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Original Article

Protein kinase D1 (PKD1) promotes angiogenesis following myocardial infarction via vascular endothelial growth factor (VEGF) pathway

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Abstract: This study is to investigate the effects of protein kinase D1 (PKD1) on the angiogenic process following myocardial infarction. Rat model of myocardial infarction was established, and endothelial progenitor cells (EPCs) were isolated from normal rats and cultured *in vitro*. These animal and cell models were administrated with PKD1, alone or together with its inhibitor. Histological detection was performed with H&E staining, and myocardial ultrastructure was evaluated with TEM. VEGF and KDR expression levels were detected with RT-PCR and Western blot analysis. Histological detection showed irregular tissue arrangement and vague cell outline in the model group, accompanied with nuclear fusion. Significant fibrosis was noted in the necrotic myocardial tissue, with rare clear and complete vessels. TEM indicated that, in the model group, the myocardial tissue was irregularly arranged, with relatively unclear intercalated disk. Intact endothelial cells were rarely seen, with severe shrinkage and significantly reduced volume. All these pathological changes could be dramatically alleviated by the treatment of PKD1, which could also be abolished by its inhibitor CID755673. Results from RT-PCR and Western blot analysis showed that, the treatment of PKD1 significantly elevated the mRNA and protein expression levels of VEGF and KDR in the myocardial tissue in the rat models of myocardial infarction. In addition, in the *in vitro* EPCs, PKD1 significantly up-regulated the mRNA and protein expression levels of VEGF and KDR. PKD1 could significantly promote the angiogenic process following myocardial infarction, which might be mediated by the VEGF signaling pathway.

Keywords: Protein kinase D1 (PKD1), myocardial infarction, angiogenesis, vascular endothelial growth factor (VEGF), endothelial progenitor cells (EPCs)

Introduction

Protein kinase D (PKD) belongs to the Ca^{2+}/calmodulin-dependent serine/threonine kinase family (including PKD1, PKD2, and PKD3), which participates in various biological processes, such as cell migration and proliferation, trans-membrane transportation, and immune responses [1, 2]. PKD1 has been recognized as one of the key regulators of tumor angiogenesis, and PKD1 antagonists have been widely used in the clinical treatment of tumors, inhibiting tumor angiogenesis and promoting tumor cell apoptosis [3]. On the other hand, the angiogenesis-promoting treatment is one of the ideal therapeutic strategies for myocardial infarction, and PKD1 might also work as a potential target in the disease treatment. In our previous study, PKD1 has been shown to be able to enhance the adhesion, migration, and proliferation of bone marrow-derived endothelial progenitor cells (EPCs) *in vitro*, and up-regulate the mRNA and protein expression levels of eNOS in these cells, suggesting its angiogenesis-promoting activity [4].

Vascular endothelial growth factor (VEGF) family members play extremely important roles in the angiogenic process after myocardial infarction. Binding of VEGF to the kinase insert domain receptor (KDR) could promote the differentiation, proliferation, and migration of endothelial cells [5]. VEGF is actively involved in the EPC-mediated angiogenic process, especially concerning the lumen formation [6, 7]. It has been demonstrated in animal models [8] and human beings [9] that, VEGF could activate the bone marrow-derived EPCs to promote

angiogenesis in ischemic myocardial injuries, and transgenic delivery of VEGF might contribute to the disease recovery. Moreover, during the angiogenic process, the VEGF receptor KDR-mediated signaling pathway would activate the mitogen-activated protein (MAP) kinase-related pathways, further inducing actin reorganization, enhancing mitosis, and promoting proliferation and migration of endothelial cells [10].

In this study, the effects of PKD1 on the angiogenic process following myocardial infarction were investigated, both *in vivo* and *in vitro*. Rat models of myocardial infarction were established, and EPCs were also isolated and cultured *in vitro*. These animal and cell models were administrated with PKD1, alone or together with its inhibitor. Pathological changes in the myocardial tissue after myocardial infarction were detected. Expression levels of VEGF and KDR were also evaluated, both in the rat models and in the EPCs.

Materials and methods

Study animals

The male Sprague-Dawley (SD) rats (SPF), 8-w old, weighing 200-240 g, were purchased from the Experimental Animal Center of Henan Province (animal production license number, SCXK Henan 2010-0002; and animal quality certification number, 1000142). These animals were kept in standard conditions with free access to food and water. All animal experiments were conducted according to the ethical guidelines of the Nanyang Institute of Technology.

