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LncRNA PEG10 aggravates cardiac hypertrophy through regulating HOXA9

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Abstract. – OBJECTIVE: To uncover the role of long non-coding RNA (lncRNA) PEG10 in the progression of cardiac hypertrophy by regulating HOXA9.

MATERIALS AND METHODS: *In vivo* cardiac hypertrophy model was established by performing transverse aortic constriction model (TAC) procedures in mice. Relative levels of PEG10, ANP and BNP in mice undergoing TAC procedures or sham operations were determined. *In vitro* cardiac hypertrophy model was established by phenylephrine (PE) treatment in primary cardiomyocytes. Relative levels of PEG10, ANP and BNP in cardiomyocytes were determined as well. Regulatory effects of HOXA9 on surface area of cardiomyocytes and relative levels of ANP and BNP were assessed. Finally, potential influences of PEG10/HOXA9 regulatory loop on cell surface area and relative levels of ANP and BNP were explored.

RESULTS: Compared with mice in sham group, those in TAC group presented higher levels of PEG10, ANP and BNP. PE treatment markedly upregulated PEG10, ANP and BNP in primary cardiomyocytes, which were downregulated by transfection of si-PEG10. Besides, surface area of cardiomyocytes was enlarged by PE treatment, which was reduced after silence of PEG10. Silence of HOXA9 presented a similar effect as that of PEG10 in cardiomyocytes. Transfection of si-HOXA9 reversed the expanded cell surface area, and upregulated ANP and BNP in cardiomyocytes overexpressing PEG10.

CONCLUSIONS: PEG10 is upregulated in hypertrophic cardiomyocytes. PEG10 aggravates cardiac hypertrophy by positively regulating HOXA9.

Key Words:
PEG10, HOXA9, Cardiac hypertrophy, ANP, BNP.

Introduction

Cardiac hypertrophy is the adaptive response to maintain the normal cardiac function at the early stage of stress stimuli. However, persistent cardiac hypertrophy is accompanied by poor cardiac remodeling, leading to increased risks of heart failure and even death[1-3]. It is generally considered that peptide hormones, growth factors and non-coding RNAs may be regulators in the progression of cardiac hypertrophy[4]. The underlying molecular mechanisms of cardiac hypertrophy are still required to be fully explored.

LncRNAs are RNA transcripts with over 200 nucleotides long and they could not encode proteins[5]. By binding to proteins, lncRNA-protein complex could regulate gene expressions at post-transcriptional level as a ceRNA[6-8]. Accumulating evidence shown differentially expressed lncRNAs in different tissues. They exert crucial functions in cellular metabolism, apoptosis, differentiation etc.[9,10]. In recent years, lncRNAs are reported to participate in the occurrence and progression of cardiovascular diseases[11-13]. LncRNA PEG10 is a well-concerned lncRNA involved in disease progression. However, its specific function in cardiac hypertrophy remains unclear.

In this study we established *in vivo* and *in vitro* cardiac hypertrophy models by performing TAC in mice and phenylephrine (PE) treatment in cardiomyocytes, respectively. We aim to clarify the role of PEG10 in the progression of cardiac hypertrophy and the underlying mechanism.

Materials and Methods

Transverse Aortic Constriction Model (TAC) in Mice

This study was approved by the Animal Ethics Committee of Sun Yat-Sen University Animal Center. 8-week-old C57BL6 mice were intraperitoneally injected with 100 mg/kg ketamine and 5 mg/kg xylazine for anesthesia. After trachea cannula, the second rib on the left side of the thoracic

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cavity was cut by a surgical scissor, and both thymuses were push aside to expose the ascending aortic arch. A 27G needle was punctured into the ascending aorta alongside with its natural growth direction. After ligation of the ascending aorta using 5-0 suture, the needle was gently pulled out. The narrowing degree of mouse ascending aorta was about 75%. Iodophor disinfection on skin and intraperitoneal administration of penicillin were performed. Mice in sham group underwent anesthesia and exposure of the ascending aortic arch without puncture and ligation.

