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Research article

LncRNA Pvt1 aggravates cardiomyocyte apoptosis via the microRNA-216/Ccnd3 axis

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A B S T R A C T

Objective: Our study aims to evaluate the role of long non-coding RNA variant translocation gene Pvt1 in cardiomyocyte apoptosis, as well as the potential targets and mechanisms involved in Pvt1-miRNA-mRNA axis.
Methods: 1.Pvt1 knockdown in cells by transfection with small interfering RNA (si-Pvt1), HL-1 cells were randomly divided into control group, hypoxia group, hypoxia + negative control group and hypoxia + si-Pvt1 group. Apoptosis-related genes expression was detected by Western blot assay, RT-qPCR and Flow cytometry assay. 2.Pvt1 knockdown model (sh-Pvt1) was established by injecting adeno-associated virus (AAV) vector shRNA-Pvt1 into the caudal vein 7 days before myocardial infarction, and echocardiography was used to measure cardiac function 7 days after myocardial infarction induced by ligation of the left anterior descending branch. HE staining was used to evaluate the pathological injury of mouse heart tissue, and the apoptotic protein expression was detected by Western blot. 3.lncRNA-related microRNAs were predicted by bioinformatics tools and further verified by dual luciferase experiment. Western blot analysis was used to identify the expression of apoptotic genes following the simultaneous transfection of si-Pvt1 and miR-216 mimics. Genes differentially expressed in hypoxia + si-NC and hypoxia + si-Pvt1 groups were identified by RNA sequencing. These genes were then compared with the target genes of miR-216 predicted by bioinformatics tools. The gene of interest Ccnd3 was excluded from the analysis. Western blot analysis was used to assess the expression of Apoptosis-related proteins in HL-1 cells co-transfected with miR-216 mimics and overexpressed Ccnd3.
Results: 1. Pvt1 was highly expressed in HL-1 induced by hypoxia, and Pvt1 knockdown can reduce cell apoptosis in hypoxia cells. 2. MI causes myocardial injury in mice, and inhibition of Pvt 11 can improve the cardiac function of mice with myocardial infarction, prevent some inflammatory cell infiltration, and reduce myocardial cell apoptosis. 3. Pvt1 acts as a sponge for miR-216 and promotes the expression of Ccnd3.
Conclusion: Pvt1 may promote myocardial infarct-induced apoptosis through the miR-216/Ccnd3 axis.

1. Introduction

Acute myocardial infarction (AMI) maintains to serve as a leading cause of morbidity and mortality on a worldwide scale [1,2]. Current medications and therapies are limited to delay the disease's course [3,4].Interruption of blood supply leads to sudden

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myocardial ischemia, causing irreversible damage to the heart in acute myocardial infarction [5]. Finding effective strategies for treatment and prevention of MI is consequently essential. The primary form of cardiomyocyte injury in MI is cardiomyocyte apoptosis. One of the major causes of arrhythmia following myocardial infarction is cardiomyocyte apoptosis, which can result in infarct size expansion and myocardial remodeling. Consequently, improving heart function during MI and the cardiac remodeling process can be achieved by inhibiting cardiomyocyte apoptosis [6].

Non-coding RNAs (ncRNAs) are a type of RNAs that lack protein-coding potential [7,8]. An increasing amount of research has demonstrated that ncRNAs make up around 98 % of the human genome. These RNAs can couple back into a larger communication network and have modulatory properties [9,10]. Long ncRNAs (lncRNAs) and microRNAs (miRNAs), the two primary forms of ncRNAs, have drawn an abundance of scrutiny. Emerging research has revealed the significance of miRNAs and lncRNAs as regulators in MI. Furthermore, in the development of MI, lncRNAs may function as competitive endogenous RNAs (ceRNA) to sequester miRNAs. Simultaneously, miRNAs often work in MI by attaching to mRNAs' 3′-untranslated region (3′-UTR) and influencing mRNA translation. Interestingly, several lncRNA-miRNA-mRNA interactions play a role in myocardial infarction [11].

Long non-coding RNAs (lncRNAs) are a notable group of non-coding RNA (ncRNA) transcripts that are longer than 200 nucleotides and only have minimal or no ability to encode proteins [12]. LncRNAs have a vital role in regulating several biological and pathological processes including cell proliferation, differentiation, apoptosis, and development [13]. A considerable amount of literature has revealed that lncRNAs serve a crucial regulatory function in several forms of cardiac development and illnesses, including coronary heart disease [3].

Long non-coding RNA Pvt1 (lncRNA Pvt1) is an oncogene located on chromosome 8q24, a recognized area associated with cancer risk. Pvt1 has been shown to be elevated in several human cancer types, including ovarian cancer, breast cancer, and non-small-cell lung cancer [14]. It has been reported that pathophysiological function of lncRNA Pvt1 in the modulation of atrial fibrosis through the lncRNA-miRNA-mRNA axis [15]. However, the possible roles and mechanisms of Pvt1 in regulating apoptosis in myocardial infarction have not been reported. The present study postulated the existence of possible ceRNA networks implicated in the death of cardiac cells triggered by myocardial infarction (MI). Within this, our team found differentially expressed lncRNA Pvt1 after atherosclerosis [16], and then the involvement of Pvt1 in cardiomyocyte apoptosis was investigated in both *in vitro* and *in vivo* models, and the underlying processes were further investigated.

2. Materials and methods

2.1. Cell culture and transfection

The HL-1 cell line produced from mice was purchased from Procell Life Science&Technology Co., Ltd. located in Wuhan, China. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10 % fetal bovine serum (FBS, PAN-Biotech GmbH, Germany) and 1 % penicillin-streptomycin (P/S) in a 37 °C incubator with 5 % carbon dioxide (CO_2) atmosphere. To make hypoxia injury *in vitro* model, the cells of the HL-1 cell line were cultured in a hypoxic incubator with 1 % O_2/5 % CO_2 whereas the cells in the incubator with 21 % O_2 were treated as control.To provide an *in vitro* model of hypoxic damage, HL-1 cell line cells were cultivated in a hypoxic incubator with 1 % O_2 and 5 % CO_2, whereas cells in an incubator with 21 % O_2 served as the control group.

