## Title

Expression Pattern and Function of Cardiac Pigment Epithelium-derived Factor During Cardiac Development

**Running title**

PEDF and Cardiac Development

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## Abstract

**Objective:** This study aims to describe the expression profile and role of cardiac pigment epithelium-derived factor (PEDF) during cardiac development.

**Methods:** Gene datasets from the Gene Expression Omnibus (GEO) database were used to analyze the correlation between cardiac PEDF expression and heart disease; western blot, immunohistochemistry, histological staining and echocardiography were used to assess the expression pattern and function of PEDF during cardiac development.

**Results:** The analysis of the data set from GEO database showed that the expression of cardiac PEDF was correlated with the occurrence and development of various heart diseases. Western blot results of different tissues of mice at 30 days postnatally showed that the abundance of PEDF expression in the heart and aorta was higher than its expression in the liver. Immunohistochemical results showed that the expression of cardiac PEDF significantly decreased after birth, mainly due to the significant decrease of PEDF expression abundance in cytoplasm. The results of histological staining and echocardiography showed that PEDF deficiency had no significant effect on cardiac structure, cardiac function and vascular hemodynamics in eight-week-old mice.

**Conclusion:** Cardiac PEDF shows high expression and dynamic changes during cardiac development, but has no effect on cardiac structure, function and vascular hemodynamics.

## Key words

Expression pattern; Pigment epithelium-derived factor; Cardiac development.

## Significance Statement

This study describes the expression pattern and function of cardiac PEDF during cardiac development, laying the foundation for further studies on the effect of cardiac PEDF in acquired heart disease.

## Introduction

Cardiovascular diseases (CVDs), represented by hypertension, coronary atherosclerosis, heart failure (HF) and arrhythmia, etc., are characterized by high incidence and high mortality rates, and are the main factors leading to death in the global population[1, 2]. At present, a number of important genetic mutations have been shown to be associated with the development of congenital heart disease, and a large number of abnormally expressed proteins have been closely associated with the development of many acquired heart diseases[3-7]. It should be noted that many genes related to acquired heart disease may be involved in cardiac development at the same time, and the abnormal expression of its encoded protein may have a hidden impact on cardiac structure and function during the cardiac development process. Therefore, to clarify the spatiotemporal expression characteristics of proteins encoded by related genes in the process of cardiac development and their effects on cardiac structure and function during cardiac development will help us define their roles in acquired heart disease more clearly.

Pigment epithelium-derived factor (PEDF) is a secreted protein with a molecular weight of 50 kD that consists of 418 amino acids, which is located at 17p13.1, contains 8 exons and 7 introns[8-11]. Although PEDF is a member of the serine protease superfamily, it does not possess the protease inhibition function[8, 12]. The current study concluded that PEDF is widely distributed in a variety of tissues, especially highly expressed in liver and adipose tissue, but its expression abundance in cardiac tissues is still more controversial[13, 14]. In addition, as a multifunctional protein, PEDF has been shown to have powerful physiological functions such as inhibition of angiogenesis, neuroprotection, metabolic regulation, and antioxidation, etc., as well as its clear protective effects in CVDs such as myocardial infarction, ischemia-reperfusion, and atherosclerosis, etc[8, 15-22]. However, whether PEDF is involved in the cardiac developmental process remains unclear.

In the present study, based on the analysis of the correlation between cardiac PEDF and multiple heart diseases, we clarified that the abundance of cardiac and aorta PEDF expression is higher to that in the liver using various molecular biological tools and confirmed that cardiac PEDF expression significantly decreases after birth accompanied by altered cardiomyocyte sublocation characteristics. Further, we confirmed that PEDF deficiency had no significant effect on cardiac structure, cardiac function and vascular hemodynamics during cardiac development using histological staining and echocardiography.

## Methods

### GEO dataset analysis

Data from GSE760, GSE19210, GSE30428, GSE4105, GSE47495 and GSE775 date sets were used to analyze the correlation between cardiac PEDF expression and the occurrence and development of different heart diseases[23-27]. All samples in above datasets are from animal models, and only the sample data of disease model group and corresponding control group are used for further analysis. For specific sample information, please see Supplementary Table 1.

### Animals

C57BL/6J wild-type mice were purchased from Laboratory Animal Center of Sun Yat-sen University. PEDF knockout (C57BL/6J background) mice were kindly provided by Prof. Guoquan Gao (Zhongshan School of Medicine, Sun Yat-sen University) and Prof. Xia Yang (Zhongshan School of Medicine, Sun Yat-sen University).

Male and female mice at 30 days postnatal were used to analyze PEDF expression differences between different tissues. Fetal, suckling or adult mice at different developmental time points were used to analyze altered cardiac PEDF expression at different developmental times. 8-week-old PEDF knockout mice and wild-type littermate controls were used to assess the effects of PEDF deficiency during cardiac development on cardiac structure, cardiac function and vascular hemodynamics.

