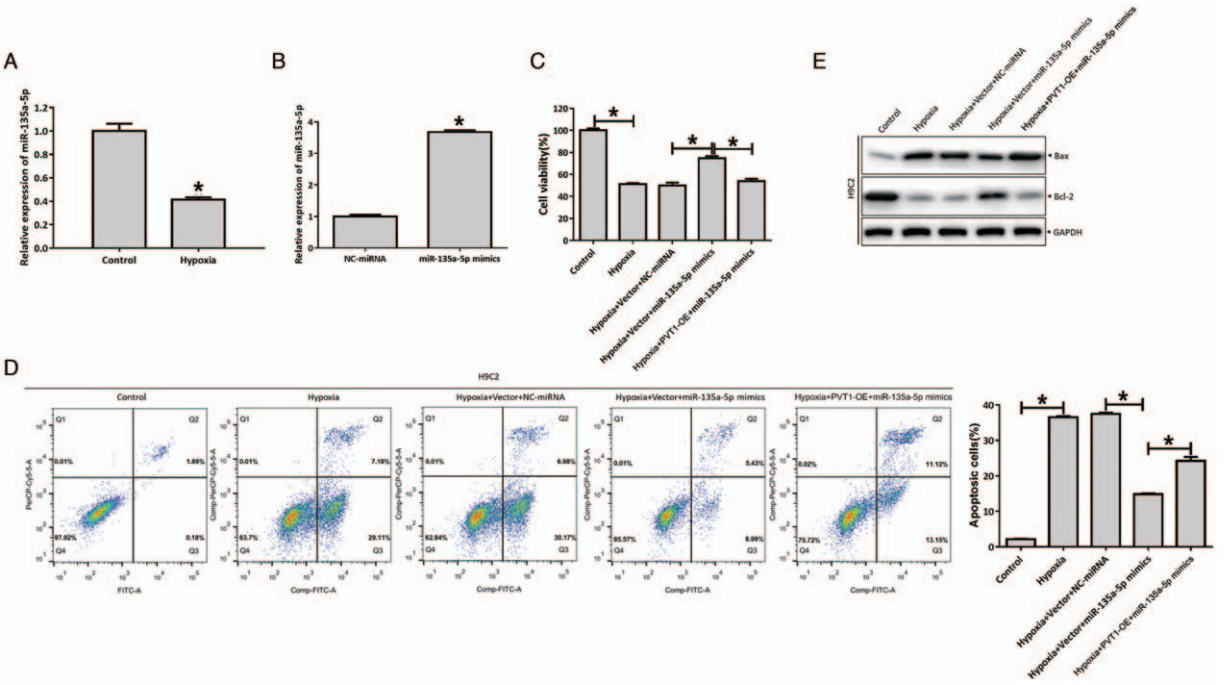

Figure 4: PVT1 association with miR-135a-5p provoked hypoxia-induced H9c2 cell apoptosis. (A) Relative expression level of miR-135a-5p in H9c2 cells following 12 h hypoxia stress treatment. (B) Relative expression level of miR-135a-5p in H9c2 cells after transfection of miR-135a-5p mimics or NC-miRNA for 48 h. Cell viability (C) and apoptosis rate (D) of H9c2 cells after transfected with the empty vector (Vector) or PVT1-OE together with NC-miRNA or miR-135a-5p following 12 h hypoxia stress treatment. (E) Bax and Bcl-2 protein detection in H9c2 cells after transfected with the empty vector (Vector) or PVT1-OE together with NC-miRNA or miR-135a-5p following 12 h hypoxia stress treatment. Data shown are the means ± SE, $^{*}P < 0.05$. Bax: Bcl-2 Associated X-protein; Bcl-2: B-cell lymphoma 2; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; miR135a-5p: MicroRNA-135a-5p; NC-miRNA: Negative control for miR-135a-5p; PVT1: Plasmacytoma variant translocation 1; PVT1-OE: Plasmids with full length cDNA of PVT1 ligated into pcDNA3.1; SE: Standard error; Vector: Empty vector (pcDNA3.1).