The small interfering RNA directed against Pvt1 (si-Pvt1) and the negative control si-NC, were designed by and purchased from Gene Pharma (Shanghai, China). The sequence of si-Pvt is 5′- GGCACUGCAAACACAAUGATT-3′, and the sequence of si-NC is 5′-UUCUCCGAACGUGUCACGUTT-3′. Briefly, the cells were inoculated into 6-well plates at a density of 1×10^6 cells per well and incubated overnight. When HL-1 cells were grown to about 50 % confluence, a mixture of LipofectamineTM3000 and Opti-MEM medium, as well as a mixture of Opti-MEM medium and siRNA, were pre-prepared repecitively. Then, these two mixes were subsequently co-incubated for 15 min at room temperature before being introduced to the cells. After transfection at 37 °C for 24 h, RT-qPCR was performed to analyze the efficiency of transfection.

The si-Pvt1-targeted small interfering RNA (si-Pvt1) and the negative control (si-NC) were developed and acquired from Gene Pharma in Shanghai, China. The si-Pvt sequence is 5′- GGCACUGCAAACACAAUGATT-3′, while the si-NC sequence is 5′-UUCUCCGAACGUGUCACGUTT-3′. In brief, the cells were added to 6-well plates at a density of 1×10^6 cells per well and allowed to incubate overnight. Following the growth of HL-1 cells to about 50 % confluence, a combination of LipofectamineTM3000 and Opti-MEM medium, as well as a combination of Opti-MEM medium and siRNA, were individually created. Next, these two mixtures were co-incubated for 15 min at ambient temperature before being added to the cells. Following a 24-h transfection at 37 °C, the effectiveness of transfection was assessed by RT-qPCR.

Next, the cells were treated in the same manner with pmirGLO-Pvt1-wt + miR216 mimics, pmirGLO-Pvt1-mut + miR216 mimics, miR216 mimics, siPvt1+ miR216 inhibitor, siPvt1+ miR-NC, pcDNA3.1-Ccnd3 (Ccnd3 overexpression plasmids) +miR216 inhibitor. Implementation of the subsequent assays was initiated 48 h after transfection. The plasmid concentration was 50 ng/ml. The Ccnd3 overexpression plasmid was manufactured by Sangon Biotechnology.

2.2. RT-qPCR

Total RNA was extracted by using the Trizol reagent (Solarbio, China). The Fast All-in-One RT Kit (ES Science, China) was employed to generate the cDNAs of mRNAs. The methodology of the quick miRNA reverse transcription kit was followed to reverse transcribe whole miRNA into cDNA using Tailing Reaction mechanism. SYBR Green (FOREVERSTAR, China) and an RT-PCR system (CFX96, Bio-Rad, USA) were employed to quantify gene expression. Normalizing against the reference genes β-actin (mRNA) and U6

(miRNA), the relative quantitative 2-ddCT method was implemented to evaluate relative gene expression. The primers are illustrated in Table 1.

2.3. Western blot analysis

The expression of pertinent proteins was determined by Western blot analysis. Cell suspensions were prepared by mixing RIPA lysate with PMSF at a ratio of 100 : 1. SDS-PAGE gels with a concentration of 12 % were generated in advance and then utilized for SDS-polyacrylamide gel electrophoresis. The following ratios were then used to dilute the antibodies, which were subsequently stored for 12 h at 4 °C:Bax(Proteintech,1:2000),Bcl-2(Proteintech,1:2000),caspase-3(Proteintech,1:1000), Ccnd3(Proteintech; 1:1000)was stored at 4 °C for 12 h, Subsequently, the samples were placed onto PVDF membranes. After blocking with 5 % non-fat milk, the membranes were subjected to overnight incubation at 4 °C with primary antibodies. Secondary antibodies were applied to the membranes at room temperature for a duration of 2 h. The signal was obtained using the Bio-Rad imaging apparatus.

2.4. Flow cytometry assay

5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) were added to 100 μl of binding buffer, and the lysed cells were resuspended. The cells were stained at ambient temperature in the absence of sunlight, and later incubated for 20 min.Apoptosis was evaluated using a flow cytometer from Beckman Coulter in Miami, FL, U.S.A.

2.5. Dual-luciferase reporter assay

The pmirGLO-Pvt1-wt vector was constructed using the lncRNA Pvt1, which has predicted binding sites for miR-216, integrated into the pmirGLO Dual-luciferase vector. Mutated binding sites were the source of the pmirGLO-Pvt1-mut. HL-1 cells were cultivated with pmirGLO-lncRNA Pvt1-wt and pmirGLO-lncRNA Pvt1-mut, and subsequently treated with miR-216 mimics. Luciferase activities were assessed 48 h post-transfection utilizing the luciferase reporter assay equipment (Promega, USA).

2.6. Animals

Male C57BL/6J mice (8–12weeks old) were purchased from experimental Animal Center of Chongqing Medical University.The Ethics Committee of Chongqing Medical University (IACUC-CQMU-2023-0082) granted prior approval for the execution of animal experimental research procedures in accordance with ethical standards. All experimental protocols adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.7. MI model was established by the left-anterior descending coronary artery (LAD) ligation

Anesthesia was administered to rodents using pentobarbital sodium (50 mg/kg). The left anterior descending artery was ligated using a 6-0 prolene suture. The success of the model was demonstrated by the left ventricle turning white.Each mouse in the sham group had identical surgical procedures without ligation. Mice were classified into two groups: AAV-sh-Pvt1 and AAV-sh-NC ($n \geq 5$). Seven days before to the myocardial infarction procedure, mice received intravenous injections of adeno-associated virus (AAV, 1×10^{11} PFU per animal).The AAV-sh-Pvt1 and AAV-sh-NC were constructed and packaged by Genechem Company. Seven days after MI operation, infarct boundary zone was harvested for the following experiments.