Isolation of Primary Cardiomyocytes

Mice were anesthetized with 75% ethanol and cut open for harvesting the heart, which was placed in D-Hanks solution. Atria were discarded, and ventricle was harvested, washed with D-Hanks for three times and cut into small pieces. Ventricular mixture was digested at 37°C for 5 min. The precipitant was digested again at 37°C for 20 min (shaken every 2 min). The mixture was centrifuged at 1000 rpm for 5 min. Subsequently, the precipitant was suspended in 2 mL of D-Hanks and centrifuged again at 1500 rpm for 10 min. The precipitant was suspended in 2 ml of medium for preparing the suspension. Incompletely digested ventricular fragments were digested and suspended in the same way. Finally, pooled suspension was cultured in a 5% CO_2 at 37°C.

Cell Treatment

Until 60% confluence, primary cardiomyocytes were treated with 100 μM phenylephrine (PE) for 36 h to induce *in vitro* cardiac hypertrophy model.

Cardiomyocytes were cultured until 60% of confluence and subjected to transfection with si-PEG10, si-NC or si-HOXA9 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 6 hours later, complete medium was replaced. Transfected cells for 24-48 h were harvested for the following experiments.

Quantitative RT-PCR

Extraction of total RNA in cells or tissues was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), qualified by an ultraviolet spectrophotometer and subjected to reverse transcription. The extracted complementary deoxyribose nucleic acid (cDNA) was applied for PCR using SYBR Green method (TaKaRa, Otsu, Shiga, Japan). Primer sequences were listed in Table I.

Immunofluorescence Staining

Cardiomyocytes were washed with phosphate-buffered saline (PBS) twice, fixed in 4% paraformaldehyde for 20 min and washed with PBS for three times. Subsequently, cells were blocked in 10% goat serum and 1% bovine serum albumin (BSA), and incubated with mouse monoclonal α-actin at 4°C overnight. After PBS wash, cells were incubated with the secondary antibody for 1 h. 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) was applied for nucleus staining. Finally, cardiomyocyte surface area was observed under a microscope and calculated.

Western Blot

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein sample was loaded for electrophoresis and transferred on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% skim milk for 2 hours, and subjected to incubation with primary and secondary antibodies. Bands were exposed by electrochemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Intergroup differences were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Table I. Primer sequences.

Gene	Primer sequences
PEG10	F: 5'-CATCCTTCCTGTCTTCGC-3' R: 5'-CCCTCTTCCACTCCTTCTTT-3'
HOXA9	F: 5'-GTGGTTCTCCTCCAGTTGATAG-3' R: 5'-AGTTGGCTGCTGGG TTATT-3'
ANP	F: 5'-CTCCGATAGATCTGCCCTCTTGAA-3' R: 5'-GGTACCGGAAGCTGTTGCAGCCTA-3'
BNP	F: 5'-GCTCTTGAAGGACCAAGGCCTCAC-3' R: 5'-GATCCGATCCGGTCT¬ATCTTGTGC-3'
GAPDH	F: 5'-CGGAGTCAACGGATTTGGTCGT-3' R: 5'-GGGAAGGATCTGTCTCTGACC-3'

Results

PEG10 was Upregulated in Mice with Cardiac Hypertrophy

We first established *in vivo* model of cardiac hypertrophy in mice by performing TAC. Primary cardiomyocytes were isolated from mice in TAC group and sham group. It is found that PEG10 was upregulated in cardiomyocytes isolated from mice in TAC group relative to controls (Figure 1A). Meanwhile, relative levels of ANP and BNP were found to be upregulated in TAC group as well (Figure 1B). It is indicated that PEG10 may be related to cardiac hypertrophy.

Knockdown of PEG10 Alleviated Hypertrophy of Cardiomyocytes

To further clarify the function of PEG10 in cardiac hypertrophy, primary cardiomyocytes were treated with 100 μM PE for 36 h to induce *in vitro* cardiac hypertrophy model. Transfection of si-PEG10 markedly downregulated PEG10 level in PE-treated cardiomyocytes (Figure 2A). PE treatment markedly enlarged the surface area of cardiomyocytes, which was reduced after transfection of si-PEG10 (Figure 2B). Both protein and mRNA levels of ANP and BNP were elevated by PE treatment, while they were downregulated by silence of PEG10 (Figure 2C, 2D). It is suggested that silence of PEG10 alleviated cardiac hypertrophy.