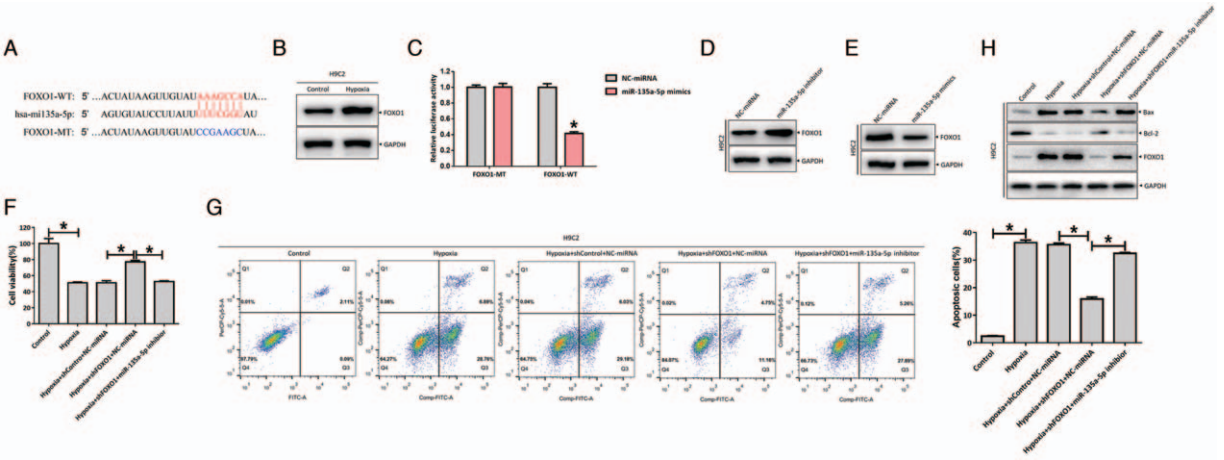

Figure 5: miR-135a-5p interacts with *FOXO1* and mediates its function in hypoxia-injured H9c2 cells. (A) Schematic representation of the predicted target site for miR-135a-5p in *FOXO1*. (B) The accumulation of FOXO1 in H9c2 cells with 12h hypoxia-stress. (C) Relative luciferase activities of luciferase reporters bearing wild-type or mutant *FOXO1* following transfection with the indicated miR-135a-5p mimics or NC-miRNA in H9c2 cells after 48 h cultivation. (D, E) The protein level of *FOXO1* in H9c2 cells after transfection with the indicated NC-miRNA, miR-135a-5p inhibitor or miR-135a-5p mimics for 48 h. (F, G) Cell viability and apoptosis rate of H9c2 cells after transfected with shControl or shFOXO1 together with NC-miRNA or miR-135a-5p inhibitor following 12 h hypoxia stress treatment. (H) Bax and Bcl-2 protein detection in H9c2 cells after transfected with shControl or shFOXO1 together with NC-miRNA or miR-135a-5p inhibitor following 12 h hypoxia stress treatment. Data shown are the means ± SE, $^{*}P < 0.05$. Bax: Bcl-2 Associated X-protein; Bcl-2: B-cell lymphoma 2; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; FOXO1: Forkhead Box O1; FOXO1-WT: Wildtype 3′-UTR sequence of *FOXO1*; FOXO1-MT: Mutated 3′-UTR sequence of *FOXO1*; H9C2: H9C2 cells; shControl: Empty vector (BLOCK-iT™ U6 RNAi Entry Vector); miR135a-5p: MicroRNA-135a-5p; NC-miRNA: Negative control for miR-135a-5p; SE: Standard error; shFOXO1: Plasmids with siRNA sequences specific for *FOXO1* subcloned into the BLOCK-iT™ U6 RNAi Entry Vector.

