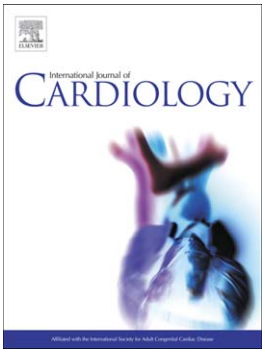


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# VEGF-A promotes cardiac stem cell engraftment and myocardial repair in the infarcted heart

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**Abstract**

**Background:** The objective of this study was to determine whether vascular endothelial growth factor (VEGF)-A subtypes improves cardiac stem cells (CSCs) engraftment and promotes CSC-mediated myocardial repair in the infarcted heart.

**Methods:** CSCs were treated with VEGF receptor (VEGFR) inhibitors, VCAM-1 antibody (VCAM-1-Ab), or PKC-α inhibitor followed by the treatment with VEGF-A. CSC adhesion assays were performed *in vitro*. *In vivo*, the PKH26-labelled and VCAM-1-Ab or PKC-α inhibitor pre-treated CSCs were treated with VEGF-A followed by implantation into infarcted rat hearts. The hearts were then collected for measuring CSC engraftment and evaluating cardiac fibrosis and function 3 or 28 days after the CSC transplantation.

**Results:** All three VEGF-A subtypes promoted CSC adhesion to extracellular matrix and endothelial cells. VEGF-A-mediated CSC adhesion required VEGFR and PKCα signaling. Importantly, VEGF-A induced VCAM-1, bit not ICAM-1 expression in CSCs through PKCα signaling. *In vivo*, VEGF-A promoted the engraftment of CSCs in infarcted hearts, which was attenuated by PKCα inhibitor or VCAM-1-Ab. Moreover, VEGF-A-mediated CSC engraftment resulted in a reduction in infarct size and fibrosis. Functional studies showed that the transplantation of the VEGF-A-treated CSCs stimulated extensive angiomyogenesis in infarcted hearts as indicated by the expression of cardiac troponin T and von Willebrand factor, leading

to an improved performance of left ventricle. Blockade of PKCα signaling or VCAM-1 significantly diminished the beneficial effects of CSCs treated with VEGF-A.

**Conclusion:** VEGF-A promotes myocardial repair through, at least in part, enhancing the engraftment of CSCs mediated by PKCα/VCAM-1 pathway.

**Key words:** VEGF-A, cardiac stem cells, myocardial infarction, VCAM-1, PKCα

Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are crucial regulators of the growth, development, and differentiation of heart and blood vessels. The therapeutic effect of VEGF is signified by the improvement of heart structure and function [1, 2]. VEGF-A, as a key angiogenesis factor of VEGF family, mainly exists in three subtypes, namely VEGF121, VEGF165, and VEGF189 (mouse VEGF less one amino acid, so the corresponding VEGF subtype VEGF120, VEGF164 and VEGF188) [3]. VEGF121 and VEGF165 are secreted into the extracellular fluid, whereas the longer isoform binds to heparin sulfate proteoglycans at the cell surface. VEGF189 is almost entirely bound to the cell surface or extracellular matrix, and is considered active after its cleavage and release from its extracellular binding site [4]. VEGF exerts its biological function by binding to tyrosine kinase receptor 1 (flt-1) and 2 (flk/KDR), which are also located on the surface of stem cells [5]. The role of different VEGF-A subtypes in engaging cardiac stem cells (CSCs) during the cardiac repair process, however, remains to be determined.

CSCs, shown as c-Kit+, Sca1+, MDR+ and other features, are found in heart tissues and are responsible for the self-renewal of myocardial tissue under physiological state and the repair of damaged myocardial tissue under pathological state [6]. Studies have shown that cardioprotective c-kit+ cells regulate the myocardial balance of angiogenic cytokines [7]. Furthermore, c-Kit+ CSCs, which can better give rise to heart tissue, offer a greater potential solution than mesenchymal stem cells (MSC) [8-10]. However, a crucial problem is the extensive loss of the cells once transplanted. Regardless of cell type, many studies have shown that the majority of cells

successfully delivered to the heart lost within the first week [11, 12]. The causes of cell loss in infarcted setting are multifactorial and are influenced by the low efficiency engraftment, coupled with the insufficient of residing signals from matrix attachments and cell-cell interactions, apart from the ischemic environment, which is devoid of nutrients and oxygen, resulting in cells apoptosis or death [12]. Recently, it has been proposed that preconditioning of MSCs with SDF-1α may result in improved cell survival and engraftment [13]. Actually, VEGF, as a key angiogenic and growth factor from MSC, are important for maintaining or improving normal cardiac function in MSC-mediated cardiac repair through promoting angiogenesis and inhibiting cardiocyte apoptosis [14, 15]. More importantly, the genetic modification and preconditioning by VEGF protein may better enhance MSC survival [16, 17]. Recent studies have shown that c-Kit+ CSCs express VEGF-R (Flt-1 and Flk/KDR) [18], and VEGF contributes to the myogenic cluster formation of MSCs and CSCs [19]. Interestingly, VEGF/VEGF-R is involved in cell adhesion and induction of VCAM-1 and ICAM-1 expression in human endothelial cells [20]. However, the effects and mechanisms underlying the function of VEGF-A subtypes in CSC adhesion and staying in injured sites are still unknown.

In this study, we have explored the characteristics and mechanism of different VEGF-A subtypes in CSC perching in damaged heart tissue by using the model of CSCs adhesion *in vitro* and *in vivo*. Improvement of CSC engraftment will ultimately allow us to address cell scalability and to make cell-based therapies more easily applicable to humans.

## Methods

The present study was conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. All animal protocols were approved by the Institutional Animal Care and Use Committee of Hubei University of Medicine.

### 2.1 Isolation and culture of c-kit+ cells from neonatal rat hearts.

CSCs were isolated from the hearts of 3-day-old Sprague–Dawley (SD) rats by a method described previously (8). Briefly, hearts were removed under aseptic conditions from rats that had been overdosed with sodium pentobarbital. Isolated myocardial tissue was cut into 1-2 $mm^3$ pieces and washed with $Ca^{2+}$- and $Mg^{2+}$-free PBS to remove blood. Heart pieces were then digested with 0.2% trypsin (Invitrogen) and 0.1% collagenase IV (Sigma) at 37°C for three times (5 min each digestion). At the end of enzymatic digestion, heart tissues were minced. Cell suspension was collected and filtered through a 70 μm cell strainer (Becton Dickson). Cells were then incubated with a rabbit anti-c-kit antibody (Santa Cruz) and separated using sheep anti-rabbit immunomagnetic microbeads (Miltenyi Biotec). Small round cells containing most of the c-kit+ population were collected. Newly isolated cardiac c-kit+ cells were seeded in 25 $cm^2$ flasks coated with 200 μg/ml fibronectin (Sigma) and grown in DMEM containing 15% FCS, 10 ng/ml basic fibroblast growth factor (bFGF), and 10 ng/ml leukemia inhibitory factor (LIF) at 37°C in an atmosphere of

5% $CO_2$ for 3 days [18]. Cells were treated with vehicle or 50 ng/ml of VEGF-A (Upstate) in 24-well plates for adhesion assays and heart implantation.