Animal model establishing and grouping

Myocardial infarction model was established by ligating the left anterior descending coronary artery (LAD), according to our previous published protocol [2]. The model rats were randomly divided into the following groups (n=8 for each group): (1) the model group, in which the model rats were subjected to saline injection; (2) the PKD1 group, in which the model rats were intraperitoneally injected with 10 mg/kg/d PKD1 (Lot No., OSP00005W; Pierce, Rockford, IL, USA) for 14 d; and (3) the PKD1+CID755673 group, in which the model rats were intraperitoneally injected with 10 mg/kg/d PKD1 and 10 mg/kg/d CID755673 (PKD1 inhibitor; Lot No., 2011756; Medchemexpress, Princeton, NJ, USA) for 14 d. In addition, another eight normal rats were used as the sham-operated group, in which the rats were threaded in the corresponding parts of the left anterior descending coronary artery instead of ligation.

Isolation, culture, and identification of EPCs

EPCs were isolated and cultured from normal rats, according to our previously published protocol [4]. These cells were incubated with EBM-2 medium (Dakewe, Beijing, China) containing 2% FBS (Baomanbio, Shanghai, China) in 6-well plates pre-coated with fibronectin, at the density of 1×10^5 cells/well. After adhesion, the cells were cultured with serum-free medium. These cells were divided into the following three groups: (1) the control group, in which the cells were treated with nothing; (2) the PKD1 group, in which the cells were treated with 100 ng/mL PKD1; and (3) the PKD1+CID755673 group, in which the cells were treated with 100 ng/mL PKD1 together with 100 ng/mL CID755673.

Reverse transcription (RT)-PCR

Total RNA was extracted from tissues or cells with Trizol. The cDNA was obtained with the reverse transcription kit (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed with the one-step RT-PCR kit (Qiagen, Hilden, Germany) on an ABI ViiA™ 7 real-time PCR detection system (ABI; Applied Biosystems, Foster City, CA, USA). The primer sequences were as follows: VEGF, forward 5'-ATGAACTTTCTGCTCTCTTGGG-3' and reverse 5'-CTCTCCTATGTGCTGGCTTTG-3'; and KDR, forward 5'-TCACGGTTGGGCTACTGC-3' and reverse 5'-AGACCTTCTGCCATCACG-3'. The 50 μL PCR system consisted of 4 μL templates, 0.5 μL Taq DNA Polymerase (TaKaRa, Dalian, Liaoning, China), 8 μL dNTP mixture (10 mM), 25 μL 5×PCR buffer, 1 μL primer each (20 nM), and 11.5 μL sterilized distilled water. Amplification condition was set as: denaturation at 94°C for 5 min, 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, for totally 30 cycles; followed by extension at 72°C for 5 min. The PCR products were subjected to 1.5% agarose gel electrophoresis, and the bands were captured and analyzed with the AlphaView SA software.

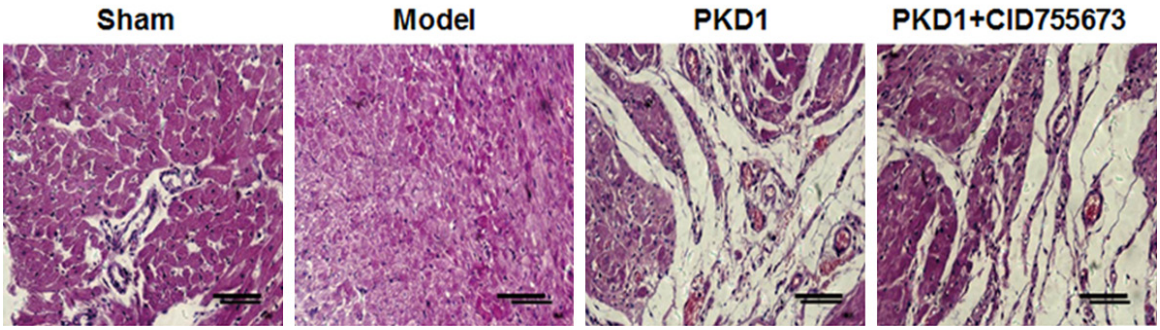

Figure 1. Effect of PKD1 on the myocardial pathomorphology in the rat models of myocardial infarction. Rat models of myocardial infarction were treated with 10 mg/kg/d PKD1, alone or with 10 mg/kg/d CID755673 for 14 d, and then the myocardial pathomorphology was detected with H&E staining. Scale bar, 50 μm.