135a-5p were screened using TargetScan (http://www.targetscan.org/). We found FOXO1, a protein that has been reported previously to cause cardiomyocyte death upon over accumulation or constitutive activation, was one of the candidate targets of miR-135a-5p.[40] To verify the interaction between miR-135a-5p and *FOXO1*, we performed the luciferase reporter assay. Our results showed that the luciferase activity of FOXO1-WT was significantly decreased whereas nearly no change was noticed in the luciferase activity of mutant *FOXO1* (FOXO1-MT) [Figure 5B]. Moreover, the accumulation of FOXO1 was significantly down-regulated by treating it

with miR-135a-5p mimics while enhanced after adding miR-135a-5p inhibitor in H9c2 cells [Figures 5C and 5D], suggesting that miR-135a-5p could directly interact with *FOXO1* and negatively regulate its expression.

To further explore the underlying link between miR-135a-5p and *FOXO1*, the viability and apoptosis of H9c2 cells were determined by transfecting shFOXO1, to knockdown *FOXO1*, or shControl (the negative control) together with NC-miRNA or miR-135a-5p inhibitor into hypoxia stressed H9c2 cells. The transfection of shFOXO1 rather than shControl alleviated hypoxia-induced H9c2 cell injury as indicated by promoted cell viability as well as decreased apoptosis [Figures 5F and 5G], which was in consistent with previous results [Figure 4], and as expected, transfection of miR-135a-5p inhibitor, instead of NC-miRNA, reversed the injury of shFOXO1 as previously induced by hypoxia which led to the decreased cell viability and enhanced apoptosis. In addition, the abnormal accumulation of Bax and Bcl-2 as induced by shFOXO1 transfection was also reversed by adding miR-135a-5p inhibitor [Figure 5H]. These results together suggested that miR-135a-5p negatively mediates hypoxia-induced H9c2 cell injury by targeting *FOXO1*.

PVT1 mediates hypoxia-induced H9c2 cell injury through miR-135a-5p/FOXO1 axis

To determine whether PVT1 regulates hypoxia-induced injury in H9c2 cells through miR-135a-5p/*FOXO1* axis. We first performed luciferase reporter assay to test whether PVT1 affects the interaction between miR-135a-5p and *FOXO1*. The decreased luciferase activity of wild-type *FOXO1* induced by miR-135a-5p was partially restored by PVT1 overexpression in H9c2 cells [Figure 6A]. Meanwhile, we examined FOXO1 accumulation when PVT1 was overexpressed or knockdown in H9c2 cells. The knockdown of PVT1 decreased FOXO1 accumulation while overexpression of PVT1 promoted the accumulation of FOXO1 [Figures 6B and 6C], suggesting that PVT1 interferes with the interaction between miR-135a-5p and *FOXO1* and, thus, positively regulate FOXO1 accumulation. We further determined whether PVT1 mediates hypoxia-induced H9c2 cell injury though the miR-135a-5p/*FOXO1* axis. The PVT1 or the empty vector, used as control, were co-transfected with shFOXO1 or shControl into hypoxia stressed H9c2 cells and then the viability and apoptosis of H9c2 cells were determined [Figures 6D and 6E]. Transfection of PVT1 instead of the empty vector aggravated hypoxia-induced H9c2 cell injury as shown by decreased cell viability and promoted apoptosis, and these results were in consistency with its role in hypoxia-induced H9c2 cell injury [Figure 2]. Additionally, as expected, knockdown of *FOXO1*, by transfecting shFOXO1 instead of NC-miRNA, reversed the PVT1 overexpression induced aggravation of hypoxia induced H9C2 injury in terms of promoted cell viability and decreased apoptosis. In addition, PVT1 overexpression induced Bax promotion and Bcl-2 reductions which were reversed by knockdown of *FOXO1* [Figure 5H]. These results together suggested that PVT1 aggravates hypoxia-induced H9c2 cell injury through the miR-135a-5p/*FOXO1* axis.