Table 1
The sequences of specific primers.

Gene name	Primer sequence (5' to 3')
Gas5	Forward: 5′- ATCCACCCTCTTGTCTGATTTTCT-3′, Reverse: 5′- AATCCACAGGGTTCAGGAAGTC-3′;
Anxa2	Forward: 5′-TGGAGAACGCCTTCCTGAAC-3′, Reverse: 5′-CCCTTGGTGTCTTGCTGGAT-3′.
Ccnd3	Forward: 5′-TGGAGAACGCCTTCCTGAAC-3′, Reverse: 5′-CCCTTGGTGTCTTGCTGGAT-3′.
Kif3	Forward: 5′-GCAAAAACTGGAGCCACAGG-3′, Reverse: 5′-CGTTGCCCAGGGTAGACAAT-3′.
Kif10	Forward: 5′-CACACATTGCTTGGTGGTGG-3′, Reverse: 5′-GGAGTCCGCTATTCTCCTGC-3′.
Rhou	Forward: 5′-CATCATCCTGGTCGGGACAC-3′, Reverse: 5′-CGGCGTCGAAAACCTCTTTG-3′.
TPD52	Forward: 5′-CTACACAGAACGTTGGTCTGC-3′, Reverse: 5′-TCAGCTCCTCTTGCTCCTCT-3′.
β-actin	Forward: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, Reverse: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′;

2.8. HE staining

Paraffin-embedded mouse heart tissues were chopped into slices and then immersed in xylene for 15 min. Subsequently, The sections were submerged in an ethanol solution with successfully decreasing concentrations for 1 min, followed by washing with distilled water. After that, the sections were subjected to HE staining, followed by soaking in ethanol solution at gradient concentration from low to high. After permeabilization in xylene, the sections were sealed with neutral resin added dropwise. Finally, pathological damage was observed under an optical microscope(Lycra, Germany).

3. Results

3.1. The expression of Pvt1 was increased in mice LAD models and hypoxia HL-1 cells

A mouse model of LAD-induced myocardial infarction (MI) and an *in vitro* model of hypoxia-induced HL-1 cell line were developed to study Pvt1 expression. The expression of Pvt1 was then identified using quantitative real-time polymerase chain reaction (qRT-PCR). Depicted in Fig. 1 is the up-regulation of Pvt1 expression seen in both *in vivo* and *in vitro* myocardial infarction model studies (Fig. 1A and B). Our study involved a comprehensive assessment of the expression of proteins associated to apoptosis in both normal cells and cells subjected to a 24-h hypoxia. The results showed that Bax and Caspase-3 expression increased significantly after hypoxic exposure, while Bcl-2 expression decreased (Fig. 1C). We hypothesise that long non-coding RNA Pvt1 is implicated in the advancement of myocardial apoptosis during myocardial infarction.

3.2. Knockdown of Pvt1 alleviates hypoxia-induced cell apoptosis in HL-1 cells

Pvt1 knockdown was accomplished by transfecting cells with si-Pvt1 (Fig. 2A). Furthermore, we investigated the impact of Pvt1 expression on cellular apoptosis. Bcl-2 was upregulated in hypoxia cells with si-Pvt1 groups, conversely Bax and caspase-3 levels were downregulated (Fig. 2B and C). Furthermore, the findings of the flow cytometry experiment provided additional confirmation of the promoting function of Pvt1 on cell apoptosis (Fig. 2D). Therefore, all the results presented offer proof that Pvt1 acts as a facilitating element in the regulation of cell death in HL-1 cells.

3.3. Pvt1 silencing improved cardiac function and reduced myocardium apoptosis in MI mice

To examine the influence of Pvt1 on MI in mice, we evaluated changes in heart function during episodes of MI after the inhibition of Pvt1. The gene expression of Pvt1 was examined in the area of infarction in isolated heart tissue. The results showed that the release of

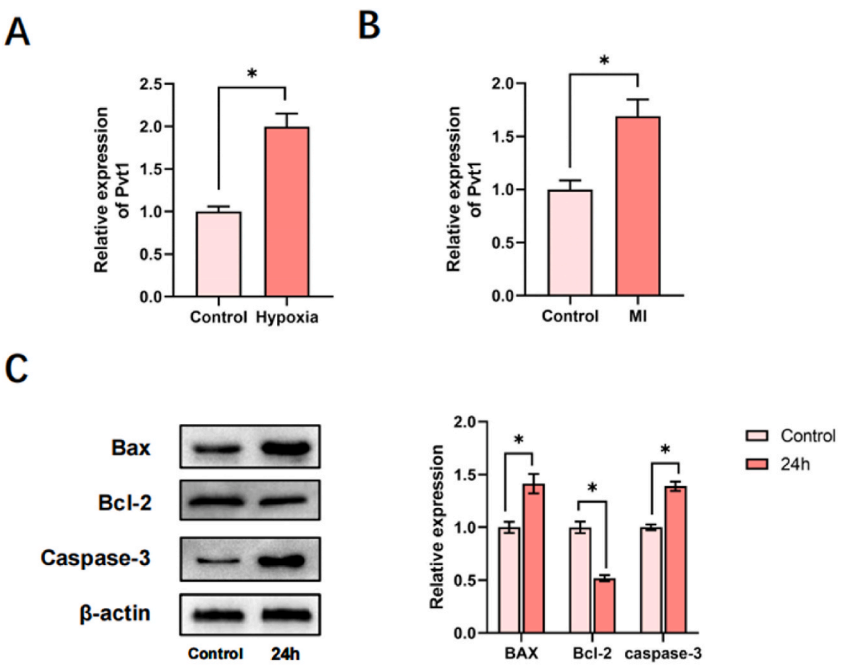

Fig. 1. Pvt1 is highly expressed in hypoxia-induced HL-1 cells and LAD-induced mice. (A,B) Pvt1 expression was examined in hypoxia-induced HL-1 cells and LAD-induced mice by RT-qPCR. (C) Quantification of proteins related to apoptosis was performed in HL-1 cells after a 24-h hypoxia treatment using Western blot analysis. *P < 0.05 Hypoxia, MI, 24h *vs.* Control.