All animals were kept under 12-hour light-dark cycles at 18-21°C with free access to water and food. All animals were fasted for 12-16 hours before sampling and testing.

### Western blot

The tissues (3 samples per group) were lysed with RIPA buffer (Beyotime, Shanghai, China) for total protein extraction. The protein concentration was determined using the BCA protein assay kit (Millipore, Bedford, MA, USA) according to the manufacturer's protocol. Equal amounts of protein

were subjected to SDS-PAGE for electrophoresis, transferred to 0.45-μm PVDF membranes (Millipore, Bedford, MA, USA), and immunoblotted with antibody against PEDF (MAB1059, Millipore, Bedford, MA,

USA). The bands were quantified using the ImageJ software program (National Institutes of Health, Bethesda, MD, USA).

**Immunohistochemistry**

Heart samples were fixed in 4% paraformaldehyde overnight followed by conventional dehydration and slicing. Then, the liver samples sections (5 μm) were blocked with 3% hydrogen peroxide and then performed at 95°C for 10 min using citrate buffer (P0083, Beyotime, Shanghai, China), then blocking steps were carried out using the QuickBlock™ Blocking Buffer (P0260, Beyotime, Shanghai, China) according to the manufacturer's instructions. After incubated with primary antibody at 4℃ overnight, the sections incubated with secondary antibody (G1210, Servicebio, Wuhan, Hubei, China) at 37℃ for 30 min. Visualization was accomplished using 3,3N-diaminobenzidine tertrahydrochloride (G1211, Servicebio, Wuhan, Hubei, China). Sections were counterstained with hematoxylin (G1004, Servicebio, Wuhan, Hubei, China). Primary antibodies against PEDF (ab227295, Abcam, Cambridge, MA, USA) was used. The mean density was quantified from 3-4 individual mice and 4-6 fields per mouse with the Image-Pro Plus software program (Media Cybernetics, Bethesda, MD, USA).

**Histological staining**

Histological staining was performed with frozen sections. The heart tissues were fixed in 4% paraformaldehyde (pH 7.4) for 24 h, dehydrated with a sucrose gradient, embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan), and serially sectioned at 5 μm. Then, the standard hematoxylin & eosin (HE) staining and Masson's Trichrome staining were performed as described in previous studies[28]. Images were captured with Leica microscope (DFC700T, Leica, Germany).

**Echocardiography and vascular ultrasound**

Echocardiography and vascular ultrasound were performed on mice using a Vevo 2100 imaging system (Visual Sonics, Toronto, Canada) as described in previous studies[28]. In detail, the mice were placed in a closed box filled with 1.5% isoflurane. After being anesthetized, the mice were immediately placed on a 37 °C thermostat to maintain normal body temperature and maintained on 0.5% isoflurane to prevent them from waking up until the end of the imaging process. For the image acquisition process, a 30-MHz probe

was used. Ultrasound images of cardiac short-axis and the aorta diameter (Ao Diam) were captured in M-Mode. Ultrasound images of the mitral valve blood flow, the peak velocity in aortic valve (AV Peak Vel) and the peak velocity in descending aorta (Desc Ao Vel) were captured in PW Doppler-Mode. The heart rate of all mice was controlled at 450-550 bpm during imaging.

**Statistical analysis**

All analyses were performed with the Statistical Package for Social Sciences version 19.0 (SPSS, Chicago. IL, USA). The data were expressed as the mean ± SD. Statistical differences between two groups were analyzed by the unpaired Student's t test and differences between multiple groups of data were analyzed by one-way ANOVA. In all statistical comparisons, a $P$ value $< 0.05$ was used to indicate a statistically significant difference.

## Results

**Cardiac PEDF expression is associated with the occurrence and development of various acquired heart diseases**

In order to clarify the correlation between cardiac PEDF expression and heart diseases, we screened several heart gene expression datasets of rodent models of acquired heart disease from GEO database, and analyzed the correlation between cardiac PEDF expression and disease progression. The analysis of GSE760 data set showed that the expression of cardiac PEDF was significantly increased in the mouse model of dilated cardiomyopathy (DCM) compared with the control mice. (Fig.1A) The analysis results of GSE19210 dataset showed that the expression of cardiac PEDF in hypertensive rat with HF was significantly higher than that in hypertensive rat without HF. (Fig.1B) The analysis results of GSE30428 dataset showed that the expression of cardiac PEDF was significantly increased in the mouse model of right ventricular (RV) hypertrophy compared to sham operated mice. (Fig.1C) The analysis of GSE4105 data set showed that the expression of cardiac PEDF was significantly increased at 2 and 7 days after ischemia-reperfusion (I/R) compared to sham operated rat. (Fig.1D) The analysis results of GSE47495 dataset showed that, compared with sham operated rat, the expression of cardiac PEDF was significantly increased in rat with large area myocardial infarction (MI), but not significantly changed in mice with small

area and moderate area MI. (Fig.1E) The analysis results of GSE775 data set showed that, compared with sham operated mice, the expression of cardiac PEDF did not change significantly within 48 hours after MI, increased significantly one week after MI, but returned to normal 8 weeks after MI. (Fig.1F)

These results confirm that the expression of cardiac PEDF is associated with the progression of various acquired heart diseases.

