Acta Pharmacologica Sinica (2011) 32: 201–208
 1671-4083/11 $32.00
www.nature.com/aps

Original Article

# Adeno associated viral vector-delivered and hypoxia response element-regulated CD151 expression in ischemic rat heart

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**Aim:** The aim of this study was to improve the delivery efficacy and target specificity of the pro-angiogenic gene CD151 to the ischemic heart.
**Methods:** To achieve the inducible expression of adeno-associated viral (AAV)-delivered CD151 gene in only the ischemic myocardium, we generated an AAV construct in which CD151 expression can be controlled by the hypoxia response element (HRE) sequence from the human Enolase gene. The function of this vector was examined in rat H9C2 cardiac myoblasts and in ischemic rat myocardium. The expression of CD151 in the areas of ischemic myocardium was confirmed at the mRNA level by real-time PCR and on the protein level by Western blot, whereas the CD151 expression in the microvessels within the areas of ischemic myocardium was detected by immunohistochemistry.
**Results:** HRE significantly enhances the expression of CD151 under hypoxic conditions or in the ischemic myocardium, and forced CD151 expression increases the number of microvessels in the ischemic myocardium.
**Conclusion:** The AAV-mediated, HRE regulated delivery of the CD151 gene shows higher expression in the ischemic myocardium and more efficiently targets CD151 to the hypoxic regions after myocardial infarction.

**Keywords:** cardiac ischemia; CD151; angiogenesis; gene therapy; gene expression; hypoxia response element

*Acta Pharmacologica Sinica* (2011) 32: 201–208; doi: 10.1038/aps.2010.205; published online 17 Jan 2011

## Introduction

The transfer of angiogenic genes to the ischemic myocardium likely provides a useful approach for the treatment of coronary artery disease[1]. The CD151 protein is a member of the tetraspanin superfamily and contains four hydrophobic transmembrane domains, two extracellular loops, and two short cytoplasmic tails[2, 3]. Tetraspanins associate both with each other and with other integral membrane proteins, including integrins and members of the immunoglobulin superfamily[4, 5]. CD151 is expressed in various cell types, including epidermal basal cells, smooth cells, skeletal cells, cardiac muscle cells, endothelial cells, epithelial cells, platelets, and Schwann cells; it is also characteristically localized in intracellular vesicles and cell adhesion structures[4, 6]. Our previous studies have shown that the direct injection of an AAV vector carrying the CD151 gene to the ischemic rat myocardium induces neovasculature formation, and the CD151 gene delivery increases the number of microvessels in an ischemic rat hind limb model[7, 8]. Further studies with a swine myocardial infarction model indicated that CD151 gene delivery induces both angiogenesis and arteriogenesis by increasing the capillary and arteriole density[9, 10]. These observations strongly suggest that CD151 can be used for gene therapy with myocardial ischemia by promoting angiogenesis.

With the continued progress of gene therapy, there have been more concerns regarding the safety of gene therapy and the routes of gene delivery. The current routes of gene delivery to the heart include direct myocardial, trans-catheter myocardial, coronary artery, and intravenous injections. Intravenous injection is a non-invasive, safe gene-import method; however, it is not possible to introduce high concentrations of exogenous genes into the ischemic myocardium to induce sufficient angiogenesis using this method. If high doses of exogenous genes are administered, there would be an increase

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Received 2010-09-02 Accepted 2010-11-02

in the exposure of the gene to the normoxic myocardium as well as other organs and also an increase in the potential side effects in non-ischemic sites such as hemangioma formation and retinopathy[11, 12]. Although such complications were not observed in our previous animal studies on CD151, it is crucial to minimize these complications for the upcoming clinical applications of CD151. Thus, for intravenous administration, it is of immediate concern to find a method that will increase CD151 expression specifically in the ischemia myocardium but not in the normoxic myocardium and other organs.

For the treatment of ischemic heart disease, an ideal control for CD151 gene expression seems to be the response to hypoxia; that is, there is high expression of the CD151 gene in the ischemic areas and low expression in the non-ischemia areas. Hypoxia-inducible factor-1 (HIF-1) is induced or upregulated under conditions of hypoxia and regulates gene expression by binding to a cis-acting hypoxia-responsive element (HRE)[13, 14]. The HRE has been found in human enolase (ENO)[15], erythropoietin (Epo)[16], and several other genes. The core consensus sequence is (A/G)CGT(G/C)C. HRE sequences of ENO and Epo have been used to regulate the expression of genes such as vascular endothelial growth factor (VEGF)[14] and suicide[17] or apoptosis[18] genes.