Discussion

MI causes severe impairment of coronary blood supply, damage to the heart muscles, and produces a range of clinical syndromes, such as myocardial ischemia and reperfusion injury, cardiac hypertrophy, prime determinant of the progression of heart failure and causes compensatory angiogenesis.

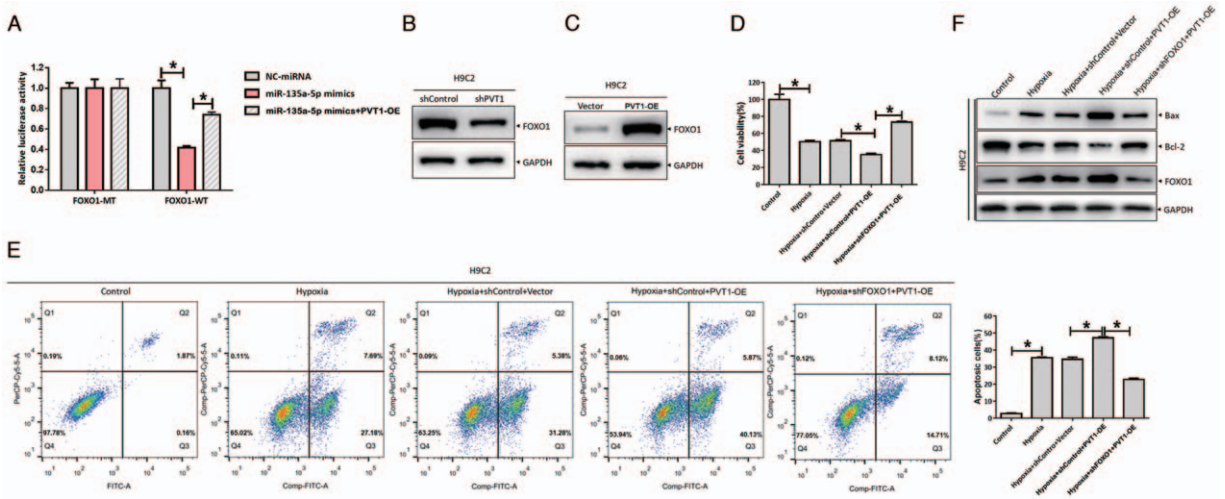

Figure 6: The functional interaction among PVT1, miR-135a-5p, and *FOXO1*. (A) Relative luciferase activities of luciferase reporters bearing wild-type or mutant *FOXO1* following transfection with miR-135a-5p mimics or miR-135a-5p mimics together with PVT1-OE in H9c2 cells after 48 h cultivation. (B and C) The accumulation of FOXO1 after transfection with shControl, shPVT1 (B) or the empty vector (Vector), PVT1-OE (C) in H9c2 cells following 48 h cultivation. (D and E) Cell viability and apoptosis rate of H9c2 cells after transfected with shControl or shFOXO1 together with the empty vector (Vector) or PVT1-OE following 12 h hypoxia stress treatment. (F) Bax and Bcl-2 protein detection in H9c2 cells after transfected with shControl or shFOXO1 together with the empty vector (Vector) or PVT1-OE following 12 h hypoxia stress treatment. Data shown are the means ± SE, $^{*}P < 0.05$. Bax: Bcl-2 Associated X-protein; Bcl-2: B-cell lymphoma 2; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; FOXO1: Forkhead Box O1; FOXO1-WT: Wildtype 3'UTR sequence of *FOXO1*; FOXO1-MT: Mutated 3'UTR sequence of *FOXO1*; H9C2: H9C2 cells; miR135a-5p: MicroRNA-135a-5p; NC-miRNA: Negative control for miR-135a-5p; PVT1: Plasmacytoma variant translocation 1; PVT1-OE: Plasmids with full length cDNA of PVT1 ligated into pcDNA3.1; SE: Standard error; shControl: Empty vector (BLOCK-iT^{TM} U6 RNAi Entry Vector); shFOXO1: Plasmids with siRNA sequences specific for *FOXO1* subcloned into the BLOCK-iT^{TM} U6 RNAi Entry Vector; Vector: Empty vector (pcDNA3.1).