**PEDF is highly expressed in the heart and aorta of postnatal mice**

To clarify the distribution of PEDF expression in each tissue, we selected male and female mice at 30 days after birth and examined the PEDF expression in several tissues including liver, mesentery, heart, lung, kidney, skeletal muscle, spleen and aorta, and analyzed the difference between the PEDF expression abundance in each tissue and the PEDF expression abundance in liver. The results showed that in male mice, PEDF expression in mesentery, lung and spleen was significantly lower than liver PEDF expression, PEDF expression in kidney and skeletal muscle was not significantly different from liver PEDF expression, while PEDF expression in heart and aorta was significantly higher than liver PEDF expression. (Fig.2A) In addition, similar expression profiles were found in female mice, with lung, skeletal muscle and spleen PEDF expression significantly lower than hepatic PEDF expression, mesenteric and kidney PEDF expression not significantly different from hepatic PEDF expression, and heart and aorta PEDF expression significantly higher than hepatic PEDF expression. (Fig.2B)

These results confirm that PEDF is highly expressed in the heart and aorta of postnatal mice, excluding the influence of sex.

**Cardiac PEDF expression decreased significantly after birth with changes in subcellular localization characteristics**

Further, to clarify the dynamic changes in cardiac PEDF expression during cardiac development, we selected cardiac tissues from different developmental times for immunohistochemical staining as a way to observe the changes in PEDF expression abundance and subcellular localization characteristics. The results showed that the expression of PEDF in mouse hearts at embryonic stage and postnatal 30 days was similar, and it was widely distributed in the cytoplasm. (Fig.3A, B) On the postnatal 30 days and the postnatal 60 days, the expression abundance of PEDF decreased significantly in the mouse heart, which was mainly

characterized by the decrease of the expression abundance in the cytoplasm. (Fig.3A, B)

These results confirm that cardiac PEDF expression is significantly reduced after birth and is predominantly characterized by a decrease in cytoplasmic PEDF abundance.

**PEDF deficiency during cardiac development has no significant effect on cardiac structure**

To further clarify the effect of PEDF deficiency on cardiac structure during cardiac development, we evaluated the cardiac structure changes using PEDF global knockout mice and wild-type littermate control. The results showed that the heart weight and heart weight/tibia length ratio of PEDF knockout mice did not change significantly compared with the wild-type littermate control mice. (Fig.4A, B) The HE staining results showed that there was no significant change in the heart structure of PEDF knockout mice compared with the wild-type littermate control mice. (Fig.4C) Masson's trichrome staining results also showed that the heart of PEDF knockout mice had no significant fibrosis changes compared with the wild-type littermate control mice. (Fig.4D)

These results confirm that PEDF deficiency during cardiac development has no significant effect on cardiac structure.

**PEDF deficiency during cardiac development has no significant effect on cardiac function**

Subsequently, we evaluated the effect of PEDF deficiency on cardiac function during cardiac development by echocardiography, both on cardiac diastolic function and on cardiac systolic function. The results showed that cardiac diastolic function was not significantly altered in PEDF knockout mice compared to wild-type littermate controls, as reflected by unchanged E/A peak ratio, isovolumetric contraction time (IVCT), and isovolumic relaxation time (IVRT). (Fig.5A-D) In addition, the ejection fraction (EF), fractional shortening (FS), left ventricular internal diameter (LVID) at the end of systole, left ventricular anterior wall (LVAW) and left ventricular posterior wall (LVPW) at the end of diastole and end of systole were not significantly altered in PEDF knockout mice compared to wild-type littermate controls, and only the LVID at the end of diastole was somewhat reduced. (Fig.5E-K) The result suggests that PEDF deficiency during cardiac development has a very weak effect on cardiac systolic function.

These results confirm that PEDF deficiency during cardiac development has no significant effect on cardiac function.

**PEDF deficiency during cardiac development has no significant effect on vascular hemodynamics**

Given that our results confirm the high expression profile of PEDF in the aorta, and that normal aortic development is closely associated with the development of heart disease, we further evaluated the effect of PEDF deficiency during cardiac development on aortic hemodynamics. Vascular ultrasound results showed no significant change in the end-diastolic Ao Diam and the end-systolic Ao diam in PEDF knockout mice compared to wild-type littermate controls. (Fig.6A-C) Further results also showed that the AV Peak Vel and the Desc Ao Peak Vel were also not significantly altered in PEDF knockout mice compared to wild-type littermates. (Fig.6D-G)

These results confirm that PEDF deficiency during cardiac development has no significant effect on vascular hemodynamics.