In this study, we use the AAV-HRE-CD151 vector, which carries one copy of the HRE from ENO, to determine the applicability and feasibility of the hypoxia-induced, HRE-driven expression of CD151 in the ischemic myocardium. Our study demonstrated that the ENO HRE can induce CD151 expression under hypoxic conditions in both cultured cardiomyocytes *in vitro* and ischemic rat myocardium *in vivo*.

## Materials and methods

### Vector construction and production

The AAV constructs carrying human CD151 or green fluorescent protein (GFP) cDNA were generated as described previously[7, 19]. Based on the previous study by Philips *et al*[20], a 68-bp HRE sequence from the human *Enolase* 1 gene was inserted into the pAAV-CD151 construct between the *Mlu*I and *BamH*I restriction sites upstream of the cytomegalovirus promotor to generate the pAAV-HRE-CD151 construct.

### Cell culture and transfection

Rat cardiac myoblasts (H9C2) were obtained from American Type Culture Collection, cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and maintained in 5% $CO_2$/95% air at 37 °C. Hypoxia conditions were achieved in the tightly sealed hypoxia chambers by repeated evacuation and filling of 5% $O_2$/1% $CO_2$/94% $N_2$ at 37 °C.

To examine the hypoxia-induced expression of CD151, H9C2 cells were cultured in 6-well plates to 70%–90% confluence and then transfected with pAAV-HRE-CD151, pAAV-CD151, or pAAV-GFP using the FuGENE HD transfection reagent (Roche, MA, Germany). Non-transfected H9C2 cells were included as a control. At 48 h after the transfection, the infected cells were split into two parts: one part was cultured under normoxic conditions (95% air, 5% $CO_2$) and the other part was cultured under hypoxic conditions (94% $N_2$, 5% $O_2$, and 1% $CO_2$) in serum-free medium for 16 h. The individual experiments were repeated ($n$=6).

### Ischemic heart model and injection of the plasmids

Adult male Sprague-Dawley Rats (Animal Use and Care Center, Tongji Medical College, Wuhan, China) with weights of 200–250 g underwent a coronary artery ligation surgery. Under the anesthesia of pentobarbital (60 mg/kg, intraperitoneally), acute myocardial infarction (AMI) was induced by ligation of the left anterior descending coronary artery. Briefly, after intubation of the trachea, an incision was made in the skin overlying the 4th intercostal space, with the overlying muscles separated and kept aside. The animals were placed under positive-pressure ventilation (frequency 65–70/min, tidal volume 3 mL), and the thoracic cavity was opened by cutting the intercostal muscles. The heart was carefully pushed to the left, and a 6–0 silk suture was looped under the left descending coronary artery near the origin of the pulmonary artery. Proper occlusion of the coronary artery resulted in an extensive transmural infarction comprising a major part of the left ventricle (LV) free wall, with small variations in size. Coronary occlusion was confirmed by the raised segment (ST) stages on the electrocardiogram and ventricular arrhythmias within the first 20–30 min after the occlusion. Twenty-four survived rats were randomly divided into four groups one day after the surgery. The rats in the control group ($n$=6) received a single dose of 1 mL saline solution by the sublingual vein, whereas the rats in the treatment groups ($n$=6 per group) were injected through the same route with a single dose of 1 mL saline solution containing 200 μg of pAAV-GFP, pAAV-CD151, or pAAV-HRE-CD151 plasmids[21, 22]. The animal protocols used were in compliance with the Guide for the Care and Use of Laboratory Animals and were approved by the Chinese Academy of Sciences.

### Western blot analysis

Cardiac myoblasts were scraped off the plates and lysed in RIPA buffer (50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Nonidet-P40, 0.5% deoxycholic acid and 0.1% SDS) and centrifuged at 14 000×$g$ at 4°C for 30 min. Tissues from the infarcted heart areas were dissected. The tissues were homogenized in 500 μL of 25 mmol/L Tris-HCl (pH 7.4) containing 1% Triton X-100, 0.1% SDS, 2 mmol/L EDTA, and 1% protease inhibitor and centrifuged at 14 000×$g$ at 4°C for 30 min. The protein concentration was measured by the Bradford method with bovine serum albumin as the standard. The cell and tissue lysates (50 μg protein) were separated in SDS-PAGE, electrically transferred to polyvinyl difluoride membranes, and probed with antibodies against CD151 (Abcam, CA, UK) and β-actin (Sigma-Aldrich, MO, USA), followed by incubation with the horseradish peroxidase-conjugated 2nd antibody and chemiluminescence detection. The intensities of the protein bands were quantified by densitometry.