Knockdown of HOXA9 Alleviated Hypertrophy of Cardiomyocytes

In PE-treated cardiomyocytes, HOXA9 level was remarkably enhanced. Transfection of si-HOXA9 could sufficiently decreased HOXA9 level, showing an effective transfection efficacy (Figure 3A). The enlarged cell surface area of cardiomyocytes following PE treatment was reduced by transfection of si-HOXA9 (Figure 3B). Moreover, silence of HOXA9 could downregulate PE-induced upregulation of ANP and BNP at both protein and mRNA levels (Figure 3C, 3D). Collectively, HOXA9 exerted a similar function as that of PEG10 in cardiac hypertrophy.

PEG10 Stimulated Cardiac Hypertrophy Through Positively Regulating HOXA9

To further uncover the role of PEG10/HOXA9 regulatory loop in cardiac hypertrophy, a series of rescue experiments were conducted. Transfection of pcDNA-PEG10 aggravated PE-induced enlargement of cell surface area, which was partially reversed by co-transfection of si-HOXA9 (Figure 4A). Besides, relative levels of ANP and BNP were upregulated in PE-treated cardiomyocytes overexpressing PEG10, which were downregulated to some extent by silence of HOXA9 (Figure 4B).

Discussion

Cardiac hypertrophy is closely associated with a wide range of cardiovascular diseases, including

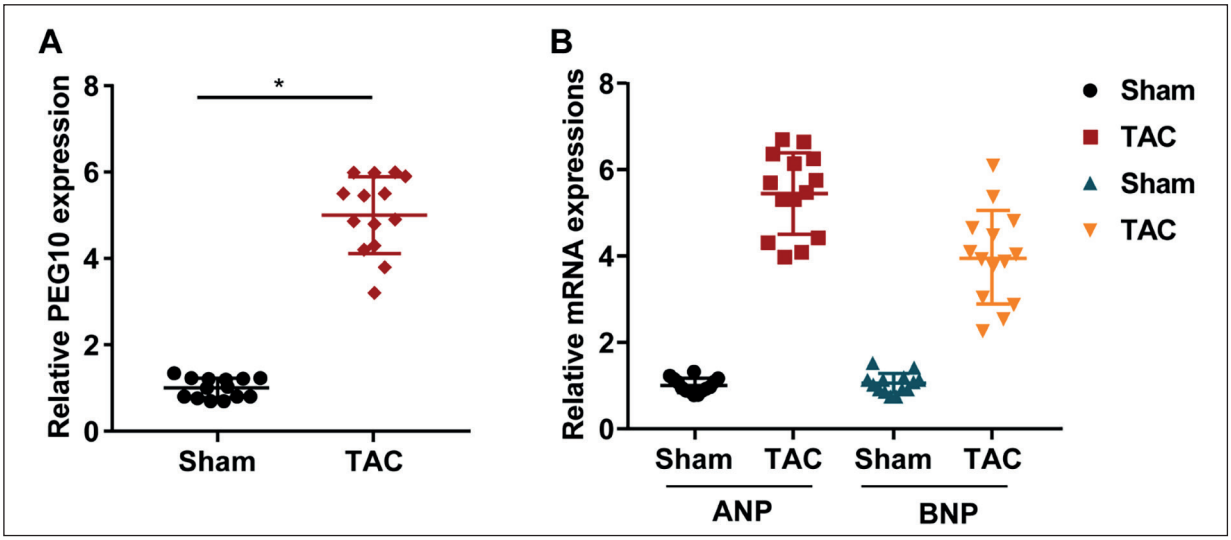

Figure 1. PEG10 was upregulated in mice with cardiac hypertrophy. ***A,*** Relative level of PEG10 in mice of sham group and TAC group. ***B,*** Relative levels of ANP and BNP in mice of sham group and TAC group.