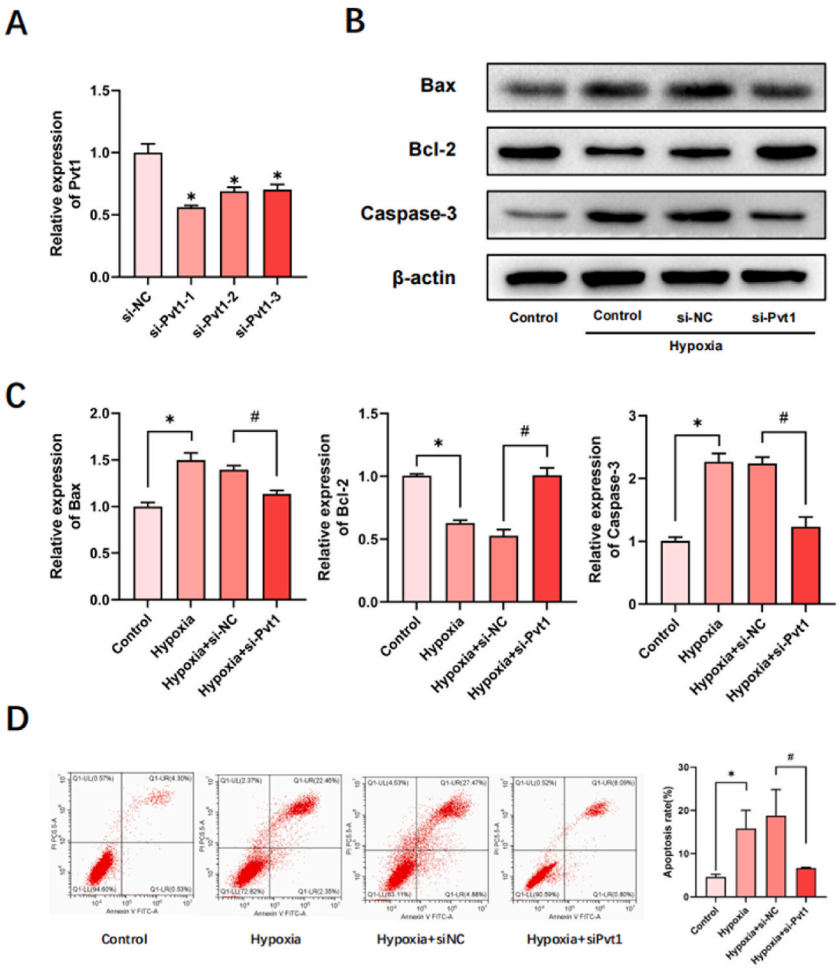

Fig. 2. Pvt1 depletion's effects on hypoxia-induced apoptosis in HL-1 cells.
(A) Verification of the silencing effectiveness of Pvt1 was conducted using RT-qPCR in hypoxia-induced HL-1 cells. (B,C)Western blot analysis was used to quantify protein levels associated to apoptosis in HL-1 cells that were depleted of Pvt1 after a 24-h period of hypoxia. (D) HL-1 cell apoptosis following Pvt1 silencing was investigated by flow cytometry analysis, following a 24-h exposure to hypoxia. *$p < 0.05$ Hypoxia *vs.* control; $^{\#}p < 0.05$ Hypoxia + si-NC *vs.* Hypoxia + si-Pvt1.

Pvt1 generated by myocardial infarction in the affected area was significantly decreased in the group that silenced Pvt1 successfully. (Fig. 3A). In comparison to the Sham group, the MI group had lower values of the left ventricular ejection fraction (LVEF) and left ventricular fraction (LVFS). However, inhibiting Pvt1 improved heart function in mice with myocardial infarction (MI), in comparison to animals who received a sh-NC as a control (Fig. 3B, C, D). Furthermore, HE staining indicated that the myocardial fibers in the Sham group were well-organized, with no presence of inflammatory cell infiltration in the stroma. Conversely, the myocardial fibers in the MI group were disordered and accompanied by the infiltration of inflammatory cells. Simultaneously, The suppression of Pvt1 led to a partial restoration of the structure of cardiac fibers and effectively prevented the invasion of inflammatory cells (Fig. 4E). These studies demonstrate that cardiac activity reduced by myocardial infarction is restored. Our findings indicate that Bcl-2 was upregulated in the AMI group with sh-Pvt1 groups, although the expressions of Bax and caspase-3 were downregulated (Fig. 3F). Combined, these results showed that decreasing the expression of Pvt1 decreased the occurrence of myocardial apoptosis in mice with AMI.

3.4. LncRNA Pvt1 regulated hypoxia cell injury via miR-216

To further explore the potential miRNA-mRNA mechanism of Pvt1, through StarBase 2.0, we noted that Pvt1 had a targeted binding site with miR-216 and predicted that miR-216 would bind at the 3′-UTR site of lncRNA Pvt1 (Fig. 4A). To validate the interaction between miR-216 and lncRNA Pvt1, a dual luciferase assay was employed to determine the presence of the aforementioned interaction. The dual luciferase activity of HEK293T cells was assessed by co-transfecting them with miR-216 mimics and either the wild type (WT) or mutant (MUT) of pmirGLO-lncRNA Pvt1. The luciferase activity in Pvt1 WT transfected cells was reduced by miR-216, as demonstrated by the dual-luciferase assay. Not in Pvt1 MUT cells, to be sure (Fig. 4B).