### Real-time quantitative PCR

Tissue samples from the infarcted heart areas were homogenized in TRIZOL solution (Invitrogen, CA, USA) and RNA was extracted from the tissue following the manufacturer's instruction. CD151 mRNA in the heart tissue was detected by real-time quantitative PCR. One microgram of total RNA was reverse-transcribed in a 20-μL reaction mixture containing 4 μL 5×PrimeScript Buffer, 1 μL Primescript RT Enzyme *Mix* I, 1 μL Oligo dT Primer (50 μmol/L), 1 μL Random 6 mers (100 μmol/L), and 9 μL RNAse-free $H_2O$ (TaKaRa, Dalian, China). The mixture was incubated at 37 °C for 15 min and 85 °C for 5 s. Then, real-time PCR was performed in an Mx3000P system (Stratagene, CA, USA) using the SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China) and 2 μL of cDNA was amplified in a 25-μL reaction mixture containing 12.5 μL of the SYBR Premix Ex Taq, 0.5 μL of the forward primer (10 μmol/L), 0.5 μL of the reverse primers (10 μmol/L), 0.5 μL of the ROX Reference Dye, and 9 μL of $dH_2O$. Amplifications were as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 58 °C for 20 s, and 72 °C for 15 s. The forward and reverse primers for CD151 are 5′-TGGGTGAGTTCAACGAGAAG-3′ and 5′-AGCCAGAAGCAGCAATTGTA-3′, respectively, and those for β-actin are 5′-CCCATCTATGAGGGTTACGC-3′ and 5′-TTTAATGTCACGCACGATTTC-3′, respectively. The reverse-transcribed cDNA samples were amplified, and cycle threshold (Ct) values were determined. The mRNA levels of CD151 were normalized to the mRNA levels of the housekeeping gene β-actin. The comparative Ct method ($2^{-\Delta\Delta Ct}$)[23] was used to analyze the differences in the level of CD151 mRNA between each group.

### Immunohistochemistry analysis and data acquisition

Heart tissue specimens were taken from the infarcted regions in the left ventricle 4 weeks after the plasmid administration, fixed in 10% formalin, embedded in paraffin, and cut into 4-μm thick sections. Some sections were stained with hematoxylin and eosin (H–E), and others were used for immunohistochemical staining with vWF (Santa Cruz, CA, USA) and SMα-actin (Sigma-Aldrich, MO, USA) antibodies for capillaries and arterioles, respectively. The densities of capillaries and arterioles were assessed according to the method previously described[10]. Five regions that are rich in blood vessels were selected, and the vessels of each region that are within 400×microscopic field (for capillary) or 100×microscopic field (for arteriole) were counted by two blinded investigators without knowledge of the group identity. The numbers of capillaries (vWF positive) and arterioles (SM α-actin positive) in each field were counted under ocular micrometers (Olympus, Tokyo, Japan), and a total of five high-powered fields (HPF) per region per heart were quantified.

### Statistical analysis

The statistical comparisons of the data were performed with a one-way analysis of variance (ANOVA), followed by the Newman-Keuls test for unpaired data. The results were expressed as the mean±standard error of the mean (SEM). A probability value of less than 5% was considered statistically significant.

## Results

### Construction of pAAV-HRE-CD151 plasmid

To induce CD151 expression in a hypoxia-dependent manner, we generated a recombinant pAAV-HRE-CD151 vector by placing the 68-bp HRE sequence from the human ENO gene between the *Mlu* I and *BamH* I restriction sites upstream of the cytomegalovirus promotor in the pAAV-CD151 plasmid. The correct insertion and intactness of the HRE sequence was confirmed by the nucleotide sequencing of the pAAV-HRE-CD151 vector (Figure 1).