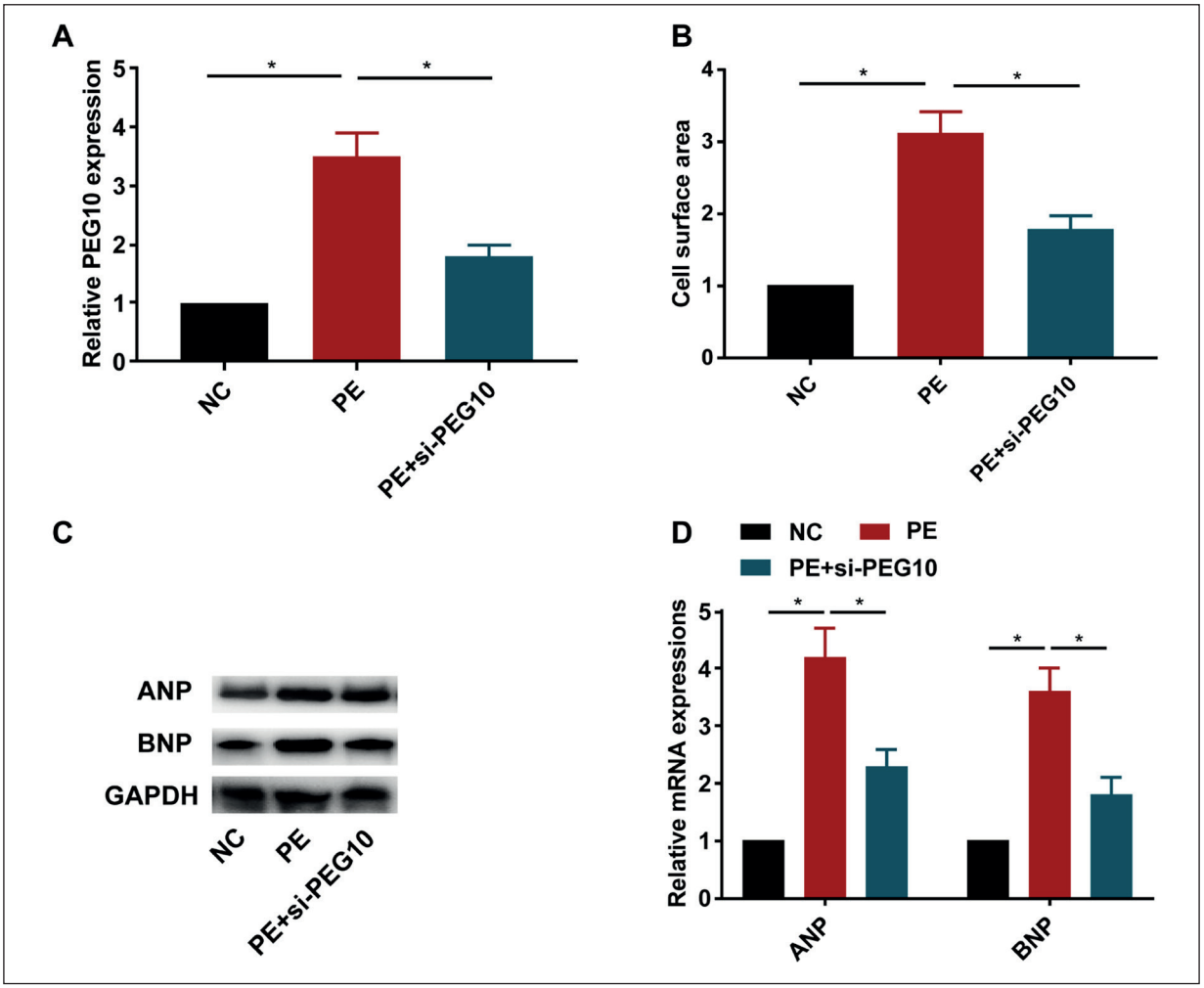

Figure 2. Knockdown of PEG10 alleviated hypertrophy of cardiomyocytes. ***A,*** Relative level of PEG10 in primary cardiomyocytes without any treatment, treated with 100 μM PE for 36 h and PE + si-PEG10. ***B,*** Cell surface area in primary cardiomyocytes without any treatment, treated with 100 μM PE for 36 h and PE + si-PEG10. ***C,*** Protein levels of ANP and BNP in primary cardiomyocytes without any treatment, treated with 100 μM PE for 36 h and PE + si-PEG10. ***D,*** The mRNA levels of ANP and BNP in primary cardiomyocytes without any treatment, treated with 100 μM PE for 36 h and PE + si-PEG10.

heart failure and sudden death[14]. Gene expression changes are the basis of pathological cardiomyocyte hypertrophy. In particular, high expressions of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) contribute to cardiomyocyte growth[15]. It is reported that non-coding RNAs are key factors in the pathogenesis of cardiac hypertrophy[16]. This study demonstrated that PEG10 was upregulated in hypertrophic cardiomyocytes both *in vivo* and *in vitro*. It is suggested that PEG10 exerts a vital role in cardiac hypertrophy.