## Discussion

In this study, we clarified the expression profile and function of cardiac PEDF during cardiac development, and confirmed that cardiac PEDF expression, which is significantly decreased after birth, still presents high expression levels, but it is not involved in the developmental process of the heart; therefore, PEDF deficiency during cardiac development does not show significant effects on cardiac structure, cardiac function and vascular hemodynamics. The significance of this study is that it excludes the effect of cardiac PEDF on cardiac development, thus contributing more to the definition of the function of PEDF in acquired heart disease.

Previous studies have demonstrated that PEDF is an important cardioprotective factor that exhibits significant protective effects in a variety of diseases, including MI and I/R[18-20]. At the same time, our analysis of several published datasets showed that cardiac PEDF showed elevated expression changes in a variety of heart diseases. This suggests that cardiac PEDF may exhibit compensatory elevated expression in the early stages of various heart diseases, thereby resisting disease progression. In the analysis of the GSE775 dataset, cardiac PEDF expression was sharply elevated at 1 week after MI and largely normalized at 8 weeks after MI, reflecting the characteristic change of cardiac PEDF expression from compensatory elevation phase to decompensated decline phase and this change possibly showing a strong correlation with

the stage of disease progression.

Liver and adipose are known to be tissues with high PEDF expression, whereas the level of PEDF expression in circulatory system tissues including heart and aorta was previously unclear[13, 14]. Our analysis of PEDF expression in multiple tissues confirmed that PEDF expression levels were significantly higher in the heart and aorta than in the liver. This further provides foundational evidence for PEDF as an important cardioprotective factor that can help us understand why PEDF is so important in cardiovascular disease.

Our results describe changes in cardiac PEDF before and after birth in mice and confirm that cardiac PEDF expression decreases dramatically within 30 days of birth and is maintained until at least 60 days postnatally. The dynamic changes in cardiac PEDF expression suggest that although PEDF during cardiac development has no significant effect on cardiac structure and cardiac function, it may be involved in some specific physiological processes. The current study shows that there are some significant alterations in physiological activities in mice before and after birth, such as cardiac energy metabolism patterns and cardiomyocyte proliferation[29-32]. Whether cardiac PEDF expression is involved in the alteration of related physiological activities remains unclear and deserves further in-depth study.

It should be emphasized that a large number of previous studies have focused on the role and mechanism of PEDF in CVDs[33]. These studies have proved that PEDF has significant protective effects in many CVDs, especially in ischemic heart disease, through antioxidant, anti-inflammatory, anti-apoptosis and other ways.[33] In terms of mechanism, the known receptors of PEDF, including adipose triglyceride lipase (ATGL) and phospholipase A2 (PLA2), play a key role in the role of PEDF in protecting cardiovascular function.[33] However, unlike our study, previous studies focused on the role of PEDF in the pathological process, and after the heart is mature, while our study focused on the heart development process under physiological conditions, which is limited to the heart development stage, and does not involve changes after the heart is mature. Therefore, although our study confirms that PEDF deficiency during cardiac development has no significant effect on cardiac structure, cardiac function and vascular hemodynamics, this feature is mainly limited to the first 8 weeks of life in mice. Whether corresponding alterations occur again after a longer period of time remains unclear. Since PEDF is known to be an important regulator of lipid metabolism and a cardioprotective factor, and since cardiac metabolic patterns are significantly altered before and after birth, it remains to be investigated whether the role of PEDF in regulating metabolism in the heart will manifest itself after a longer period of time[18, 34, 35, 36].

## Conclusion

The expression abundance of cardiac PEDF decreased significantly after birth, accompanied by changes in subcellular localization characteristics, but it was still higher than the expression of liver PEDF. Nevertheless, cardiac PEDF has no effect on cardiac structure, cardiac function and vascular hemodynamics during cardiac development.

## Conflicts of interest

The authors declare no conflict of interest.

## CRediT authorship contribution statement

**Xing-hui Li**: Conceptualization, Investigation, Formal analysis, Writing original draft. **Yan-di Wu**: Investigation. **Tong-sheng Huang**: Investigation, Formal analysis. **Teng Wu**: Investigation, Formal analysis. **Xin-lu Fu**: Investigation, Formal analysis. **Jiang Qian**: Investigation. **Yan Zou**: Investigation. **Cong-hui Shen**: Investigation. **Shi-jie Xiong**: Investigation. **Zi-qi Feng**: Investigation. **Hui-ting Zheng**: Investigation. **Yuan-jun Ji**: Investigation. **Wei-bin Cai**: Conceptualization, Writing original draft.