**2.2 Cell labeling.**

For transplantation studies, CSCs were labeled with a cell tracker dye PKH26 using a PKH26 Red Fluorescent Cell Linker Kit (Sigma) according to the manufacturer's instructions. The efficiency of PKH26 labeling was evaluated by Flow Cytometry [18].

**2.3 *In vitro* CSC migration assay.**

CSCs were collected and seeded on the top wells of a transwell insert (Millipore, USA) at a density of $2\times10^4$ cells/well in 200 μl of 2% FCS-contained DMEM. 600 μl of 2% FCS DMEM containing VEGF-A (20 ng/ml) were added to the bottom wells of the transwell plates (pore size, 8 μm). 2% FCS DMEM was used as a control. For the inhibition experiments, CSCs were pre-incubated with VEGFR1 inhibitor (2 nM of AMG706 (AMG), selleckchem), VEGFR2 inhibitor (70 nM of VEGFR2-I, Merck, NO: 676480) for 1 h before VEGF-A treatment. CSCs were cultured at 37°C in a humidified atmosphere of 5% $CO_2$ for 24 h. Transwell inserts were removed, and migration rate was quantified by counting the cells migrating to the bottom wells and averaging the cell numbers from 5 high-power fields (200×) per chamber as observed by phase contrast microscopy. CSC migration index was calculated to express the induced migration using the following equation: Migration index=induced

migration/Random migration. Each assay was carried out in triplicate [18].

**2.4 CSC adhesion assay using extracellular matrix (ECM)-mediated adhesion system *in vitro.***

Cell adhesion assays were conducted according to the method described previously [21]. 24-well culture plates were pre-treated with 200 μl poly-L-lysine (0.1%) at 4 ℃ overnight. After removing the poly lysine, the plates were placed at 37 ℃for 1 h. CSCs were treated similarly as in section 2.3 above and then labeled with PKH26. PKH26-labeled CSCs ($1.0\times10^5$ cells/ml) in 200 μl of 2% FCS DMEM were added into each well and incubated at 37°C in an atmosphere of 5% $CO_2$ for 24 h. After removing the culture medium and washing slightly with PBS for 2 times, the cells were fixed with 4% paraformaldehyde. The CSCs adhering onto the poly-L-lysine-coating plates were imaged under fluorescence microscope, and the cell numbers from 5 high-power fields (200×) of three independent experiments were analyzed with Image Pro 6.0 software. Adhesion efficiency was calculated using the following formula: Adhesion index = cell numbers of the treated group/control group.

**2.5 CSC adhesion assay using endothelial cell-mediated adhesion *system in vitro.***

Cell adhesion to endothelial cells was assayed using the procedure described previously [22]. CSCs were pre-incubated with VEGFR1 inhibitor (2 nM of AMG706 (AMG)), VEGFR2 inhibitor (70 nM of VEGFR2-I), anti-VCAM-1, anti-ICAM-1 antibodies (10 μg/ml, Santa Cruz), or a PKCα antagonist (Gŏ6976, 6 nmol/L) for 1 h

before VEGF-A treatment (50 ng/ml) as described above. Human umbilical vein endothelial cells (ECs) were cultured in 24 well plates for 48 h. When ECs reached 80-90% confluence, the medium was changed to 2% FCS DMEM containing $1.0\times10^5$ cells/ml of PKH26-labeled CSCs and incubated at 37°C in an atmosphere of 5% $CO_2$ for 24 h. After removing the culture medium and washing slightly with PBS for 2 times, the cells were fixed with 4% paraformaldehyde and labeled with 4',6-diamidino-2-phenylindole(DAPI, 5 μ g/ml) followed by gentle washing with PBS for 2 times. CSCs adhering on the endothelial cells were imaged under fluorescence microscope and analyzed similarly as in Section 2.5.

**2.6 Myocardial infarction and CSC implantation.**

Myocardial infarction (MI) was achieved by ligation of the left anterior descending coronary artery (LAD) as previously described [18]. Briefly, SD rats (280-300 g) were anaesthetized with ketamine (50 mg/kg, ip) and xylazine (10 mg/kg, ip). Tracheal ventilation with room air was carried out by using a ventilator (HX-300, Tai-meng Instruments, China). Following left lateral thoracotomy at the fourth intercostal space, the LAD was ligated. Before chest closure, infarction was confirmed by observation of injury demarcation with blanching of the myocardium as well as electrocardiography. Rats were grouped randomly.

Seven days after MI induction, the rat thorax was reopened, and $2\times10^5$ CSCs in serum-free medium (50 μL) were injected with a 30-gauge tuberculin syringe into the site of infarcted myocardium. To manipulating cell properties, CSCs were pre-treated

with vehicles, VCAM-1 antibody (10 μg/ml, Santa Cruz), or a PKC antagonist (Gŏ6976, 6 nmol/L) for 1 h prior to 8 h of VEGF-A treatment. Then the cells were labeled with PKH26 cell tracker dye, washed with DMEM for 3 times, and collected for heart implantation. In sham-operation group, rats underwent identical surgery but without ligation of the coronary artery. Penicillin (150,000 U/ml, IV) was given before each procedure. Buprenorphine hydrochloride (0.05 mg/kg, sc) was administered twice a day for the first 48 h after the procedure.

**2.7 Measurement of CSC engraftment efficiency in the infarcted rat heart.**

Three days after the CSC implantation, hearts were collected for testing assay the CSC engraftment efficiency *in vivo*. Heart tissues were fixed in 4% paraformaldehyde and embedded in optimum cutting temperature compound (Fisher Scientific). Serial transverse sections (5 μm) were cut across the longitude axis of the heart and mounted on slides. After a brief wash in 1xPBS, heart sections were observed under fluorescence microscope, the mean red fluorescence intensity of implanted CSCs labeled with PKH26 were analyzed. Meanwhile, the whole left ventricle was collected to detect the expression of CSC marker genes Nkx2.5, GATA4, and c-Kit by using real-time PCR.