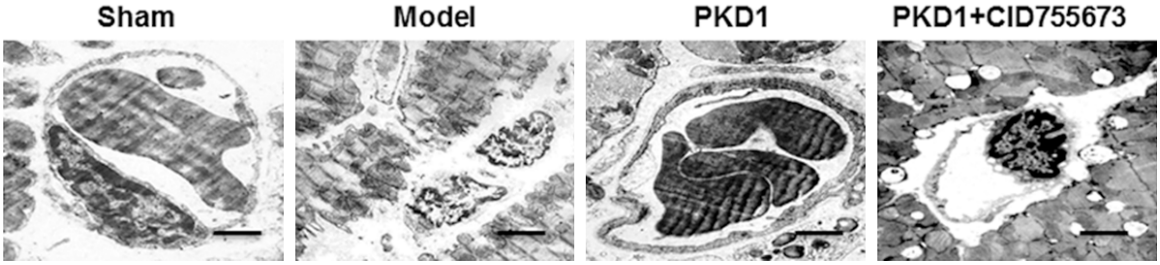

Figure 2. Effect of PKD1 on the myocardial ultrastructure in the rat models of myocardial infarction. Rat models of myocardial infarction were treated with 10 mg/kg/d PKD1, alone or with 10 mg/kg/d CID755673 for 14 d, and then the myocardial ultrastructure was detected with TEM. Scale bar, 2 μm.

Western blot analysis

EPCs were harvested and homogenized with the Next Advance Bullet Blender Storm tissue homogenizer (Next Advance, New York, NY, USA), and then subjected to centrifugation at 4°C at 12,000× g for 2 min. Protein concentration was determined with the Bradford method. 20 μg protein was separated with 12% SDS-PAGE, and electronically transferred onto a nitrocellulose membrane. After blocking with 5% fat-free milk for 2 h, the membrane was incubated with rabbit anti-rat anti-VEGF polyclonal primary antibody (1:1000 dilution; Hengye Biotechnology, Tianjin, China), or rabbit anti-rat anti-KDR polyclonal primary antibody (1:1000 dilution; Hengye Biotechnology), at 4°C overnight. The membrane was then incubated with goat anti-rabbit IgG (1:2000 dilution; Boster, Wuhan, Hubei, China) at room temperature for 1 h. After exposure in a dark room, the protein bands were scanned and analyzed with the AlphaView SA software. β-actin was used as interference control.

Histological examination

Apical myocardial tissue was obtained and frozen in liquid nitrogen. After fixed with 10% formalin, the tissue was cut into 4-μm sections on an ERM-3100 machine (Haosilin Technology Co., Ltd., Suzhou, Jiangsu, China). After washing, the section was subjected to the H&E staining and observed under an optical microscope.

Transmission electron microscopy (TEM)

Apical myocardial tissue was obtained from the rat models. After washing with saline, the tissue was immersed with 2.5% glutaraldehyde, and quickly cut into 0.5-mm^3 cubes with the double-sided blade. After fixed with 2.5% glutaraldehyde for 3 h, the tissue was subsequently subjected to 1% osmium tetroxide treatment, gradient dehydration, epoxy resin and acetone embedding, and then cut into 50-nm ultrathin sections. After staining with uranyl acetate-lead citrate, the sections were observed with TEM.