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## Figure legends

**Figure 1. Correlation between cardiac PEDF expression and heart disease.** A. Cardiac PEDF expression between DCM and control mice based on GSE760 data set. B. Cardiac PEDF expression between hypertensive with HF and hypertensive without HF rat based on GSE19210 data set. C. Cardiac PEDF expression between RV hypertrophy and sham mice based on GSE30428 data set. D. Cardiac PEDF expression between I/R and sham rat based on GSE4105 data set. E. Cardiac PEDF expression in MI rat with different infarct size based on GSE47495 data set. F. Cardiac PEDF expression in MI mice with different time after infarct based on GSE775 data set. DCM, dilated cardiomyopathy; HF, heart failure; RV, right ventricular; I/R, ischemia-reperfusion; MI, myocardial infarction. Data are expressed as the mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

**Figure 2. Expression level of PEDF in different tissues.** A. PEDF expression levels in different tissues of male mice at postnatal 30 days. (n=3) B. PEDF expression levels in different tissues of female mice at postnatal 30 days. (n=3) Data are expressed as the mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

**Figure 3. Cardiac PEDF expression at different time points of cardiac development process.** A. Immunohistochemical staining results. B. Quantitative results of immunohistochemical staining. (n=3-4) Data are expressed as the mean ± SD. *, $p < 0.05$.

**Figure 4. Effect of PEDF deficiency on cardiac structure during cardiac development.** A. Heart weight. (n=5) B. Heart weight/ tibia length ratio. (n=5) C. HE staining. D. Masson's trichrome staining.

**Figure 5. Effect of PEDF deficiency on cardiac function during cardiac development.** A. Mitral valve rheography. B. E/A ratio. (n=4) C. IVCT. (n=4) D. IVRT. (n=4) E. Echocardiography images in short-axis. F. EF. (n=4) G. FS. (n=4) H. LVID at the end of diastole. (n=4) I. LVID at the end of systole. (n=4) J. LVAW. (n=4) K. LVPW. (n=4) IVCT, isovolumetric contraction time; IVRT, isovolumic relaxation time;

EF, ejection fraction; FS, fractional shortening; LVID, left ventricular internal diameter; LVAW, left ventricular anterior wall; LVPW, left ventricular posterior wall. Data are expressed as the mean ± SD. *, $p < 0.05$.

**Figure 6. Effect of PEDF deficiency on vascular hemodynamics during cardiac development.** A. Ultrasound images of Ao Diam measurement. B. Ao Diam at the end of diastole. (n=4) C. Ao Diam at the end of systole. (n=4) D. Ultrasound images of AV Peak Vel measurement. E. AV Peak Vel. (n=4) F. Ultrasound images of Desc Ao Peak Vel measurement. G. Desc Ao Peak Vel. (n=4) Ao Diam, aorta diameter; AV Peak Vel, peak velocity in aortic valve; Desc Ao Vel, peak velocity in descending aorta. Data are expressed as the mean ± SD.