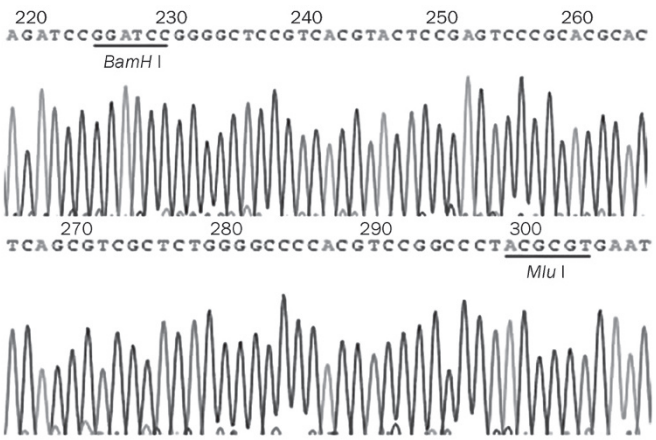


**Figure 1.** Identification of pAAV-HRE-CD151 by nucleotide sequencing. The sequence between the *BamH* I and *Mlu* I restriction sites is the HRE reverse complementary sequence (68 bp).

### HRE-induced *in vitro* CD151 expression in cardiac myoblasts

To determine if the HRE enhancer can conditionally induce AAV-mediated CD151 gene expression under hypoxic conditions, we expressed the plasmids of pAAV-HRE-CD151, pAAV-CD151, and pAAV-GFP in H9C2 cardiac myoblasts. Under the hypoxic conditions, CD151 protein expression was significantly increased in the pAAV-HRE-CD151 group compared to the pAAV-CD151, pAAV-GFP, and non-transfected cardiac myoblast groups ($P<0.01$). Under the normoxic conditions, CD151 protein expression was increased significantly in the pAAV-HRE-CD151 group compared to the pAAV-GFP and non-transfected cardiac myoblast groups ($P<0.01$); however, there was no significant difference in CD151 expression between the pAAV-HRE-CD151 and pAAV-CD151 groups. These results indicated that the HRE of ENO enhanced the AAV vector-mediated CD151 expression in cultured cardiac myoblasts under the hypoxic condition (Figure 2). In all experiments, an equal number of cells were lysed from each group to level the total protein loading, and actin expression was used to normalize the CD151 expression in each group.

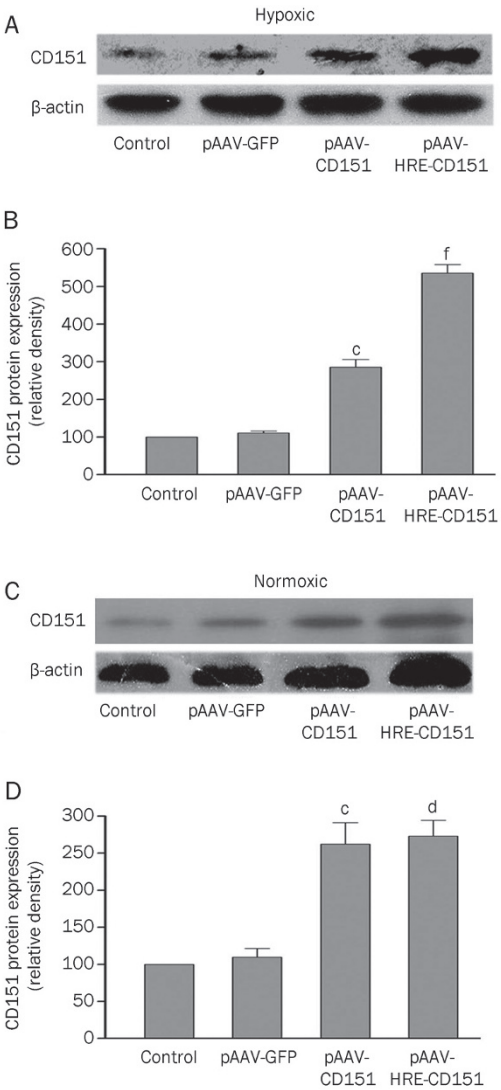


**Figure 2.** Hypoxic induction of CD151 expression in H9C2 cells after CD151-expressing vector transfection. (A) Western blot analysis of CD151 expression in hypoxic conditions. β-actin was used as an internal control. (B) Quantitative analysis of CD151 protein expression. The mean density of CD151 in the control group was defined as 100%. Data are presented as means±SEM ($n$=4–6 per group). $^{c}P$<0.01 *vs* control and pAAV-GFP groups. $^{f}P$<0.01 *vs* the pAAV-CD151 group. (C) Western blot analysis of CD151 expression in normoxic conditions. β-actin was used as an internal control. (D) Quantitative analysis of CD151 protein expression. The mean density of CD151 in the control group was defined as 100%. Data are presented as means±SEM ($n$=4–6 per group). $^{c}P$<0.01 *vs* control and pAAV-GFP groups, $^{d}P$>0.05 *vs* the pAAV-CD151 group.