Long non-coding RNA (lncRNA) paternally expressed 10 (PEG10) locates on human chromosome 7q21.3 and spans from 94,656,325 bp to 94,669,695 bp[17]. PEG10 is a gene normally expressed during placental development. A recent study[18,19] illustrated that the placental gene PEG10 promotes the proliferative ability of neuroendocrine prostate cancer. In this paper, silence of PEG10 reversed PE-induced reduction in the surface area of cardiomyocytes. Meanwhile, the knockdown of PEG10 downregulated heart failure markers ANP and BNP, indicating that PEG10 promoted cardiac hypertrophy.

HOX (Homebox) encodes homeodomain protein products that are transcriptional factors with a common protein fold structure. HOX is classified into four clusters, namely HOXA, HOXB, HOXC and HOXD[20]. HOX gene regulates and

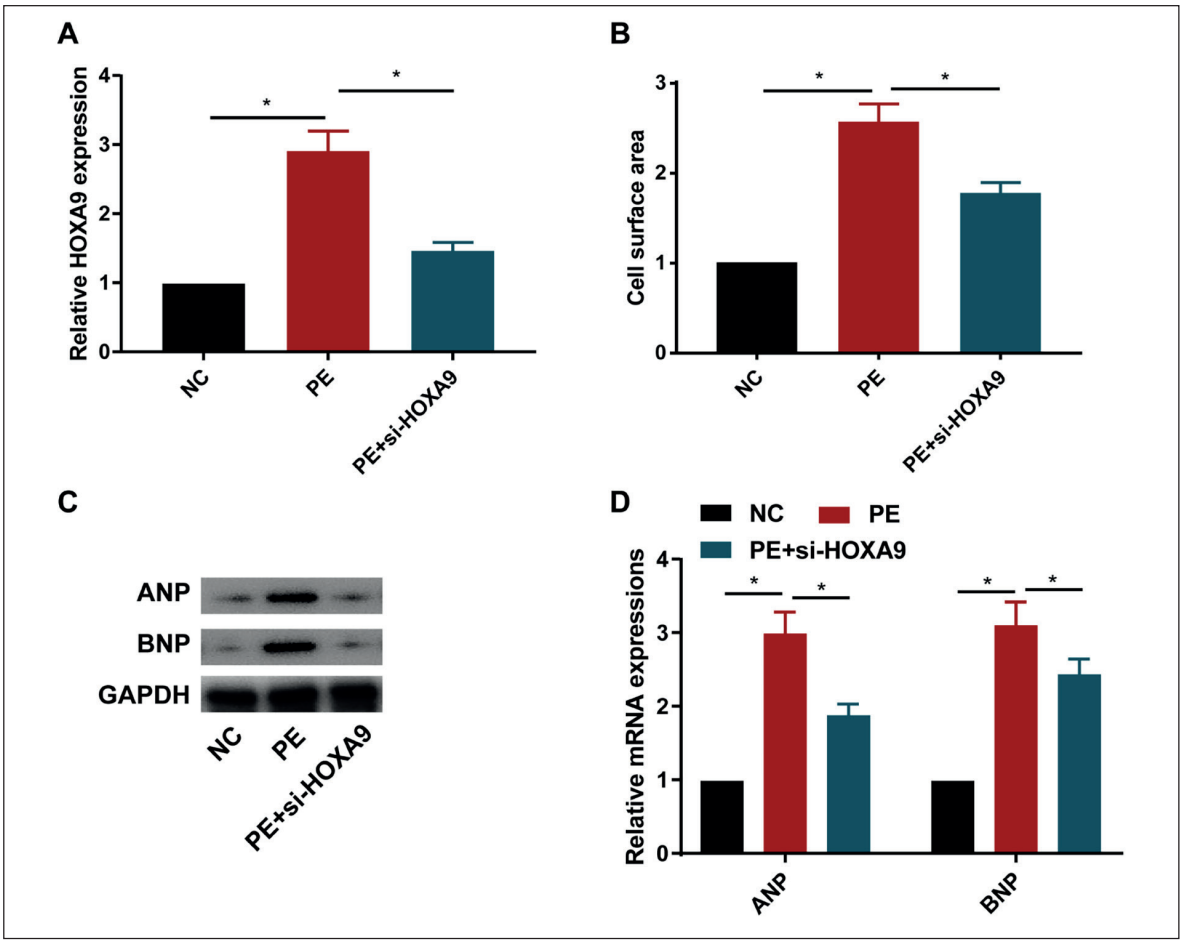

Figure 3. Knockdown of HOXA9 alleviated hypertrophy of cardiomyocytes. ***A,*** Relative level of HOXA9 in primary cardiomyocytes without any treatment, treated with 100 μM PE for 36 h and PE + si-HOXA9. ***B,*** Cell surface area in primary cardiomyocytes without any treatment, treated with 100 μM PE for 36 h and PE + si-HOXA9. ***C,*** Protein levels of ANP and BNP in primary cardiomyocytes without any treatment, treated with 100 μM PE for 36 h and PE + si-HOXA9. ***D,*** The mRNA levels of ANP and BNP in primary cardiomyocytes without any treatment, treated with 100 μM PE for 36 h and PE + si-HOXA9.