https://doi.org/10.55415/deep-video-2023-0027.v1

## Figures

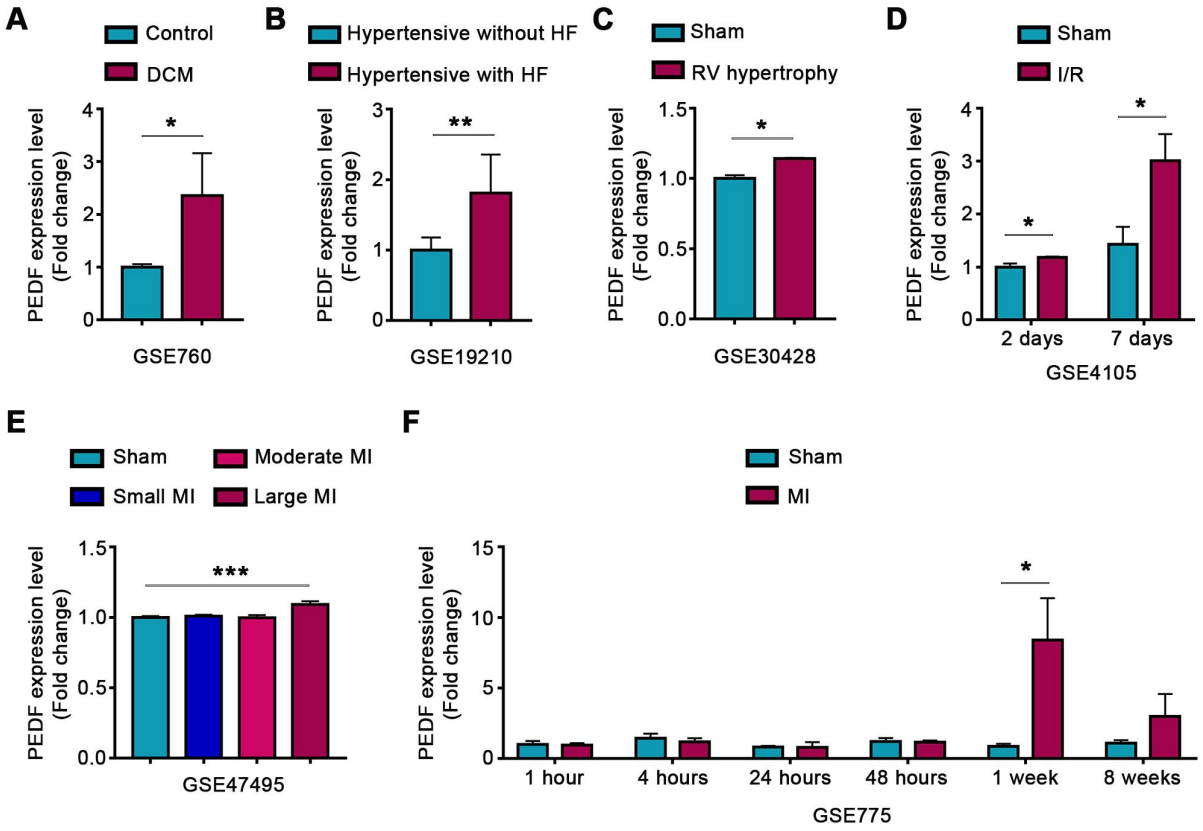


**Fig.1**

https://doi.org/10.55415/deep-video-2023-0027.v1

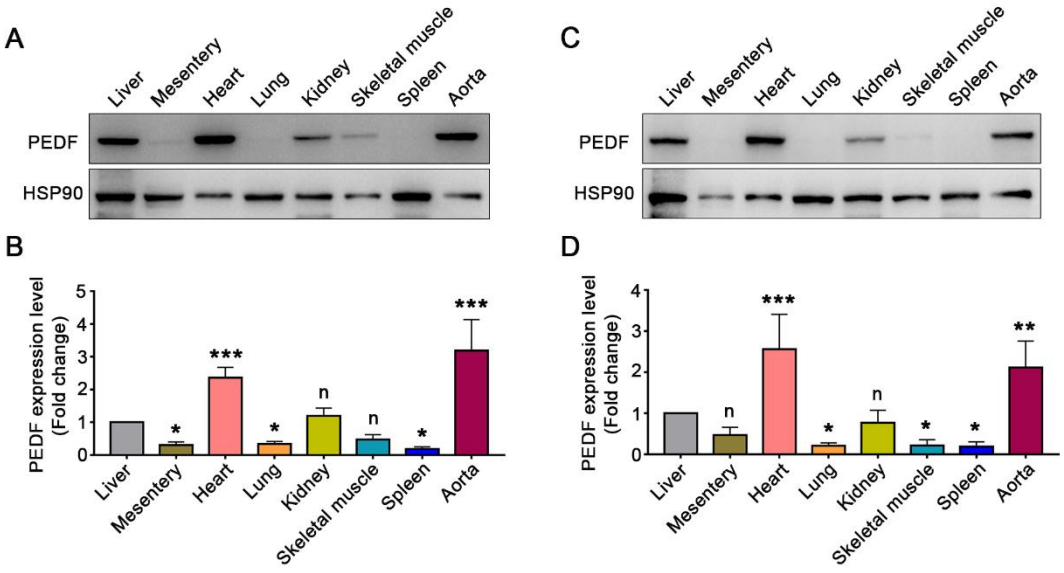