### Induction of CD151 expression by HRE in the ischemic myocardium

The ENO HRE-driven *in vivo* expression of CD151 was assessed by intravenously injecting pAAV-GFP, pAAV-CD151, and pAAV-HRE-CD151 vectors into the ischemic rat heart. CD151 expression was analyzed at the mRNA level using real time PCR. The CD151 level in the ischemic hearts of the pAAV-HRE-CD151 group was markedly higher than that in the saline control and pAAV-GFP groups ($P$<0.01) (Figure 3). In the same model, the CD151 level in the pAAV-HRE-CD151 group was higher than that in the pAAV-CD151 group ($P$<0.01). Consistent with the results *in vitro*, ENO HRE significantly enhances CD151 expression in the ischemic myocardium *in vivo*.

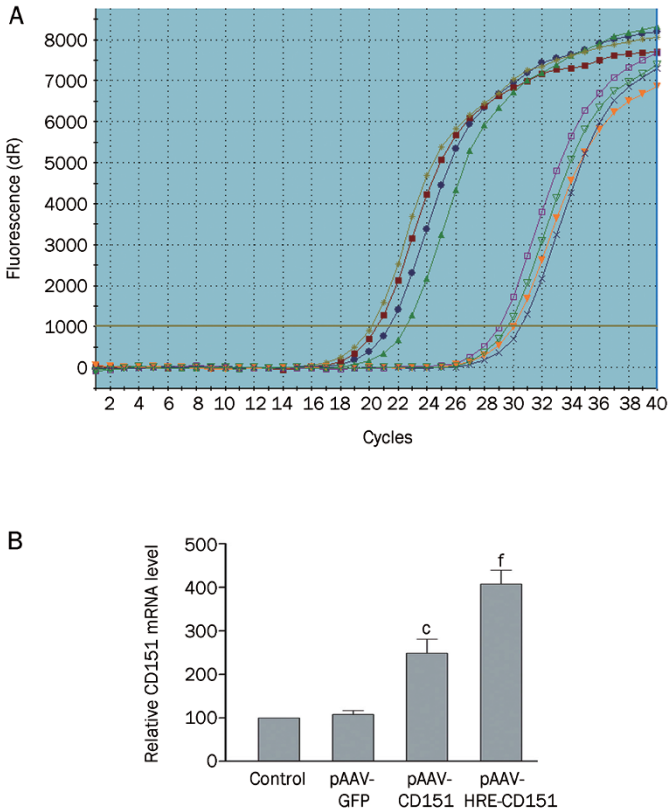


**Figure 3.** Induction of CD151 expression by HRE in the ischemic myocardium after CD151 gene delivery. Four weeks after coronary artery ligation, samples were collected from the same heart regions. (A) Real-time PCR amplification plots of CD151 and β-actin. (B) The mRNA levels for CD151 and β-actin after gene delivery. β-Actin was used as an internal control. In each group, the CD151 mRNA level was normalized to the β-actin mRNA level. The data are presented as the ratio of the treated group to the saline control group, which was defined as 100%. The results are presented as means±SEM ($n$=4–5 per group); $^{c}P$<0.01 between the pAAV-CD151 group and the saline control and pAAV-GFP groups. $^{f}P$<0.01 between the pAAV-HRE-CD151 group and the saline control, pAAV-GFP, or pAAV-CD151 groups.