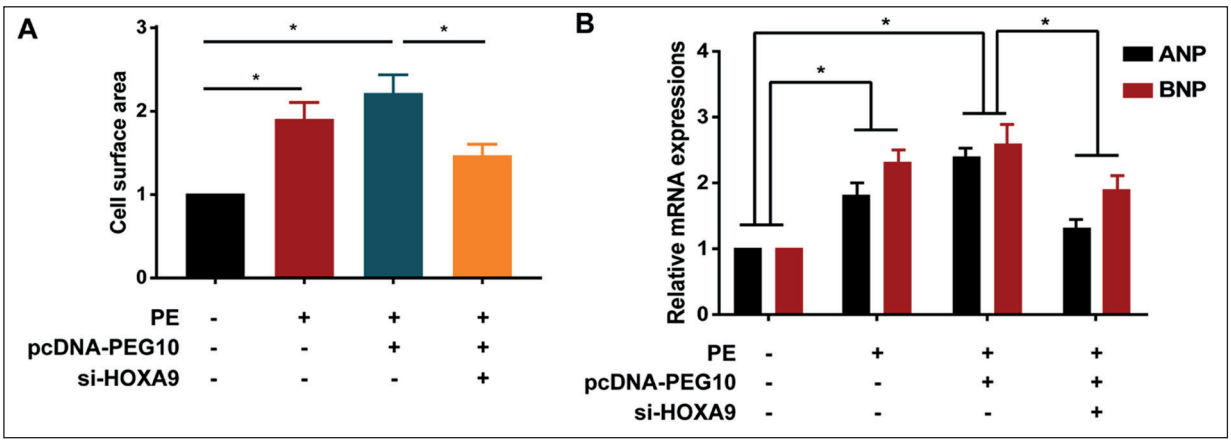

Figure 4. PEG10 stimulated cardiac hypertrophy through positively regulating HOXA9. ***A,*** Cell surface area in primary cardiomyocytes without any treatment, treated with 100 μM PE for 36 h, PE + pcDNA-PEG10 and PE + si-HOXA9. ***B,*** Relative levels of ANP and BNP in primary cardiomyocytes without any treatment, treated with 100 μM PE for 36 h, PE + pcDNA-PEG10 and PE + si-HOXA9.

determines different types of cell differentiation during embryonic development[21,22]. The HOXA9 gene maps to the chromosome 7p15.2. Its aberrant expression is involved in the occurrence of many solid tumors and hematopoietic malignancies[23]. Our results illustrated that knockdown of HOXA9 could alleviate cardiac hypertrophy. Notably, PEG10 aggravated cardiac hypertrophy through positively regulating HOXA9 level.

Conclusions

PEG10 is upregulated in hypertrophic cardiomyocytes. PEG10 aggravates cardiac hypertrophy by positively regulating HOXA9. It is considered that PEG10 may be utilized as a drug target for clinical treatment of cardiac hypertrophy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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