In the present study, the cardiomyocyte cell line H9c2 was cultured under hypoxic condition to simulate myocardial infarction *in vitro,* and the biological function of PVT1 on hypoxia-induced H9c2 cell injury was analyzed by determining cell viability and apoptosis. In addition, the biological function of miR-135a-5p was also detected. miR-135a-5p mimics and inhibitor were used to transfect H9c2 cells to study their effect on cell viability and apoptosis. Furthermore, the target gene of miR-135a-5p was explored and confirmed with luciferase reporter assay. Hypoxia-induced H9c2 cells showed cell viability inhibition and promotion of cell apoptosis, and we found the hypoxia-induced injury in H9c2 cells was aggravated by overexpressing PVT1. Previous studies have suggested the role of PVT1 in cardiovascular diseases. PVT1 proliferates vascular endothelial cells by directly decreasing the expression of miR-190a-5p and serves as an effective diagnostic biomarker in the chronic heart failure.[41] PVT1 positively regulates cardiac hypertrophy and shown an upregulation of 2.5 fold in hypertrophic hearts after transverse aortic constriction for four weeks.[42] PVT1 was also shown regulates atrial fibrosis, commonly associated with congestive heart failure, by acting as a sponge for miR-128-3p.[43] In this study we found PVT1 mediates hypoxia-induced H9c2 cell injury through sponging miR-135a-5p.

miR-135a-5p was previously found to be involved in ischemic heart diseases, the expression level of miR-135a-5p was significantly decreased both in the rat I/R injury group and H9c2 cells that subjected to hypoxia/reoxygenation condition, and overexpression of miR-135a-5p significantly inhibited I/R-induced cell apoptosis, demonstrating that miR-135a-5p protects against myocardial I/R injury in rats by downregulating protein tyrosine phosphatase 1B.[44] miR-135a-5p is upregulated and aggravates sepsis induced myocardial depression by activating p38 MAPK/NF-kB pathway.[45] miR-135a-5p increased 2.9 fold in the rat heart under prolonged (after 10 days of treatment) hypoxic condition.[46] miR-135a-5p was also shown five-fold ($P < 0.001$) increase in coronary artery disease.[47] In another study miR-135a-5p was shown to regulate H_2O_2-induced apoptosis in H9c2 cells via targeting Bcl-2 protein.[48] Although the downstream factors of miR-135a-5p were explored in these reports mentioned above, the upstream factors of miR-135a-5p remains obscure. Here, our results indicated PVT1 mediates miR-135a-5p expression in hypoxia-induced H9c2 cells by sponging miR-135a-5p, meantime, *FOXO1* was identified as a new target of miR-135a-5p in regulating the process of hypoxia-induced H9c2 cell injury.