Western blot analysis demonstrated that the levels of human CD151 proteins extracted from ischemic myocardium were increased significantly in the pAAV-CD151 and pAAV-HRE-CD151 groups compared with the saline control and pAAV-GFP groups ($P$<0.01, Figure 4). In contrast, the expression of CD151 proteins in the pAAV-HRE-CD151 group was higher than that in the pAAV-CD151 group ($P$<0.01). There was no significant difference in the level of CD151 proteins between the saline control and pAAV-GFP groups ($P$>0.05). These

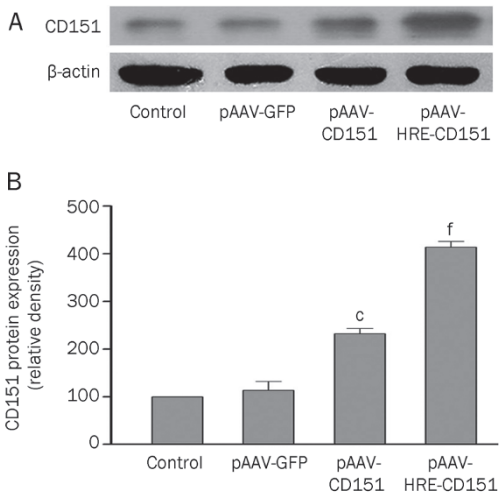


**Figure 4.** Enhancement of CD151 expression by HRE in the ischemic myocardium after CD151 gene delivery. Four weeks after coronary artery ligation, tissue samples were collected from the same heart regions. (A) Western blot analysis for CD151 and β-actin. β-Actin serves as an internal protein loading control. (B) Quantification of CD151. The density of the CD151 band in the saline control group was arbitrarily defined as 100 units, and the densities in other groups are the relative levels to 100 units. Data are presented as means±SEM ($n$=6−8). $^{c}P$<0.01 between the pAAV-CD151 group and the saline control and pAAV-GFP groups. $^{f}P$<0.01 between the pAAV-HRE-CD151 group and the saline control, pAAV-GFP, or pAAV-CD151 groups.

results indicate that AAV-mediated CD151 gene delivery promotes CD151 protein expression in myocardial tissue and that ENO HRE can induce higher CD151 expression in the ischemic myocardium *in vivo*.

### Analysis of arteriole and capillary density

The formation of capillaries and arterioles was evaluated by vWF and SM α-actin staining, respectively, at 4 weeks after the plasmid injections. Higher microvessel densities were found in the pAAV-HRE-CD151 and pAAV-CD151 groups and less neovascularization was found in the saline control and pAAV-GFP groups (Figure 5). There were no significant differences in the numbers of microvessels ($P$>0.05) between the saline control and pAAV-GFP groups. In contrast, the capillary and arteriole densities in the pAAV-HRE-CD151 group were markedly higher than that in the pAAV-CD151 group ($P$<0.01). These findings indicate that the ENO HRE-induced higher CD151 expression is correlated with more neovascularization in the ischemic myocardium. This further supports our earlier observation that CD151 gene delivery increases the formation of capillaries and arterioles.

## Discussion

Despite the success of bypass surgery and angioplasty, many patients have a limited opportunity to accomplish revascularization in the ischemia myocardium because of diffused coronary artherosclerosis, occluded bypass grafts, or excessive risk of surgery. Hence, ischemic heart disease remains the leading cause of morbidity and mortality in the world. Therapeutic angiogenesis, using angiogenic growth factors or cytokines to stimulate collateral blood vessel formation, has been tested as an alternative treatment[14, 24, 25]. The pro-angiogenesis factors that have been investigated include CD151, VEGFs[1, 7], FGFs[25], and angiopoietins[26]. As a member of the tetraspanin family, CD151 plays a role in regulating endothelial cell (EC) motility and angiogenesis *in vitro*[4, 27]. A recent study demonstrates that the mouse lung ECs (MLECs) from CD151-null mice display marked reduction in the angiogenesis-related endothelial events including migration, spreading, invasion, Matrigel contraction, tube and cable formation, and spheroid sprouting[28]. Conversely, overexpression of CD151 in human umbilical vein ECs enhances cell proliferation, migration, and capillary tube formation on Matrigel[29]. We have reported that CD151 gene delivery increases the number of microvessels in a rat myocardial ischemia model and an ischemic rat hindlimb model[7, 8]. Further studies have underlined that CD151 gene delivery promotes angiogenesis in a pig myocardial ischemia model and demonstrated that CD151-induced neovascularization effectively enhances the myocardial perfusion and markedly ameliorates the regional myocardial dysfunction based on $^{13}$N-$NH_3$ PET imaging and echocardiography[9, 10]. All these observations strongly suggest that CD151 can be useful for gene therapy to target myocardial ischemia by promoting angiogenesis.