**2.8 Total RNA isolation and quantitative PCR (qPCR).**

Total RNA in the whole left ventricle was isolated using Trizol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed by the iScript Select cDNA Synthesis Kit

(Bio-Rad) in accordance with the manufacturer's instructions. qPCR was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN) in accordance to the manufacturer's instructions. GAPDH levels were taken for normalization and fold change was calculated using $2^{-\Delta\Delta Ct}$. Primer sequences are provided in Table 1.

**2.9 Immunostaining.**

Cultured CSCs were fixed in 4% paraformaldehyde. Immunostaining was performed with specific primary antibodies: mouse anti-rat CD31 (1:500, Abcam), rabbit anti-rat c-kit (1:250, Santa Cruz), mouse anti-rat GATA4 (1:250, Santa Cruz), mouse anti-rat Flk1 (1:200, Santa Cruz), goat anti-rat Flt1 (1:200, Santa Cruz), or mouse anti-rat troponin T (Cardiac isoform, cTnt, 2µg/ml, Neomarkers). The immunostaining was visualized with corresponding fluorescent antibodies. The nucleus was stained with DAPI (50 µg/ml).

For heart tissues, heart sections were incubated in a blocking buffer (PBS containing 1% fetal calf serum (FCS) and 0.1% Triton X-100) at room temperature for 1 h. Incubations in antibodies (diluted 1:250 in blocking buffer) were carried out at 4°C overnight for primary antibodies, and room temperature for 1 h for secondary antibodies. The primary antibodies used were: mouse anti-rat cardiac troponin T and rabbit anti-rat von Willebrand Factor-VIII (vwFVIII, 1:250, Santa Cruz). The secondary antibodies were FITC-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG (Santa Cruz).

The number of vwFVIII-positive vessels was counted in a similar way to that for the

capillary density (18). Five fields from each section (n=3) were randomly selected. The number of capillaries was counted manually by two pathologists who were blind of the experiment design. Capillary density was calculated as mean number of capillaries per high power field (HPF) (200×).

**2.10 Flow Cytometry analysis.**

CSCs were collected and washed twice and incubated in the dark at 4°C for 40 min with fluorescein isothiocyanate-labeled ICAM-1 (sc-1511) or VCAM-1 (sc-8304) antibody. Matching isotype antibodies (BD Biosciences) served as controls. Cells were analyzed by Flow Cytometry using a Coulter Epics XL-MCL$^{TM}$ Flow Cytometer (Beckman Coulter). Each analysis included 50,000 events.

**2.11 Western blot.**

Heart tissues were homogenized on ice in 0.1% Tween-20 homogenization buffer containing protease inhibitors. 20 μg of proteins were resolved in 7.5% SDS-PAGE gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). After being blocked with 5% nonfat milk, the membrane was incubated with primary antibody (1:1000 dilution) for 90 min followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG, anti-goat IgG, 1:10000 dilution; Santa Cruz). Protein expression was visualized by enhanced chemiluminescence reaction (Amersham Pharmacia Biotech) and measured by densitometry. The rabbit polyclonal antibody raised against VCAM-1 (1:500,

sc-8304), goat polyclonal antibody raised against ICAM-1 (1:500, sc-1511) were used to identify the protein expression of VCAM-1 and ICAM-1, respectively.

**2.12 Measurement of hemodynamic parameters.**

Measurements of haemodynamic parameters were carried out 28 days after CSC implantation as previously described [18]. Rats were anaesthetized with pentobarbital sodium (60 mg/kg, ip). The carotid artery and femoral artery were isolated. Two catheters that were filled with heparinized (10 U/ml) saline solution and connected to a Statham pressure transducer (Gould, Saddle Brook, NJ, USA) were planted into the carotid artery and femoral arteries. The carotid arterial catheter was advanced into the LV to record ventricular pressure. The femoral artery catheter was inserted into an isolated femoral artery and used to monitor mean arterial pressure (BP) and heart rate. These haemodynamic parameters including left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and rate of rise and fall of ventricular pressure ($+dP/dt_{max}$ and $-dP/dt_{max}$) were monitored simultaneously and recorded on both a thermal pen-writing recorder (RJG-4122, Nihon Kohden, Japan) and an FM magnetic tape recorder (RM-7000, Sony, Tokyo, Japan).

**2.13 Histological and Morphometric analyses of the infarcted hearts.**

28 days after the CSC implantation, the wet weight of rats was measured. After the left ventricular (LV) function was assessed, the hearts were removed, and the infarction size and fibrosis area were measured. For detection of the scar tissue in the

infarcted myocardium with different treatments, LV myocardium was fixed in 10% formalin, cut transversely, embedded in paraffin, and stained with Masson's trichrome solution. Transverse sections (8 μm) were randomly obtained from the level of papillary muscle and were scanned for the scar areas. The ratio of scar length to LV circumferences of the endocardium and epicardium was determined and expressed as a percentage to of the infarct size. In addition, bright field microscopy was performed on hematoxylin and eosin- stained histological section to identify and quantify islands of myofibers in the LV scar tissue. Collagen content was calculated based on Masson's staining and expressed as a percentage of the infarcted and peri-infarction area (n=6). Sixteen separate high-power fields (200×) in each section were photographed under a light microscope with the same exposure time. The collagen volume fraction (CVF) was calculated as a percentage of the infarcted and peri-infarction area using Image Pro 6.0 as described previously [18].

**2.14 Statistical analyses.**

Data are presented as mean ± SD. Statistical significance between two groups was determined by paired or unpaired Student's t-test. Results for more than two experimental groups were evaluated by one-way ANOVA to specify differences between groups. $P < 0.05$ was considered as a significant difference.

**Results**

**The characteristic of CSCs**

To detect the purity of CSCs isolated using the c-kit immunomagnetic microbeads, the CSCs were analyzed by flow cytometry, and the c-kit positive cells accounted for 95% of the total cells sorted (Supplemental sFig 1A). The cells appeared small, round, and not attached to the culture plate (Supplemental sFig 1B). In addition to c-kit, these cells also expressed Nkx2.5, GATA4, MEF2C, CXCR4, Flk-1, Flt-1, NRP-1, and αMHC (Supplemental sFig 1C and sFig 2). A weak expression of CD34, CD44, CD90 and CD45 was also detected in these cells (Supplemental sFig 1D). The expression of NRP-1 and CXCR4 was moderate while the expression of Flk-1 and Flt-1 was high, confirming the CSC phenotype of the isolated cells [23].