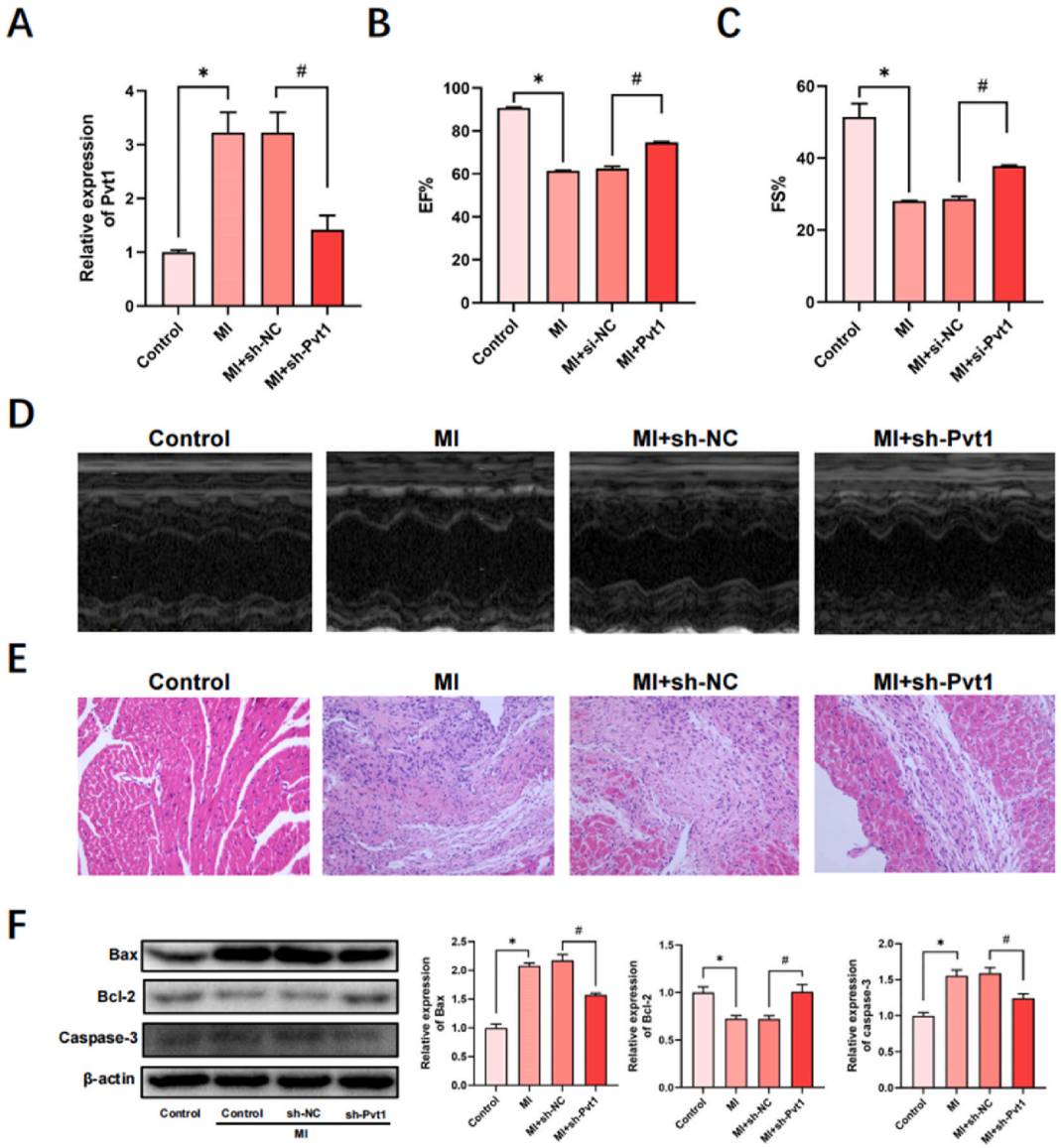

Fig. 3. Pvt1 silencing improved cardiac function and reduced myocardium apoptosis in MI mice. Normal mice served as control group, whereas animals induced with LAD were administered AAV-sh-Pvt1 or AAV-sh-NC. (A) Quantification of Pvt1 expression using RT-qPCR in cardiac tissues of mice driven by LAD. (B, C, D) evaluation of the EF (B), FS(C) of mice following Pvt1 interference via echocardiography(D). (E) structure of myocardium of mice observed via HE staining. (F) Measurement of Bax, Bcl-2, and Caspase-3 expression in cardiac tissues in mice treated with Pvt1 interference. *$p < 0.05$ MI *vs.* Control; $^{\#}p < 0.05$ MI + sh-NC *vs.* MI + sh-Pvt1.

To verify whether lncRNA Pvt1 is involved in cardiomyocyte apoptosis by interacting with miR-216, we first performed RT-qPCR, and the results demonstrated a decrease in the expression of miR-216 in the group treated with the miR-216 inhibitor, as compared to the muR-NC group (Fig. 4C). Subsequently, apoptosis-related protein expression was detected, and Bax and Caspase-3 protein levels were increased and Bcl-2 level was inhibited after co-transfection of miR-216 inhibitor in Pvt1-inhibited HL-1 cells under hypoxia treatment (Fig. 4D and E).