The adeno-associated virus (AAV) vector belongs to the nonpathogenic member of the parvovirus family. It has several advantages over other viral vectors such as low immunogenicity, a long-term expression of transgene, and an ability to infect both dividing and nondividing cells[30]. Our previous studies have also found that AAV is an efficient vector for the *in vivo* transfer and sustained expression of the CD151 gene in the myocardium. AAV-mediated CD151 gene transfer provides heart with effective and long-lasting protection from acute myocardial infarction-induced injuries.

A potential problem associated with exogenous gene therapy is that different dosages and durations of the exogenous gene stimulation may yield different results. A relatively low level of the angiogenic factor in the myocardium is insufficient to induce angiogenesis, because transient exposure to the exogenous genes in humans was insufficient to trigger and maintain a therapeutically meaningful angiogenic response in clinical trials, especially when patients also had an extensive atherosclerotic disease[31]. The administration or expression of angiogenic factors for the short term and/or at low levels either could not stimulate myocardial angiogenesis in initial clinical trials and induced only a short-lived angiogenic response[32] or led to nonfunctional, hypo-perfused capillaries that regress when the levels of angiogenic factors drop[32]. For the treatment of ischemic heart disease, angiogenic therapy is aimed at stimulating functional and sustainable new blood vessels. However, an excessively high-level expression of the angiogenic factor *in vivo* may result in side effects in non-ischemic sites such as excessive angiogenesis, which will lead