**VEGF-A promoted CSC migration *in vitro***

To determine the effect of different VEGF-A subtypes on CSC migration, we performed transwell-based migration assays. As shown in Supplemental sFig 3A-3B, the migration index of CSCs was increased in a dosage-dependent manner with the treatment of VEGF121, VEF165 or VEGF189. VEGF-A appeared to promote CSC migration in a VEGF receptor (VEGF-R)-dependent manner because both VEGF-R1 and VEGF-R2 inhibitors attenuated the CSC migration as shown in Supplemental sFig 4A-4B. Interestingly, VEGF-R inhibitors did not completely block the CSC migration induced by VEGF-A, suggesting that other pathways may also be involved.

**VEGF-A promoted CSC adhesion *in vitro***

In a Polylysine lysine-coated adhesion system, we found that the adhesion index of CSC was increased dosage dependently with the treatment of VEGF121, VEGF165, or VEGF189 (Fig 1A). Similar to CSC migration, VEGF-A-induced CSC adhesion was also VEGF receptor-dependent. Both VEGF-R1 and VEGF-R2 inhibitors blocked the VEGF-A-induced CSC adhesion (Fig 1B). To mimic the CSC adhesion to endothelial cells *in vivo*, we further assessed the CSC adhesion *in* an endothelial cells-mediated adhesion system. As shown in Fig 2A-2B, the CSC adhesion to endothelial cells was increased in a dosage-dependent manner with either VEGF121, VEGF165, or VEGF189 treatment. The adhesion index reached the maximum when cells were treated with 20 ng/ml of VEGF121 or VEGF165 and 10 ng/ml of VEGF189, respectively.

**PKC-α was involved in VEGF-A-mediated CSCs adhesion *in vitro***

Our previous studies have shown that PI3K/Akt was involved in VEGF-mediated CSC migration [23]. To test if VEGF-A-mediated CSC adhesion to ECs involved PI3K/Akt, we pre-treated CSCs with a specific inhibitor of PI3K/Akt, i.e., Wortmannin prior to the cell adhesion assay. As shown in Fig 3A-3B, Wortmannin only attenuated the effect of VEGF121 but not VEGF165 and VEGF189 on CSC adhesion, suggesting that additional signaling pathways may be involved in VEGF-A induced CSC adhesion. Since PKC-α is known to regulate cell adhesion [26], we detected if PKC-α is involved in VEGF-A-induced CSC adhesion by treating CSCs with PKC-α inhibitor Gŏ6976. We found that Gŏ6976 blocked the effects of

VEGF-A-mediated CSC adhesion, suggesting an important role of PKC-α in CSC adhesion (Fig 3A-3B). It appeared that blocking PKC-α had a greater effect on the function of VEGF-121 than VEGF165 or 189 (Fig 3A-3B).

**VEGF-A induced VCAM-1 expression via VEGFR and PKCα**

ICAM-1 and VCAM-1 play important roles in the cell-cell adhesion [24-27]. To determine if VEGF-A-mediated CSC adhesion to ECs was related to ICAM-1 and VCAM-1, we detected ICAM-1 and VCAM-1 expression on CSCs with Flow cytometry (Fig 4A-4D) and western blot (Fig 4E-4L). VEGF-A induced VCAM-1 but not ICAM-1 expression. Importantly, blockade of VEGFR and PKCα inhibited VCAM-1 expression, suggesting that VEGF-A induced VCAM-1 expression through VEGFR/PKCα pathway.

**VCAM-1 was essential for the VEGF-A-mediated CSC adhesion to ECs *in vitro***

Since VCAM-1 expression was regulated by VEGF-A in CSCs, we sought to determine if VCAM-1 is important for VEGF-A-induced CSC adhesion. As shown in Figure 5, similar to the effect of blocking VEGFR, which inhibited the CSC adhesion (Fig 5A and 5B), blockade of VCAM-1 with its specific antibody attenuated VEGF-A-mediated CSC adhesion (Fig 5A and 5C), consistent with the effect of VEGF-A on VCAM-1 expression (Fig 4).

**PKC-α/VCAM-1 was essential for VEGF-A-mediated CSC adhesion *in vivo***

To explore if VEGF-A-mediated CSC adhesion affects its residing in the site of infarcted myocardium, we performed CSC engraftment in infarcted heart and observed the successful adhesion of VEGF-pretreated CSCs in the sites of infarcted myocardium (Fig 6A). VEGF165 treatment appeared to promote a greater accumulation of CSCs than untreatment group. Importantly, VCAM-1 and PKCα played a critical role in CSC residing in the myocardium because VCAM-1 antibody or PKC-α inhibitor Gŏ6976 caused a significant reduction in accumulation of CSCs in the infarcted hearts (Fig 6A). The effect of PKC-α/VCAM-1 on CSC adhesion was also confirmed by the reduced expression of CSC marker genes c-kit, Nkx2.5 and GATA4 (Fig 6C-6E). These data demonstrate that VEGF-A induces CSC adhesion in vivo via PKC-α/VCAM-1.

**VEGF-A-activated CSCs promoted angiomyogenesis *in vivo***

To determine the function of CSC in vivo, we tested if CSCs differentiate to cardiomyocytes and endothelial cells. As shown in Fig 7A and 7B, VEGF-165 promoted the differentiation of CSC into cardiomyocytes (Fig 7A) and endothelial cells (Fig 7B) *in vitr*o as shown by their marker protein expression. To determine if VEGF-A-activated CSCs induce angiomyogenesis in infarcted myocardium and if VEGF functions through PKC-α/VCAM-1, VEGF-A-activated CSCs were pre-treated with VCAM-1-antibody (VCAM-1) or PKC-α inhibitor Gŏ6976 and were implanted in the infarcted myocardium. The angiogenesis was evaluated by the density of microvessel expressing vwFVIII, a mature endothelial cell marker. We found that

VEGF-A-activated CSCs significantly increased the blood vessel formation (Fig 7C), which was blocked by VCAM-1-antibody and PKC-α inhibitor Gŏ6976 (Fig 7C). These data demonstrate that VEGF-A-activated CSCs enhanced angiomyogenesis in infarcted myocardium dependent of PKC-α and VCAM-1.