3.5. miR-216 is a downstream molecule of Pvt1 and regulates Ccnd3

An investigation into the precise regulation mechanism of Pvt1 was conducted by identifying the downstream miRNAs of Pvt1 in mice using the starBase database. Furthermore, the target genes of the mi-216 were inferred using the miRWalk database. Concurrently, differentially expressed mRNAs involved in the regulation of the hypoxic state were identified by RNA-sequencing. Initially, The cells were divided into two groups: the hypoxia + si-NC group and the hypoxia + si-Pvt1 group. Subsequently, we conducted transcriptome analysis on the cell samples from each group. Using a criteria of $|\log FC| > 1$ and an adjusted p-value <.05, we conducted

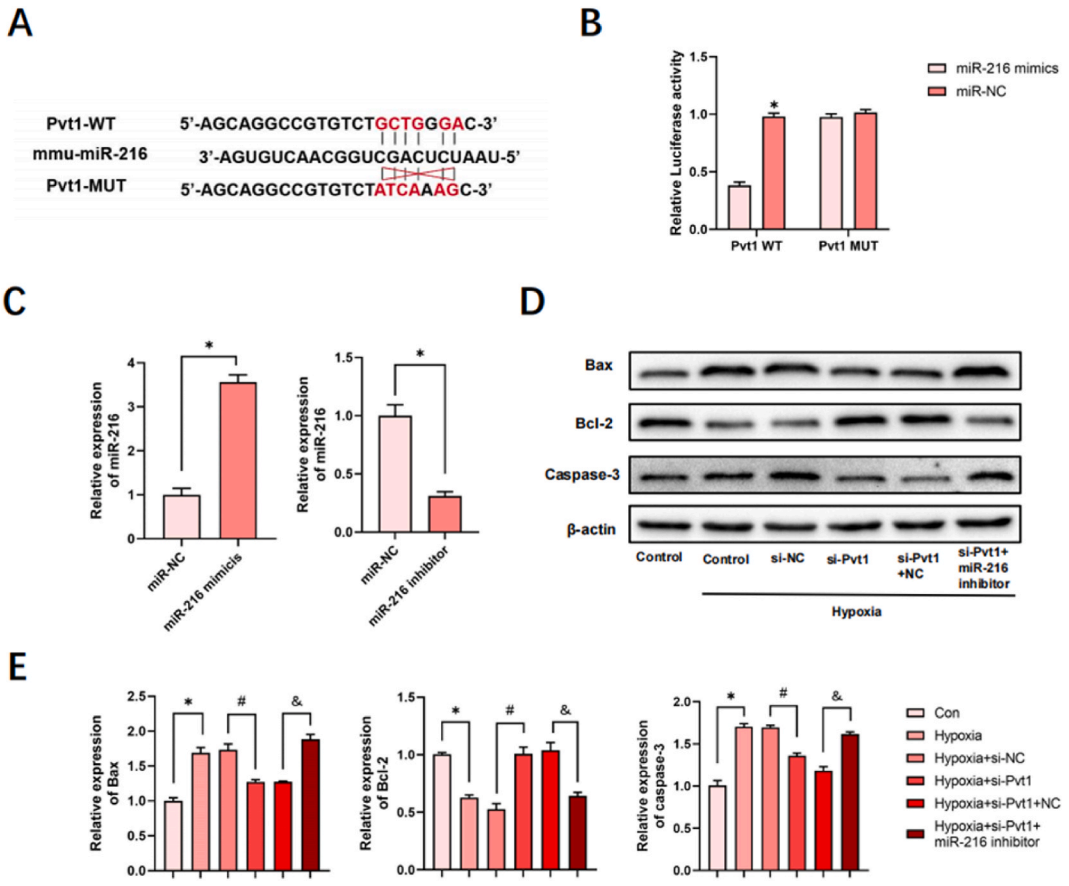

Fig. 4. miR-216 interacts with Pvt1 to regulate apoptosis.
(A) Binding sites between Pvt1 and miR-216. (B) Luciferase activities of both Pvt1-WT and Pvt1-MUT were observed in HEK 293T cells following the increase of miR-216 levels. (C) The RT-qPCR method was used to quantify the upregulation and downregulation efficiency of miR-216. (D) After transfection with siPvt1+NC and siPvt1+miR-216 inhibitors, apoptosis-related protein levels were quantified in HL-1 cells that were depleted of Pvt1 and exposed to 24 h of hypoxia using Western blot analysis. *$p < 0.05$ miR-216 mimics, miR-216 inhibitor vs. miR-NC; Hypoxia vs. control; Hypoxia *vs.* control; $^{\#}p < 0.05$ Hypoxia + si-NC *vs.* Hypoxia + si-Pvt1; &$p < 0.05$ Hypoxia + si-Pvt1+miR-216 inhibitor *vs.* Hypoxia + si-Pvt1+miR-NC.

differential analysis and identified 447 genes that were substantially downregulated in si-Pvt1 samples (Fig. 5A). Subsequently, the downregulated genes were compared with the target genes of miR-216 described earlier (Fig. 5B). The resulting candidate genes were reviewed to identify genes of interest. Analyzed quantitatively, the expression of Ccnd3 was markedly upregulated in the hypoxia + siPvt1 group. (Fig. 5C). Importantly, Ccnd3 was shown to be highly expressed. Hence, Ccnd3 was chosen as the focus of the study. In conclusion, Pvt1 may interact with miR-216 to regulate Ccnd3 in cells experiencing hypoxia.

To further explore the downstream mRNA mechanism, we overexpressed Ccnd3(pcDNA3.1-Ccnd3) after transfection of miR-216 mimics, and detected the expression of apoptotic genes by Western blot. Our study revealed that increasing the expression of miR-216 might enhance the expression of Bcl-2. Nevertheless, the production of Bcl-2 in apoptosis was restored by the upregulation of Ccnd3. However, Bax and caspase-3, which were suppressed by 216 mimics, were increased as a result of Ccnd3 overexpression (Fig. 5D and E). In conclusion, Pvt1 may bind to miR-216 to manipulate Ccnd3 in hypoxic cells.

4. Discussion

Increasing data suggests that people with congenital heart disease (CHD) are often exposed to stress due to decreased oxygen supply and increased burden from various cardiac abnormalities, leading to high rates of adverse health outcomes and death [17]. Chronic hypoxia is prevalent in individuals with coronary heart disease and has a role in the development of coronary heart disease in a multifaceted manner [18].Hypoxia is a pathological process that causes aberrant changes in morphological and functional structure by depriving tissues and cells of sufficient oxygen supply through the oxygen barrier. The myocardium is particularly susceptible to hypoxic environments [19]. Studies have shown that hypoxia induces cardiomyocyte injury by increasing apoptosis, so it is of great significance to study the endogenous cytoprotective mechanism of cardiomyocytes against hypoxic injury [20].We observed a substantial increase in the expression of Pvt1 in hypoxia-induced HL-1 cells and LAD-induced MI mice.