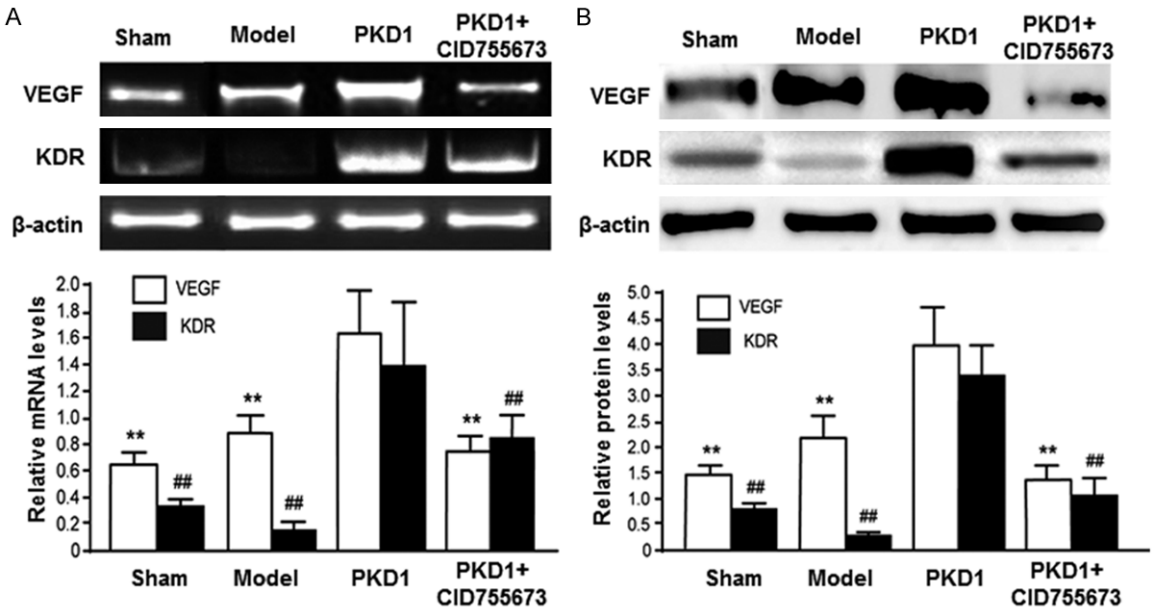

Figure 3. Effect of PKD1 on the expression levels of VEGF and KDR in the myocardial tissue in rat models of myocardial infarction. Rat models of myocardial infarction were treated with 10 mg/kg/d PKD1, alone or with 10 mg/kg/d CID755673 for 14 d. Then the mRNA and protein expression levels of VEGF and KDR were detected with RT-PCR (A) and Western blot analysis (B), respectively. For the VEGF expression, compared with the PKD1 group, $^{**}P<0.01$; for the KDR expression, compared with the PKD1 group, $^{\#\#}P<0.01$.

Statistical analysis

Data were expressed as mean ± SD. SPSS 16.0 software (IBM software, Somers, NY, USA) was used, and one-way ANOVA with Tukey's correction was performed for statistical analysis. $P< 0.05$ was considered statistically significant.

Results

Effect of PKD1 on myocardial pathomorphology in rat models of myocardial infarction

To investigate the effect of PKD1 on the myocardial pathomorphology in the rat models of myocardial infarction, H&E staining was performed. Our results showed that, in the control group, regular tissue morphology and clear cell outline were observed. However, in the model group, irregular tissue arrangement and vague cell outline were observed, accompanied with nuclear fusion (with the magnification of 200×). Significant fibrosis was noted in the necrotic myocardial tissue, and morphologically clear and complete vessels were rarely seen. Compared with the model group, in the PKD1 group, the myocardial tissue was relatively normal-arranged, and the cell outline was more clear and consistent. On the other hand, in the PKD1+CID755673 group, similar morphological features were observed as the model group (**Figure 1**). These results suggest that, PKD1 treatment could significantly alleviate the pathological changes in the myocardial tissue following myocardial infarction.

Effect of PKD1 on myocardial ultrastructure in rat models of myocardial infarction

The myocardial ultrastructure in the rat models of myocardial infarction was then detected with TEM. Our results showed that, in the control group, the myocardial tissue was neatly arranged, with clear intercalated disk and intact endothelial cell morphology. However, in the model group, the myocardial tissue was irregularly arranged, with relatively unclear intercalated disk. Intact endothelial cells were rarely seen, with severe shrinkage and significantly reduced volume. Compared with the model group, in the PKD1 group, the morphology of myocardial tissue was clear and regular, and most vascular endothelial cells were intact, with smooth cell membrane. Pericytes could also be observed in the PKD1 group. In the PKD1+CID755673 group, similar results were observed as the model group, including shrinking endothelial cells, vague cell membrane, dis-