**VEGF-A-activated CSCs improved cardiac structure and function**

To determine if VEGF-165 repairs cardiac structural damage and functional impairment caused by myocardial infarction, VEGF-A-activated CSCs pretreated with VCAM-1-antibody (VCAM-Ab) or PKC-α inhibitor Gŏ6976 (PKCα-I) were implanted in the infarcted myocardium of ischemic hearts. Masson's staining revealed that blockade of VCAM-1 or PKC-α caused more collagen accumulation in the infarcted and peri-infarction areas 28 days post-MI compared to the control (CSCs and VEGF–CSCs) groups(Fig 7D). Semi-quantitative analysis indicated that the collagen volume fraction in the infarcted area was significantly decreased in VEGF treated group than the vehicle group, but VCAM-1-antibody or PKC-α inhibitor abolished the beneficial effect of VEGF-A. Moreover, the animals that received an injection of VEGF-CSCs had a significantly reduced infarct size (Fig 7E). Functional analysis showed that LV function including LVSP, LVEDP, $+dP/dt_{max,}$ and $–dP/dt_{max}$ was significantly improved with implantation of CSCs treated with VEGF-A (Fig 7G-7J). However, VCAM-1 antibody or PKC-α inhibitor Gŏ6976 significantly reduced VEGF-CSC-improved LV function (Fig 7G-7J).

**Discussion**

Stem cells therapy is currently a promising strategy in repairing MI. The loss of transplanted cells in injured sites, resulting from low efficiency engraftment, is still a curial problem for clinical effects of cells transplantation [11] although MSCs pretreated with epidermal growth factor have showed a better adhesion ability in infarcted myocardium [22]. In this study, we demonstrated that CSCs activated *in vitro* with VEGF-A amplified CSC engraftment, increased angiomyogenesis in myocardium, and improved cardiac function of infarcted heart. To our knowledge, this is the first report showing the importance of adhesion of CSCs in the infarcted heart.

Recent studies have indicated the importance of CSCs in the repair of damaged myocardium. However, the molecular mechanisms controlling CSC engraftment and migration in the ischemic myocardium are unknown [31, 32]. Cell adhesion molecules are involved in the regulation of cell adhesion and migration and wound healing [27]. Interestingly, the expression of integrins, fibronectin, LFA-1, ICAM-1 and VCAM-1 is increased after myocardial infarction [33, 34]. Endogenous or transplanted CSCs expressing α 4 β 1 integrin, connexin 43, connexin 45, N-cadherin and E-cadherin [35] need to adhere and reside in the sites of injured heart to repair the injured heart through these ligand-receptor interactions. In fact, LFA-1 and VLA-4 have been shown to be involved in endothelial progenitor cells homing to ischemic tissue [36, 37]. $CD34^+$ hematopoietic stem cells (HSCs) homing to ischemic hearts is

mainly controlled by SDF-1α/CXCR4 axis [39, 40]. Recent data have shown that the circulating MSCs adhesion to cardiac microvascular endothelium was a VCAM-1-dependent process [38]. β1-integrin, not CXCR4 regulates intramyocardial trafficking and engraftment of MSCs in the ischemic myocardium [33]. Our previous study has revealed that both VEGF-R and CXCR4 are involved in CSC migration into the infarcted heart [18, 23]. Different from MSCs, HSCs and EPCs, CSC adhesion is attributed to VCAM-1. VEGF-A treatment enhanced CSC adhesion to ECs and the sites of infarcted heart through induction of VCAM-1, not ICAM-1, which required PKCα signaling. This may be due to different expression levels of different receptor/ligand, e.g., CXCR4, VCAM-1, ICAM-1, LFA-1 and VLA-4 in CSCs comparing to other stem cells. For example, the levels of VCAM-1, ICAM-1, and CXCR4 in MSCs are much lower compared with HSCs, EPCs and CSCs [9, 23, 39-45]. Moreover, ICAM-1, VCAM-1 mRNA expression was much lower in HSCs, EPCs, cardiosphere-derived CSCs [9, 46], but higher in cells sorting-derived CSCs [9, 23]. Together, these results suggest that different stem cells may use distinctive classes of surface adhesion receptors to establish functional interactions with resident cells or ECM in infarcted heart, thereby differentially influencing intramyocardial homing and engraftment.

VEGF has been shown to be important for the mobilization of CSCs and redirection of their differentiation toward cardiovascular cell types [18, 23, 28 and 29]. Recent study indicates that VEGF is required for the attachment and transendothelial migration of MSCs [30]. In our study, VEGF enhanced adhesion of CSCs to ECs via

VEGF-R *in vitro*. Inhibition of VEGF-R in CSCs blocked the adhesion of CSCs to ECs, demonstrating the importance of VEGF-R activation. VEGF-A treatment induced the expression of VCAM-1 on CSCs and amplified adhesion of CSCs to ECs in vitro and engraftment of CSCs to the ischemic area. The increased adhesion of CSCs produced the beneficial effect on cardiac function and angiogenesis through increasing numbers of cells engraftment to the injured areas of the heart. Although our results support this concept, other possibilities also exist. First, it is possible that VEGF treatment increases survival of the CSCs [16, 28], and if more cells survive *in vivo*, then we would observe more in the infarcted myocardium. Second, treatment of CSCs with VEGF could enhance their "crosstalk" with resident cells, eg, cardiocytes, smooth muscle, endothelial cells, and facilitate their differentiation into myocardial and vascular cells [18, 29], or protect resident cells from apoptosis and death [17]. We have no way of special addressing this possibility although enhanced crosstalk may contribute to the mechanims of VEGF facilitation of the CSCs therapeutic.

Interestingly, SDF-1α/CXCR4 axis regulates HSC adhesion to ECs through LFA-1/I-CAM-1 and VLA4/VCAM-1, thus affecting HSC migration and acrossing the endothelial cell layer, and homing of HSCs [43, 47, and 48]. Hypoxic preconditioning also enhances the benefit of cardiosphere-derived CSC therapy for treatment of MI by inducing CXCR4 expression [46]. Our previous study has shown that condition medium from MSCs induces SDF-1α expression in cardiomyocytes and CXCR4 expression in CSCs, which is attributed to the VEGF in condition medium from MSCs. Both VEGF and SDF-1α promote endogenous CSC mobilization and

migration into the MI site to repair the injured heart [23]. Therefore, VEGF, PKC-α, VCAM-1, and CXCR4 are likely to form an integrated system in the regulation of CSC functions in the cardiac repair after the infarction.

**Acknowledgement**

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**Figure legends**

**Figure 1. Effect of VEGF-A subtypes on CSC adhesion to extracellular matrix.** The adhesion assay was performed as described in method. (A) Quantitative analysis for adhesion of CSCs pretreated with different dosages of VEGF-A subtypes. $^{*, \&, \#}p<0.05$ vs other dose of VEGF-A (n=15). (B) Effect of VEGFR1 or VEGFR2 inhibitor on VEGF-A-induced CSC adhesion. $^{*}p<0.05$ vs VEGFR inhibitor-treated

groups (n=3).