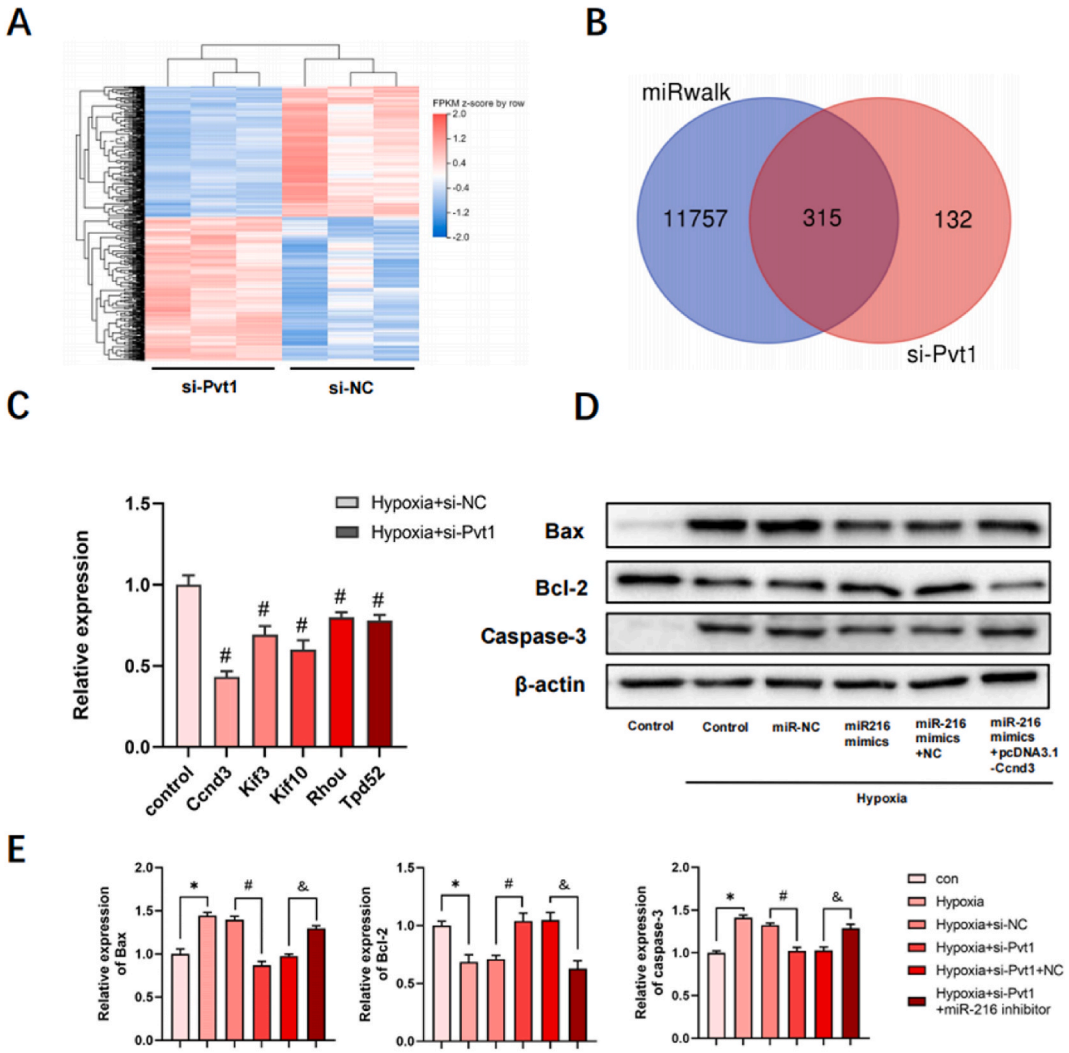

Fig. 5. Ccnd3 and miR-216 synergistically regulates apoptosis *in vivo*. The mRNAs that were differentially expressed were screened using RNA-sequencing. (A) Screening of differentially expressed mRNAs was accomplished through RNA-sequencing. between Hypoxia + si-NC group and Hypoxia + si-Pvt1 group. (B)Intersection of the downregulated genes identified in the RNA-sequencing and the downstream genes of miRNA predicted using the miRWalk database. (C) Identification of the most differentially expressed long non-coding RNAs using qRT-PCR. (D) Protein levels associated to apoptosis were quantified in HL-1 cells depleted of Pvt1 following the co-transfection of miR-216 inhibitor and pcDNA3.1-Ccnd3 in cells exposed to 24 h of hypoxia by Western blot analysis. $^{*}p < 0.05$ Hypoxia *vs.* control; $^{\#}p < 0.05$ Hypoxia + si-Pvt1 *vs.* Hypoxia + si-NC; & $p < 0.05$ Hypoxia + miR-216 mimics + NC *vs.* Hypoxia + miR-216 mimics + pcDNA3.1 Ccnd3.

Further investigation has conclusively shown the regulatory function of long non-coding RNAs (lncRNAs) in the progression and progression of several cardiovascular disorders [21]. Previous studies have demonstrated that long non-coding RNA (lncRNA) NEAT1 is upregulated in myocardial tissue affected by ischemia/reperfusion. Conversely, reducing lncRNA NEAT1 has a potential protective effect against damage to cardiomyocytes induced by hypoxia/reoxia [22]. LncRNA ANRIL has been definitively demonstrated to enhance the survival of hypoxia-induced H9c2 cells and provide protection against hypoxia-induced H9c2 cell damage during acute myocardial infarction [23]. Preserving H9c2 cell viability, decreasing cell apoptosis, and blocking cytochrome *c* release are mechanisms by which LncRNA ROR mitigates hypoxia-induced damage to H9c2 cells [24].