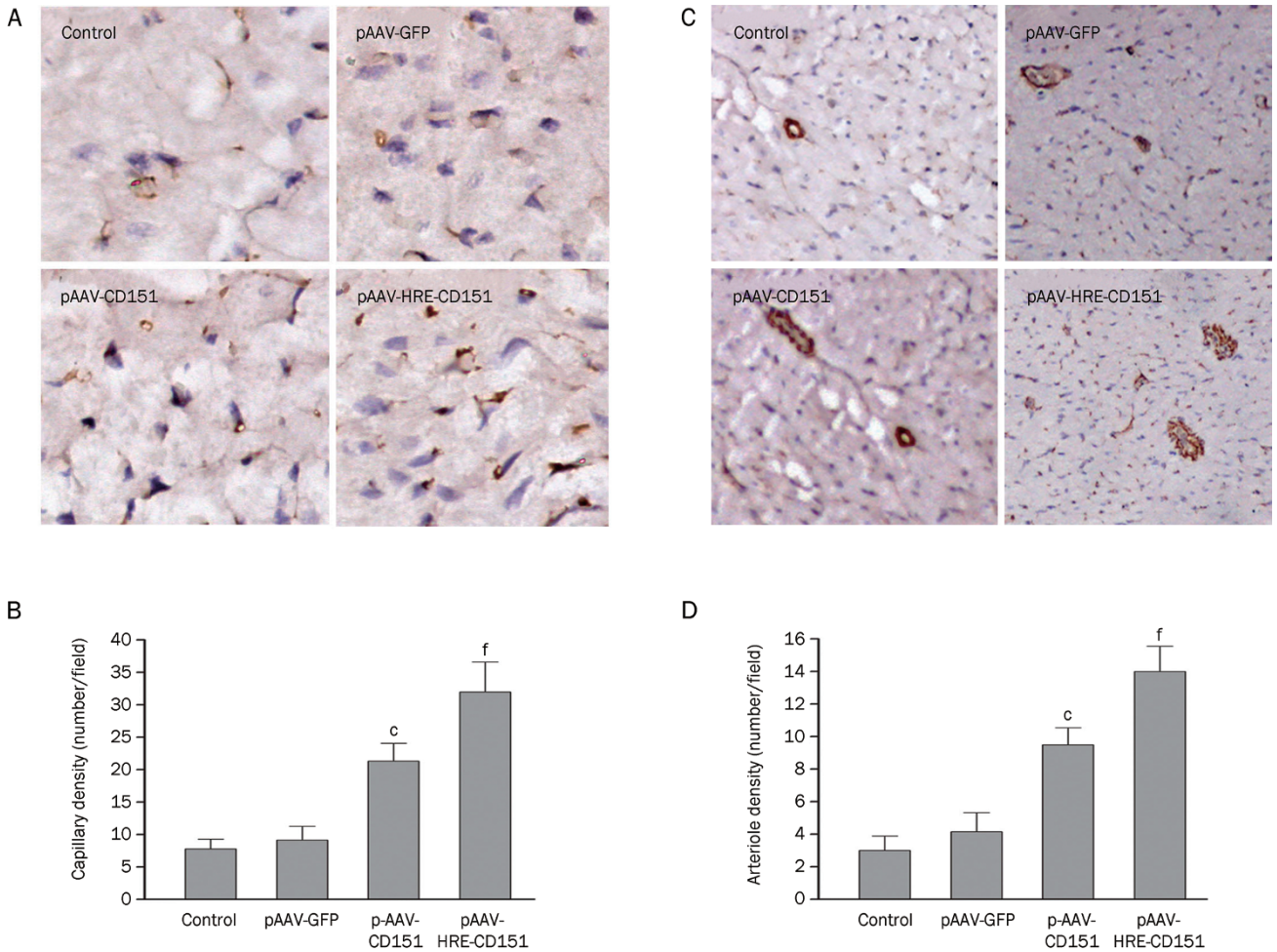


**Figure 5.** Overexpression of CD151 increases the densities of capillaries and arterioles. (A) Immunohistochemical staining of capillaries with antibody to von Willebrand factor (vWF). The ECs are stained in brown. (B) Quantification of the density of myocardial capillaries. (C) Immunohistochemical staining of arterioles with antibody to SM α-actin. The smooth muscle cells are indicated in brown. (D) Quantification of the density of myocardial arterioles. Data are projected as means±SEM ($n$=4−6 per group). $^{c}P$<0.01 between the pAAV-CD151 group and the saline control and pAAV-GFP groups. $^{f}P$<0.01 between the pAAV-HRE-CD151 group and the saline control, pAAV-GFP, or pAAV-CD151 groups.

to adverse consequences in the retina, synovium, and other areas[12, 33]. Thus, long-lasting and reasonably high levels of exogenous gene stimulation are needed for clinical success. More importantly, it is crucial to increase the exogenous gene expression only in the ischemic myocardium without significantly affecting the global level of this gene in the body.

One ideal mechanism to regulate the exogenous gene expression in the ischemic heart is to allow the expression of this gene in response to hypoxia. Earlier studies demonstrated that an increased level of HIF-1α in the ischemic myocardium was found as early as 2 days after the coronary artery occlusion and lasted until 2 weeks later[14]. Therefore, the HIF-1α/HRE is an ideal regulatory system to be used for controlling exogenous gene expression in the ischemic myocardium. To maintain a higher level of CD151 expression in the ischemic regions and meanwhile reduce the side-effects, we chose to inject a lower level of the CD151 gene and make use of HRE to induce higher CD151 gene expression in the hypoxic area. Consequently, the angiogenesis induced by uncontrolled and persistently expressed exogenous gene can be avoided by HRE-controlled CD151 expression. We chose to use ENO HRE in our study because Phillips *et al* showed that HRE from ENO yielded the best induction of gene expression under hypoxic conditions[20]. CD151 expression under the control of a HRE enhancer and CMV promoter exhibited marked upregulation under hypoxic conditions. This upregulation is likely mediated by HIF-1α in cardiomyocytes because H9C2 cells express HIF-1α in hypoxia[14]. In accordance with higher CD151 expression, the formation of capillaries and arterioles in the ischemic myocardium was also elevated under the HRE enhancer. These findings together suggest that hypoxia enhances CD151 expression in the ischemic myocardium in an HRE-dependent manner and that higher CD151 gene expression in turn results in increased neovascularization.

In conclusion, we have demonstrated that ENO HRE enhances the AAV vector-mediated CD151 gene expression in

cultured cardiomyocytes *in vitro* and ischemic rat myocardium *in vivo*. These results further demonstrate that the HIF-1/HRE axis is a useful system for controlling CD151 expression in the treatment of ischemic heart disease and potentiates the efficacy of CD151-based gene therapy.

**Acknowledgements**

This work was supported by a grant from the National Natural Science of China (No 30670856).

**Author contribution**

Xiao-lin HUANG and Zheng-xiang LIU designed the study and handled funding; Quan WEI, Jing-yang LIN, and Yu-jie FEI performed the research, analyzed the data and drafted the manuscript; Xin A ZHANG contributed the reagents and analytical tools and reviewed and revised the manuscript.

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