**Fig.2**

https://doi.org/10.55415/deep-video-2023-0027.v1

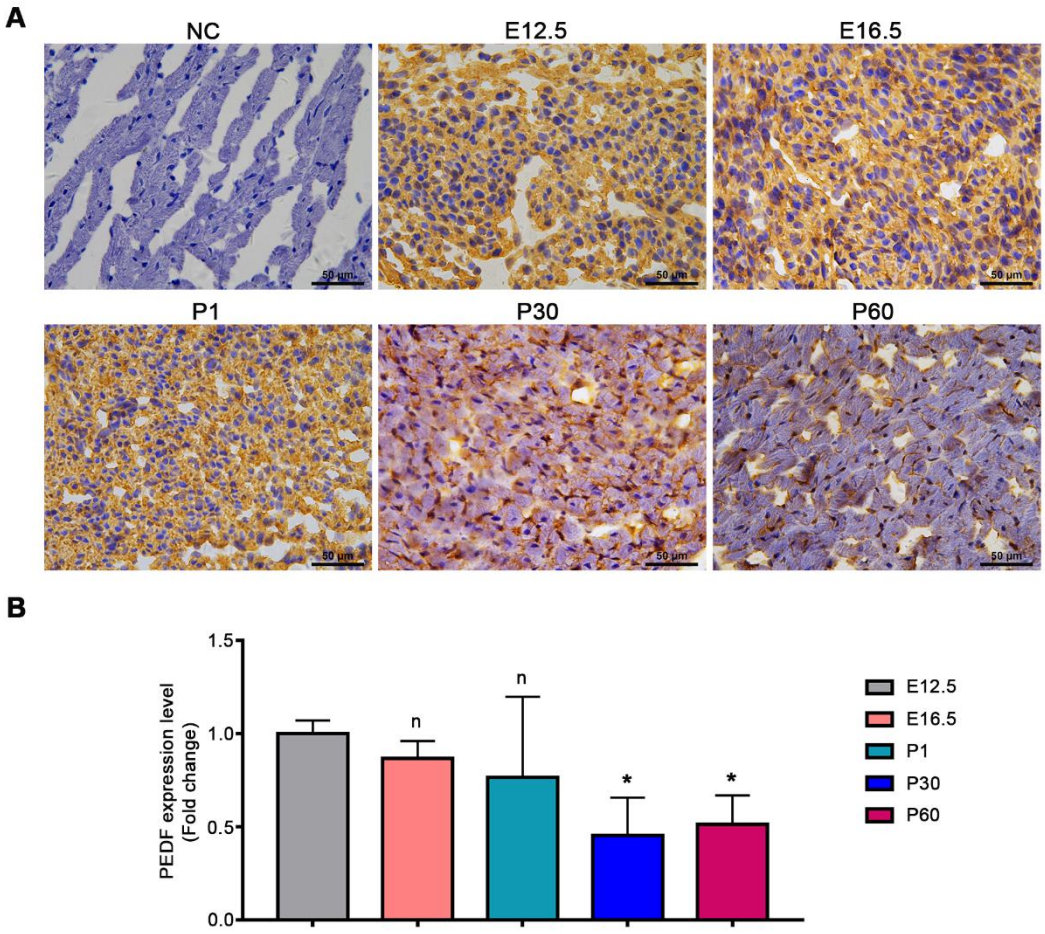


**Fig.3**

https://doi.org/10.55415/deep-video-2023-0027.v1

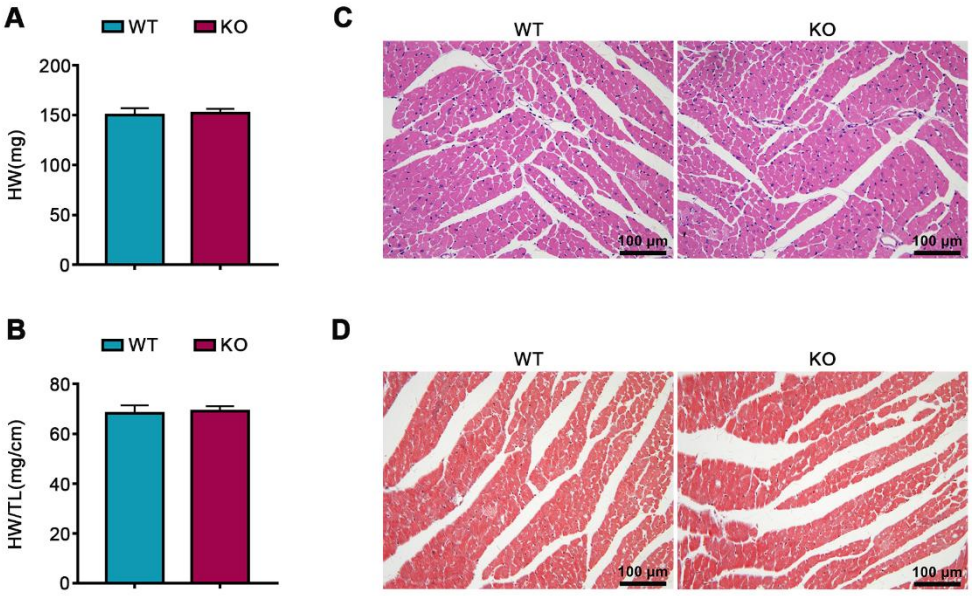