Pvt1 is a cancer-linked gene situated on chromosome 8q24, a widely recognized area associated with the risk of cancer. Recently, a growing body of research has focused on investigating the involvement of Pvt1 in a broader spectrum of human diseases [25]. Li et al. elucidated that the interaction between the long non-coding RNA Pvt1 and 4EBP1 enhances the development of cutaneous squamous cell carcinoma [26]. Tong et al. revealed an upregulation of Pvt1 expression in H9c2 cells treated with ischemia/reperfusion (H/R), and Pvt1 knockdown could improve myocardial ischemia-reperfusion injury by inhibiting Gasdermin D-mediated pyroptosis of cardiomyocytes [27]. Our study consistently provides evidence that hypoxia leads to upregulation of Pvt1 in cardiomyocytes. Functional experiments provided additional evidence that the knockdown of Pvt1 greatly reduced the apoptosis triggered by hypoxia in HL-1

cells. These results suggest that the upregulation of Pvt1 expression induced by hypoxia could function as a defense mechanism against hypoxic injury in cardiomyocytes. Furthermore, we showed that decreasing the expression of Pvt1 greatly enhanced cardiac function and decreased cardiomyocyte death following myocardial infarction in mice.

There is a considerable amount of data suggesting that a considerable proportion of long non-coding RNAs (lncRNAs) have a role in controlling the progression of cardiovascular illnesses by mean of the ceRNA mechanism. Certain long non-coding RNAs (lncRNAs) have the ability to operate as ceRNAs by binding to miRNAs through sponging-like mechanisms, therefore controlling their production and activity [28,29] Competition binding of certain miRNAs by CeRNA is regarded as a significant way by which lncRNAs control biological activity [30]. Accumulating data suggests that lncRNAs are crucial in cardiovascular disorders, such as organ damage and fibrosis, by functioning as ceRNAs that attach to miRNAs [31]. Serum extracellular vesicles containing lncRNA MIAT specifically interact with miR-485–5p by a sponge-like mechanism, leading to atrial damage, inflammation, fibrosis, and facilitating atrial remodeling and atrial fibrillation [32]. LncRNA up-regulation DCRF enhances the rate of cardiac fibrosis, damage, and cardiomyocyte autophagy by serving as an intermediary between ceRNA and miR-551b-5p signaling pathways [33].

To investigate the molecular mechanism by which Pvt1 knockdown impact hypoxia-treated HL-1 cells, we confirmed that Pvt1 contains a potential binding site for miR-216 using Starbase database. Subsequent luciferase reporter assay demonstrated that The direct interaction between Pvt1 and miR-216 resulted in the negative regulation of miR-216 expression in HL-1 cells. Similarly, Previous studies have demonstrated that Pvt1 functions as ceRNA for miR-140–5p in lung cancer during chemoresistance produced by hypoxia [34]. Consistent with the findings of other investigations, we have shown that the expression of miR-216 was reduced following exposure of HL-1 cells to hypoxia. Moreover, the suppression of miR-216 nullified the impact of Pvt1 suppression on apoptosis triggered by hypoxia in HL-1 cells. These findings indicate that suppressing Pvt1 downregulates the damage to HL-116 cells caused by hypoxia via increasing the expression of miR-216. Interestingly, a recent paper showed that miRNA-216 specifically targets PTEN in HK-2 cells, and inhibiting PTEN leads to the prevention of apoptosis triggered by H/R [35].

The mechanism of miR-216 was investigated by target gene prediction, and the results showed that Ccnd3 was a potential target gene of miR-216. Pathological cardiomyocyte hypertrophy arises from an elevated demand on the heart caused by conditions such as hemodynamic stress or myocardial illness [36]. Excessive pressure on the heart, without relief, can result in hypertrophy compensating and ultimately cause systolic failure. The hypertrophic heart has several anomalies that may contribute to decompensation, ultimately resulting in heart failure. Cardiac hypertrophy is often linked to an upregulation of embryonic gene expression and a concomitant downregulation of adult gene expression [37,38]. D-type cyclins are enhanced in cardiac hypertrophy [39–42], implicating them as potential targets for therapeutic strategies during pathophysiological processes. The onset of pressure overload-induced left ventricular hypertrophy was accompanied by a transient upregulation of protein levels of cyclin D2, cyclin D3, CDK4, and CDK6 in left ventricular (LV) tissues and isolated cardiomyocytes, as demonstrated by expression analysis [40]. In our study, after co-transfection of miR-216 mimics and pcDNA3.1-Ccnd3, Increased production of Bax and caspase-3, which were suppressed by 216 mimics, was observed following overexpression of Ccnd3, suggesting that Pvt1 is involved in hypoxia-induced injury by mediating the miR-216/Ccnd3 axis.

Taken together, our study demonstrated that Pvt1 binds to miR-216 to attenuate its inhibitory effect on its target Ccnd3, thereby promoting cardiomyocyte apoptosis. Our study provides new promising targets and therapeutic directions for the treatment of myocardial infarction and other hypoxic heart diseases.

Ethics declaration

The animal experimental research procedures were carried out in rigorous adherence to ethical standards and obtained prior authorization from the Ethics Committee of Chongqing Medical University (IACUC-CQMU-2023-0082). All experimental protocols were carried out following the guidelines outlined in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Data availability statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.Original data was available as supplementary file.

CRediT authorship contribution statement

Yu Hu: Writing – original draft, Visualization, Validation, Software, Methodology, Data curation, Conceptualization. **Minghao Luo:** Writing – review & editing, Resources, Formal analysis. **Yuzhou Xue:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Dingyi Lv:** Supervision, Resources, Formal analysis, Conceptualization. **Longxiang Huang:** Writing – review & editing, Methodology, Conceptualization. **Xiang Li:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Jian Shen:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38261.

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