**Figure 2. Effect of VEGF-A subtypes on CSC adhesion to endothelial cells.** The adhesion assay was performed as described in method. (A) Representative image of VEGF-A-induced CSC adhesion to endothelial cells. The CSCs were treated with different dosages of VEGF-121/165/189. (B) Quantitative analysis of CSC adhesion. $^{*, \&, \#}p<0.05$ vs. other VEGF treatment groups (n=3).

**Figure 3. VEGF-A-mediated CSC adhesion to endothelial cells was PKC-α-dependent.** The adhesion assay was performed as described in method. CSCs were pretreated with vehicle (control), PKC-α (Go6976), or PI3k/Akt inhibitor (Wortmannin or WM) as indicated prior to the treatment with VEGF-A subtypes. (A) Representative image of VEGF-A-induced CSC. (B) Quantitative analysis for CSC adhesion. $*p<0.05$ vs control (DMSO) group (n=3).

**Figure 4. PKC-α was required for VEGF-A-induced VCAM-1 expression in CSCs.** (A) Representative cell counts of VCAM-1- or ICAM-1-expressing cells treated with VEGF-A subtypes as measured by Flow Cytometry. (B) Quantitative analysis of the numbers of ICAM-1-positive CSC by Flow Cytometry. $*P>0.05$ vs control group (n=3). (C) Quantitative analysis of the numbers of VCAM-1-positive CSC (VCA-1) by Flow Cytometry. $^{\#, *, \&}p<0.05$ vs VEGF-untreated cells (-); $*p<0.05$ vs VEGF-121 or VEGF-189 treatment group (n=3). (D) The effect of VEGF receptors

and PKC-α on VCAM-1-positive cells treated with VEGF-165 as assayed by Flow Cytometry. CSCs were treated with VEGF type I receptor (AMG706, AMG), type II receptor (VEGF-R2-I), or PKC-α inhibitor (PKC-α-I) prior to VEGF-165 treatment. *$p$<0.05 vs control group; $^{\#}p$<0.05 vs VEGF165-treated group without inhibitor (n=3). (E-F) VEGF-A regulated the VCAM-1, but not the ICAM-1 protein expression in CSCSs. (E) Western blotting for ICAM-1 and VCAM-1 expression in CSCs. (F) Quantitative analysis of ICAM-1 and VCAM-1 expression by normalized to α-Tubulin. n=3, $^{\#,*,\ \&}p$<0.05 vs VEGF-untreated group; *$p$<0.05 vs VEGF-121 or VEGF-189-treated group (n=3). (G-H) The effect of VEGF receptors on the VEGF-165-induced VCAM-1 protein expression in CSCs. CSCs were treated with VEGF type I receptor (AMG) or type II receptor inhibitor (VEGF-R2-I) prior to VEGF-165 treatment. VCAM-1 expression was analyzed by Western blot (G) and quantified by normalized to α-Tubulin (H). *$p$<0.05 vs VEGF-untreated group. $^{\#,\ \&}p$<0.05 vs VEGF-165-treated group (n=3). (I-L) The effect of PKC-α on the VEGF-165-induced VCAM-1 protein expression. CSCs were treated with PKC-α inhibitor (PKC-α-I) prior to the VEGF-165 treatment. VCAM-1 expression was analyzed by Western blot (I) and quantified by normalized to α-Tubulin (L). *$p$<0.05 vs. VEGF165-untreated group. $^{\&}p$<0.05 vs VEGF-165-treated group (n=3).

**Figure 5. VEGF-A-mediated CSC adhesion to endothelial cells was dependent of VEGFR, ICAM-1 and VCAM-1.** The adhesion assay was performed as described in method. CSCs were pretreated with VEGFR inhibitors (AMG706, AMG, or VEGFR2

inhibitor, VEGFR2-I), ICAM-1 or VCAM-1 antibody (Ab) as indicated prior to the treatment with VEGF-A subtypes. (A) Representative images of VEGF-A-induced CSC adhesion. (B) Quantitative analysis for CSC adhesion shown in A. *$p$<0.05 vs VEGFR inhibitor, ICAM-1-Ab or VCAM-1-Ab-pretreated group (n=3).

**Figure 6. VEGF-A promoted CSC engraftment in the infarcted hearts through via VCAM-1 and PKC-α signaling.** CSC engraftment was performed as described in method. (A) Representative image of CSC engraftment in the infarcted myocardium. CSCs were treated with or without VCAM-1 antibody (VCAM-1-Ab) or PKC-α inhibitor (Go6976) prior to the treatment with VEGF165 (VEGF-CSCs). Immunostaining was performed to detect cTnt expression and merged with PKH26-labeled CSCs. (B-E) Quantitative analysis for the efficiency of CSC engraftment in the infarcted myocardium by detecting mean intensity of red fluorescence (indicative of PKH-26 labeled CSCs) and the expression of CSC markers. CSCs were treated with VEGF165 for 8 h following the treatment with VCAM-1 (10 μg/ml) antibody (VCAM-Ab) or PKC-α inhibitor (PKC-α-I) for 1 h. The PKH26-labelled CSCs were then implanted into the infracted heart. Three day after cell transplantation, hearts were collected for detection of the CSC engraftment efficiency. (B) Mean red fluorescence intensity. $^{*}p$<0.05 vs. CSC group; $^{\#,\&}p$<0.05 vs. VEGF-CSCs group (n=5). (C-E) The expression of CSC markers c-Kit (C), Nkx2.5 (D), and GATA4 (E) was detected by real time PCR. n=3, $^{*}p$<0.05 vs. CSC group; $^{\#,\&}p$<0.05 vs. VEGF-CSCs group (n=6).