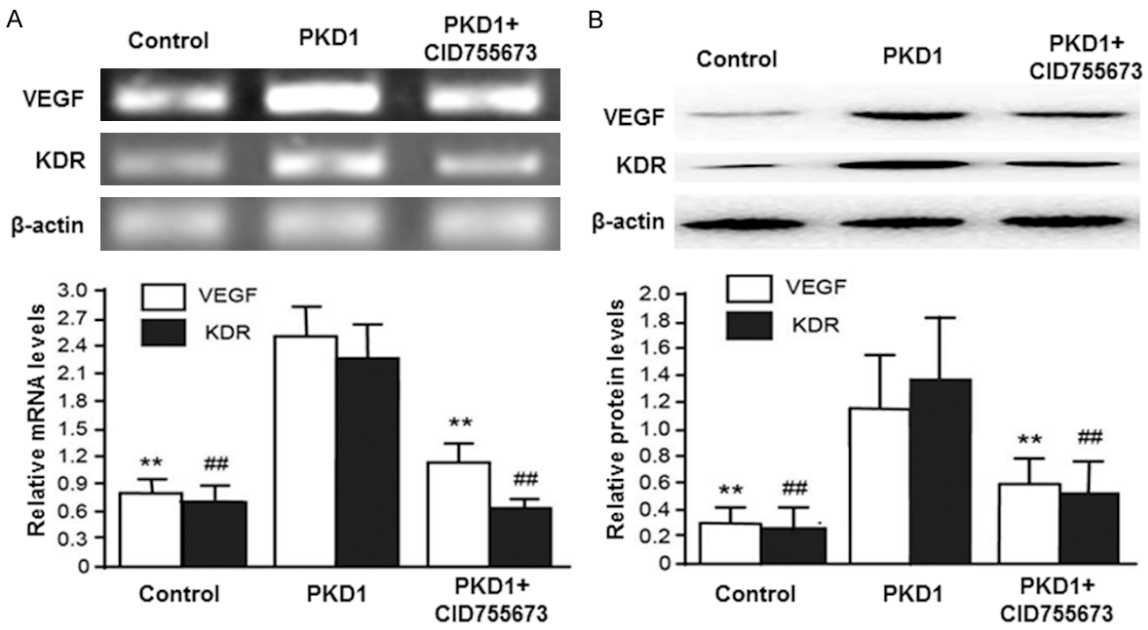

Figure 4. Effect of PKD1 on the expression levels of VEGF and KDR in the EPCs. EPCs were treated with 100 ng/mL PKD1, alone or together with 100 ng/mL CID755673. Then the mRNA and protein expression levels of VEGF and KDR were detected with RT-PCR (A) and Western blot analysis (B), respectively. For the VEGF expression, compared with the PKD1 group, $^{**}P<0.01$; for the KDR expression, compared with the PKD1 group, $^{\#\#}P<0.01$.

appearing pericytes, and reduced intact endothelial cells (**Figure 2**). These results suggest that, PKD1 treatment could significantly alleviate the altered myocardial ultrastructure in the rat models of myocardial infarction.

Effect of PKD1 on myocardial VEGF and KDR expression levels in rat models of myocardial infarction

To investigate the effect of PKD1 on the VEGF and KDR expression in the myocardial tissue in rat models of myocardial infarction, the mRNA and protein expression levels were detected with RT-PCR and Western blot analysis, respectively. Our results from the RT-PCR showed that, compared with the control group, the VEGF mRNA expression level was slightly increased (P>0.05), while the KDR mRNA expression level was slightly decreased (P>0.05), in the model group. Moreover, compared with the model group, the mRNA expression levels of both VEGF and KDR were significantly elevated in the PKD1 group (both P<0.01). However, in the PKD1+CID755673 group, the mRNA expression levels of VEGF and KDR were significantly lower than the PKD1 group (P<0.01) (**Figure 3A**).

Similar results were observed for the Western blot analysis. Compared with the control group, the VEGF protein expression was slightly elevated (P>0.05), while the KDR protein expression level was slightly declined (P>0.05), in the model group. Compared with the model group, the protein expression levels of both VEGF and KDR were significantly increased in the PKD1 group (both P<0.01), which were significantly decreased by the treatment of CID755673 (both P<0.01) (**Figure 3B**). Taken together, these results suggest that, the PKD1 treatment could significantly elevate the mRNA and protein expression levels of VEGF and KDR in the myocardial tissue in the rat models of myocardial infarction.