**Fig.4**

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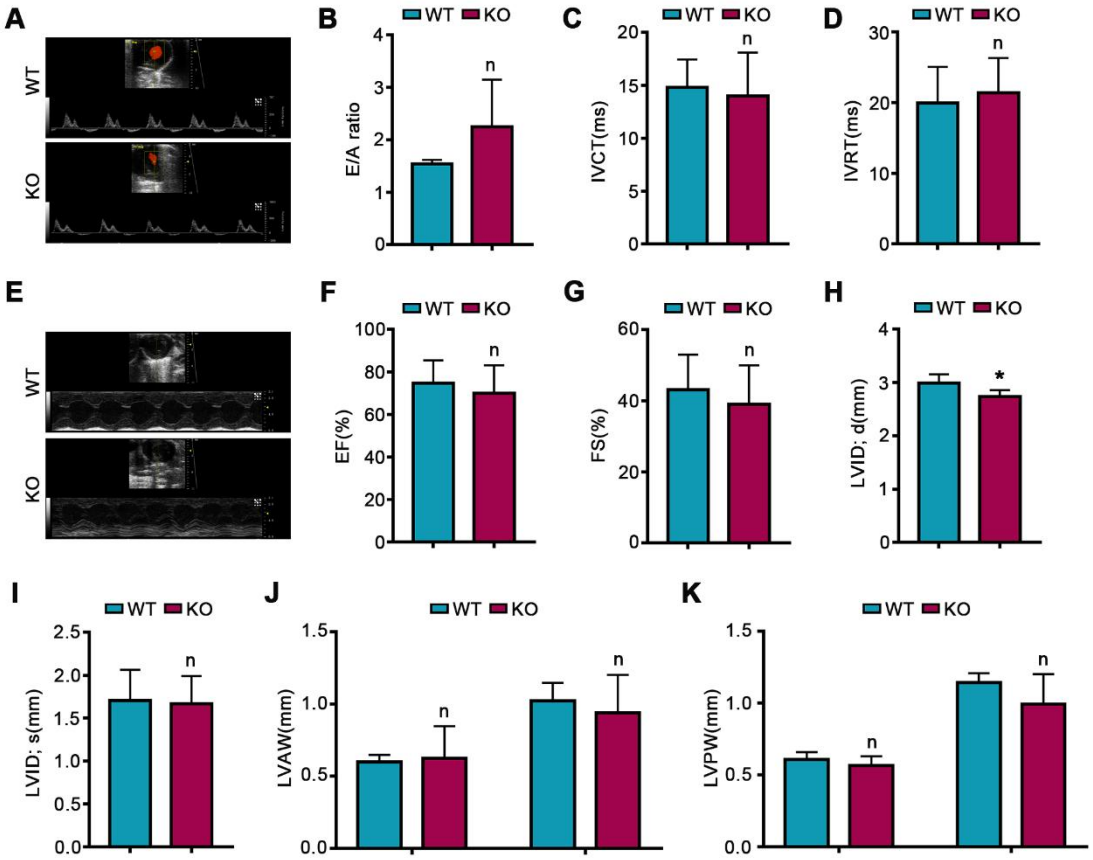


**Fig.5**

https://doi.org/10.55415/deep-video-2023-0027.v1

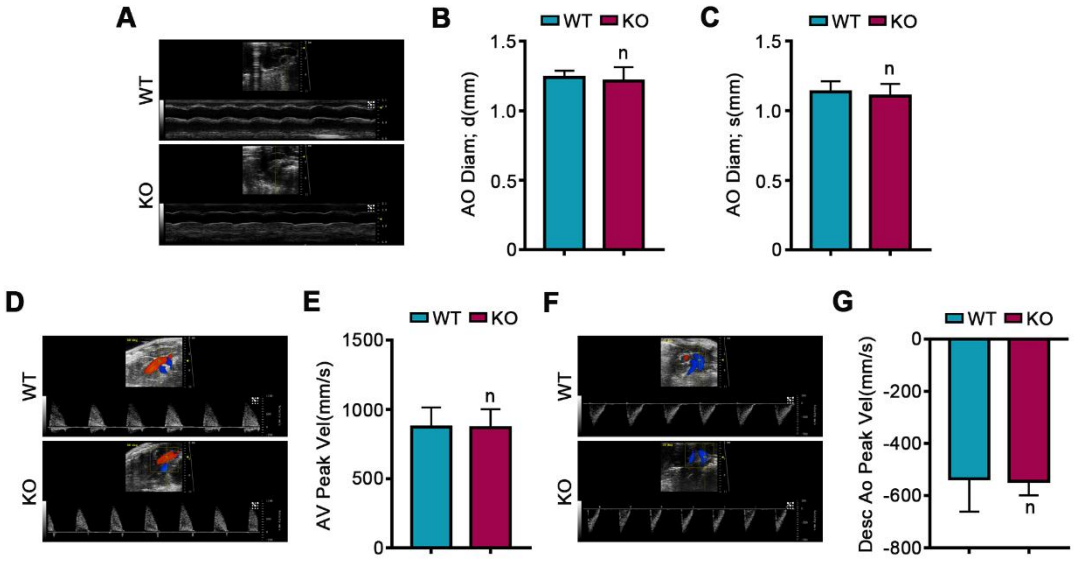


**Fig.6**