**Figure 7. VEGF-A-CSC transplantation improved heart function.** (A-B) VEGF165 promoted the differentiation of CSC into cardiomyocytes and endothelial cells *in vitro*. The differentiation of cardiomyocytes and endothelial cells were detected by the expression of cTnt (A) and endothelial marker CD31 (B), respectively (400×). (C) Quantitative analysis of blood vessel density in infarcted areas of rat hearts after CSC transplantation. CSCs were treated with or without VCAM antibody (VCAM-Ab) or PKCα inhibitor (PKCα-I) prior to the treatment of VEGF (VEGF-CSC) as indicated. CSC transplantation was performed as described in Methods. **P<0.001* vs non-transplantation (Ctrl) or CSC group; $^{\#,\ \&}$*P<0.01* vs. VEGF-CSC group (n=6). (D) Representative heart sections with Masson's trichrome staining (400 × ). VEGF attenuated cardiac fibrosis by PKCα/VCAM. (E) Quantification of collagen content in the infarcted hearts. **P*<0.05, vs. CSC group; $^{\&,}$ $^{\#}$*P*<0.01 vs. VEGF-CSC group (n=6). (F) Quantification of infarction sizes. **P*<0.05, vs. CSC group; $^{\&,\ \#}$*P*<0.01 vs. VEGF-CSC group (n=6). (G-J) VEGF-CSC improved the left ventricular (LV) function of infarcted hearts. LV function was measured under the baseline resting conditions 28 days after treatment. (G) Left ventricular systolic pressure (LVSP). (H) Left ventricular end-diastolic pressure (LVEDP). (I, J) Rate of rise and fall of ventricular pressure ($+dP/dt_{max}$ (I) and $-dP/dt_{max}$ (J). **P*<0.05, vs. CSC group; $^{\&,\ \#}$*P*<0.01 vs. VEGF-CSC group (n=6).