Presently, PVT1 was found to regulate hypoxia-induced myocardial cell injury by acting as a sponge of miR-135a-5p and PVT1 overexpression aggravated hypoxia-induced H9c2 cell injury via down-regulation of miR-135a-5p. Previous reports suggested lncRNAs exert their biological function by sponging miRNAs. For example, lncRNA Cardiac hypertrophy related factors regulates cardiac hypertrophy *in vivo* and *in vitro* by acting as an endogenous sponge of miR-489, which targets myeloid differentiation primary response gene 88.[44] lncRNA *GAS5* functions as tumor suppressor though sponging and negatively regulates the expression of miR-21.[49] lncRNA GAS5 inhibits the proliferation of cardiac fibroblast via phosphatase and tensin homologue/matrix metalloprotease-2 signaling pathway though negatively regulating miR-21.[50] lncRNA myocardial infraction-associated transcript overexpression in H9c2 cells dramatically reduce the miR-150 expression and enhances cardiac hypertrophy.[51] Cardiac apoptosis-related lncRNA suppresses anoxia-induced mitochondrial fission and apoptosis by targeting miR-539 and *PHB2*.[52] Mitochondrial dynamic related lncRNA inhibits mitochondrial fission and apoptosis by down-regulating the expression of miR-361 and further regulating the processing of miR-484 primary transcript.[53] lncRNA H19 suppresses miR-19b expression which targets *sox6* and thus inhibits cell proliferation and enhances apoptosis during late-stage cardiac differentiation.[54] miR-188-3p regulates *ATG7* and suppresses autophagy and suppresses autophagy and myocardial infraction.[55] lncRNA necrosis-related factor targets miR-873 to regulate programmed necrosis and myocardial injury caused by I/R.[56] Presently, our investigation demonstrated that PVT1 mediates hypoxia-induced H9c2 cell injury by sponging miR-135a-5p which targets *FOXO1* and negatively regulates its expression, which makes PVT1 and miR-135a-5p promising novel diagnostic and therapeutic targets for ischemic heart disease. Nevertheless, there are limitations in our study, as the biological function of PVT1, miR-135a-5p, and *FOXO1* was determined *in vitro*, further *in vivo* research will be necessary to confirm their role in MI.

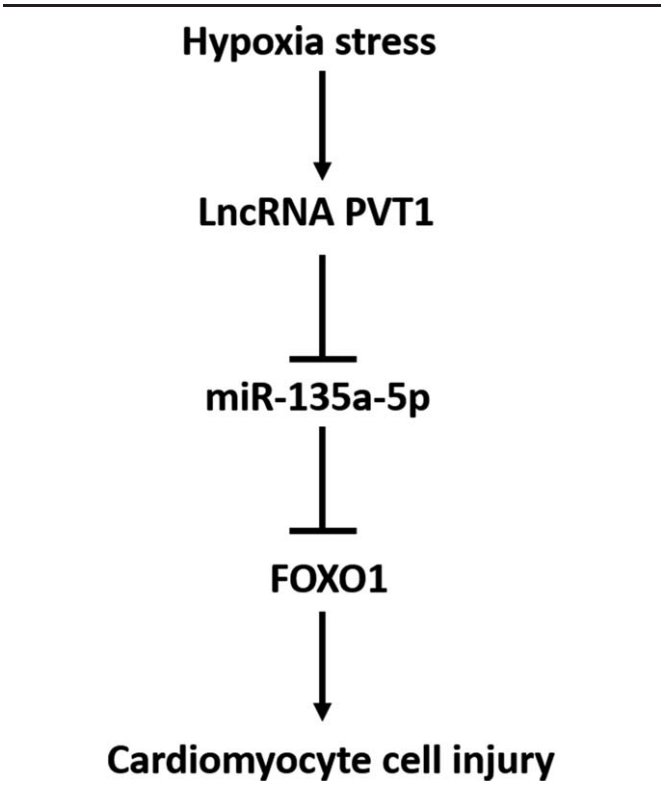

Figure 7: Schematic representation of factors involved in hypoxia-induced cardiomyocytes. FOXO1: Forkhead box O1; LncRNA PVT1: Long non-coding RNA plasmacytoma variant translocation 1; miR135a-5p: MicroRNA-135a-5p.

In conclusion, we found that PVT1 was induced under hypoxic conditions and knockdown of PVT1 alleviated hypoxia-induced H9c2 cell apoptosis. Furthermore, we have illustrated that PVT1 suppresses the expression of miR-135a-5p and miR-135a-5p negatively regulated hypoxia induced injury by targeting *FOXO1*. Altogether, PVT1 triggered hypoxia-induced H9c2 cell injury via miR-135a-5p/*FOXO1* axis [Figure 7], and PVT1-miR-135a-5p/*FOXO1* axis might constitute as a diagnostic and prognostic biomarker for hypoxia-induced cardiovascular disease.

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Conflicts of interest

None.

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