Effect of PKD1 on VEGF and KDR expression levels in EPCs

The effect of PKD1 on the mRNA and protein expression levels of VEGF and KDR in EPCs in vitro was then investigated. Our results from the RT-PCR and Western blot analysis showed that, compared with the control group, the PKD1 treatment significantly elevated the mRNA and protein expression levels of VEGF and KDR in the EPCs (both P<0.01). However, when the EPCs treated with PKD1 was subjected to the

treatment of CID755673, the mRNA and protein expression levels of VEGF and KDR were significantly declined in these cells ($P<0.01$) (**Figure 4**). In line with the in vivo results, these results suggest that, the PKD1 treatment could increase the mRNA and protein expression levels of VEGF and KDR in EPCs.

Discussion

In our previous report, PKD1 has been demonstrated to be able to improve the proliferation, adhesion, migration, and angiogenesis ability of rat bone morrow-derived EPCs [4]. The proliferation, adhesion, and migration of bone marrow-derived EPCs have been recognized as natural responses to ischemic tissue injuries, including myocardial infarction, which significantly promote angiogenesis in the lesions. In this study, our results further showed that, PKD1 significantly up-regulated the expression of VEGF and KDR in the bone marrow-derived EPCs. VEGF plays important roles in the angiogenic process after myocardial infarction. The interaction between VEGF and KDR promotes the differentiation, proliferation, and migration of endothelial cells [5]. VEGF is actively involved in the EPC-mediated angiogenic process, especially concerning the lumen formation [6, 7]. Meanwhile, VEGF has also been shown to modulate the interaction between endothelial cells themselves, as well as the interaction between endothelial cells and matrix, which might increase the vascular permeability and contribute to the lumen formation [11]. Taken together, these findings suggest that, the effects of PKD1 on the proliferation, adhesion, migration, and angiogenesis ability of rat bone marrow-derived PKD1 might be closely associated with VGEF and its receptor KDR.

Ischemic myocardial lesions induced by myocardial infarction would stimulate and activate the angiogenesis signal, growth factor, NO, and the endothelial cells. Moreover, pericytes detach from the blood vessel walls, which increases the vascular permeability and enhance the plasma protein extravasation, providing necessary matrix components for the migration of loosely connected endothelial cells [12]. Thereafter, under the regulation of so-called teloblasts (a special type of endothelial cells), microvascular bud would appear and develop along with the proliferation of endothelial cells, probably ending up with the lumen formation [12]. The accumulation of pericytes reinforces the stability of the newly formed lumen, which is regulated by VEGF and KDR [12].

In this study, our results showed that, in rat models of myocardial infarction, PKD1 significantly alleviated the changed myocardial tissue morphology, and increased the microvascular density. Moreover, our results from TEM showed that, PKD1 significantly induced the proliferation of endothelial cells and pericytes, which might be necessary for the angiogenic process in the myocardial tissue. Moreover, the contraction ability of myocardial cells is controlled by the interaction between the newly generated endothelial cells and the remaining normal cells in the myocardial tissue [13], which could exert the protective effects on the myocardial cells under myocardial infarction, in an NO-dependent manner [14]. Furthermore, the treatment of PKD1 significantly up-regulated the expression levels of VEGF and KDR in the myocardial tissue in the rat models of myocardial infarction. In the ischemic myocardial tissues after myocardial infarction from human beings or rodent models, hypoxia and/or distraction would rapidly activate the VEGF signal via HIF-1, promoting the lumen formation of new blood vessels [15, 16]. These findings suggest that, the angiogenesis-promoting effects of PKD1 might be mediated by the VEGF signaling pathway. Previous studies have shown that, VEGF derived from the endothelial cells could induce the activation of PKD1, which is important for the proliferation and migration of endothelial cells, as well as the angiogenic process [17]. In this study, our results demonstrated that, PKD1 significantly up-regulated the mRNA and protein expression levels of VEGF in EPCs. Therefore, the interaction between PKD1 and VEGF might be a potential target for the treatment of ischemic heart diseases.

In conclusion, our results showed that, PKD1 could significantly alleviate the pathological changes in the myocardial tissue in the rat models of myocardial infarction. Moreover, PKD1 significantly elevated the mRNA and protein expression levels of VEGF and KDR in the myocardial tissue and in the *in vitro* EPCs. Our results suggest that PKD1 could significantly promote the angiogenic process following myocardial infarction. These findings might provide evidence for the therapeutic strategies targeting on PKD1 and VEGF for the treatment of ischemic heart diseases in clinic.

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Disclosure of conflict of interest

None.

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