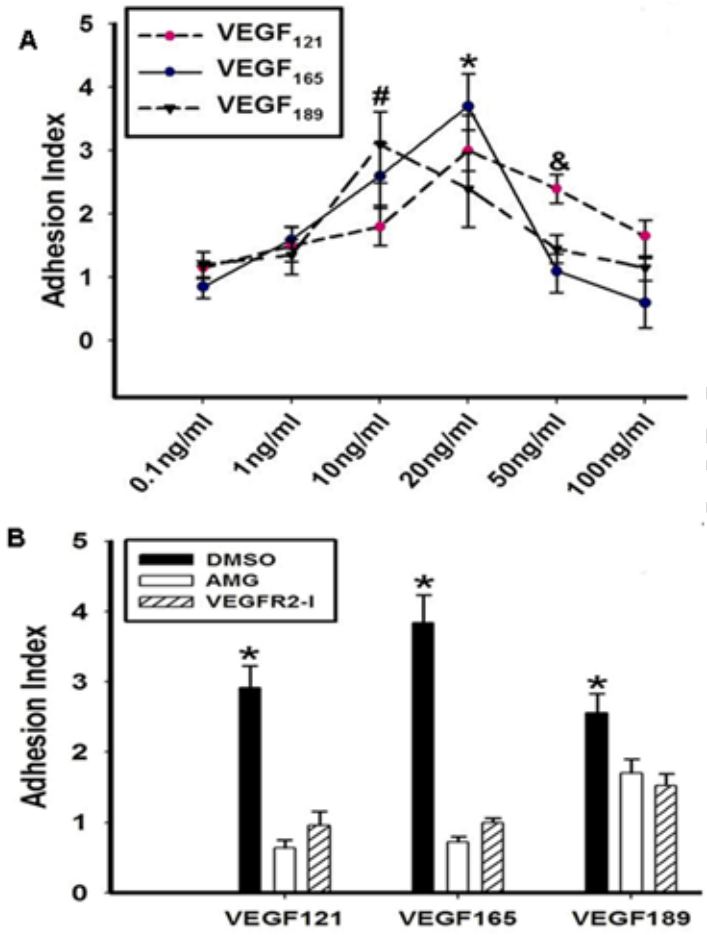



Fig. 1

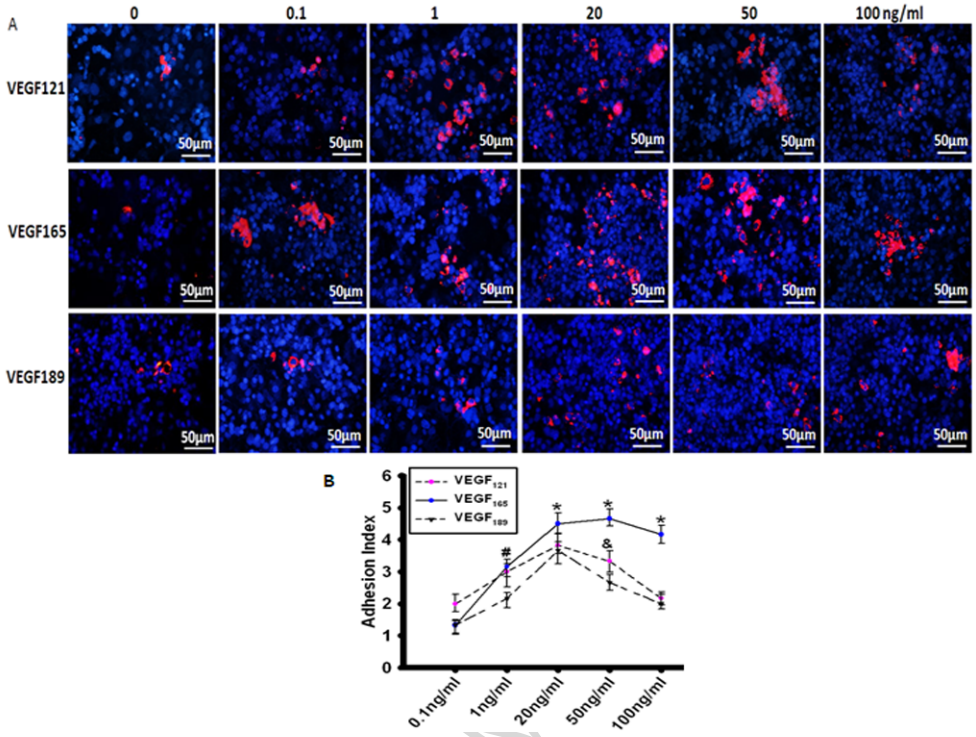



Fig. 2

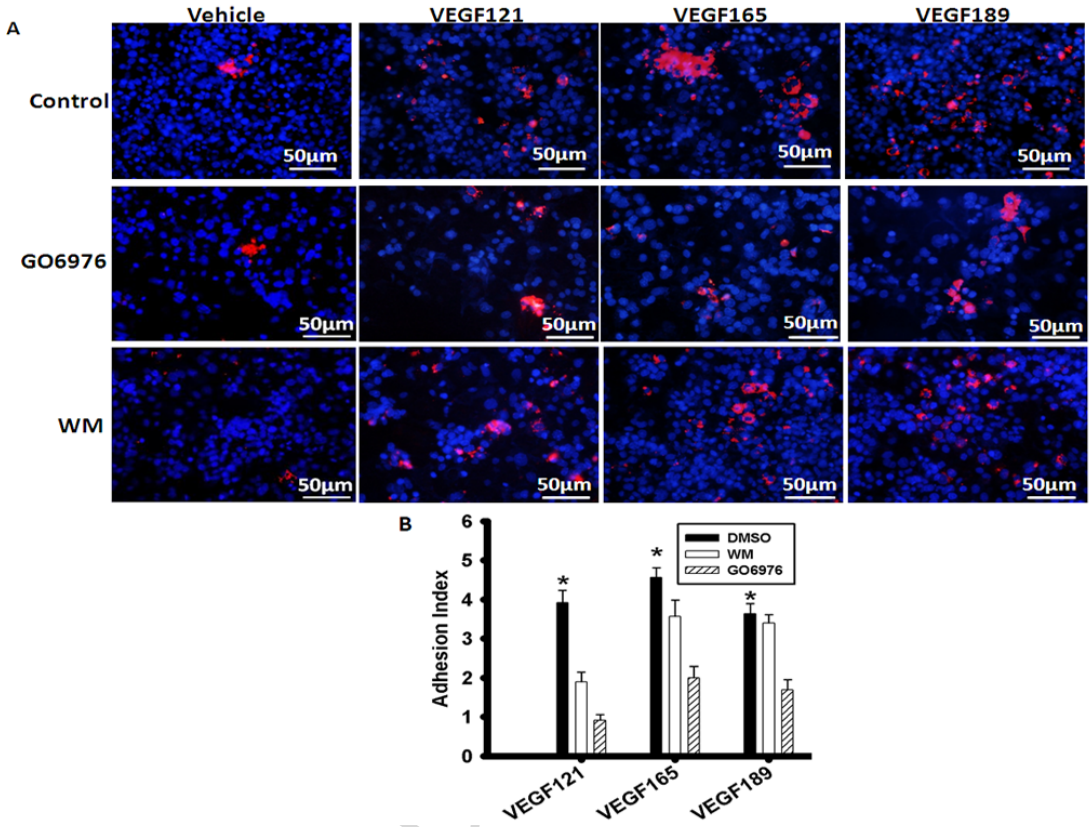



Fig. 3

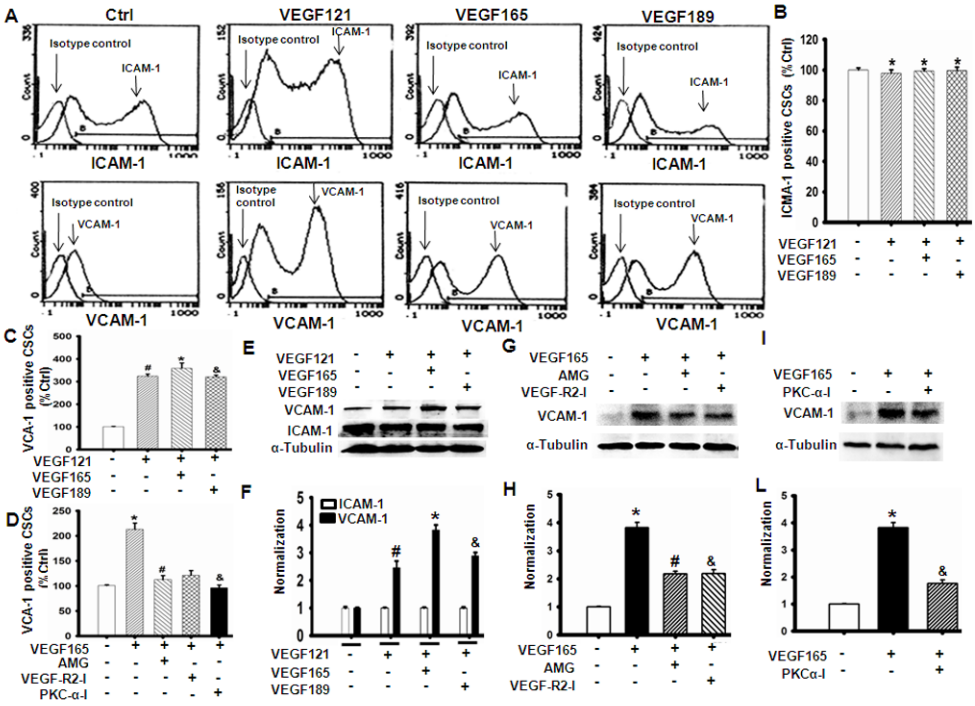



Fig. 4

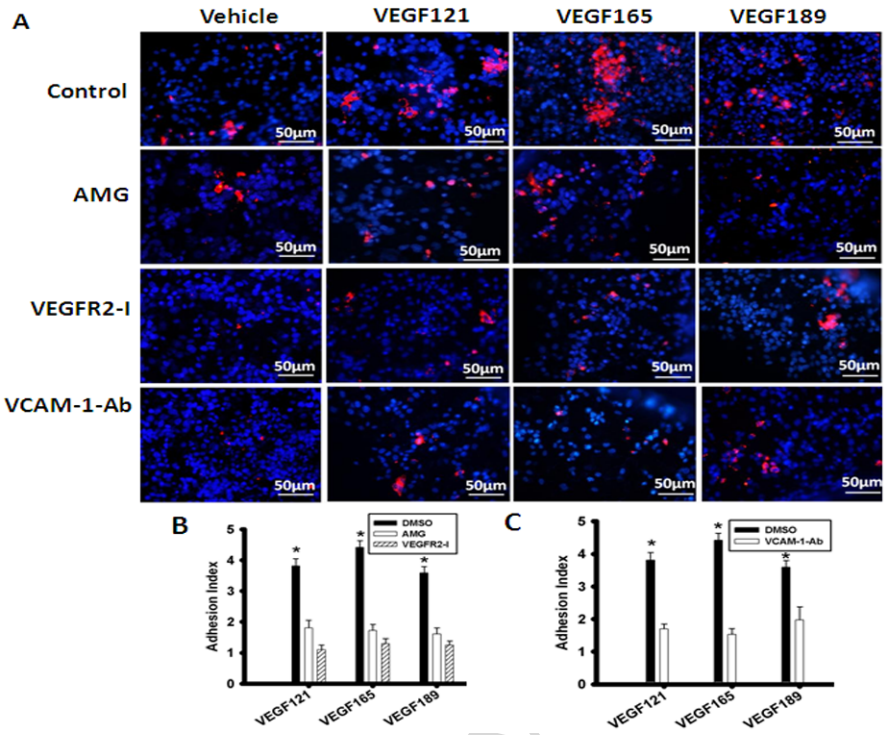



Fig. 5

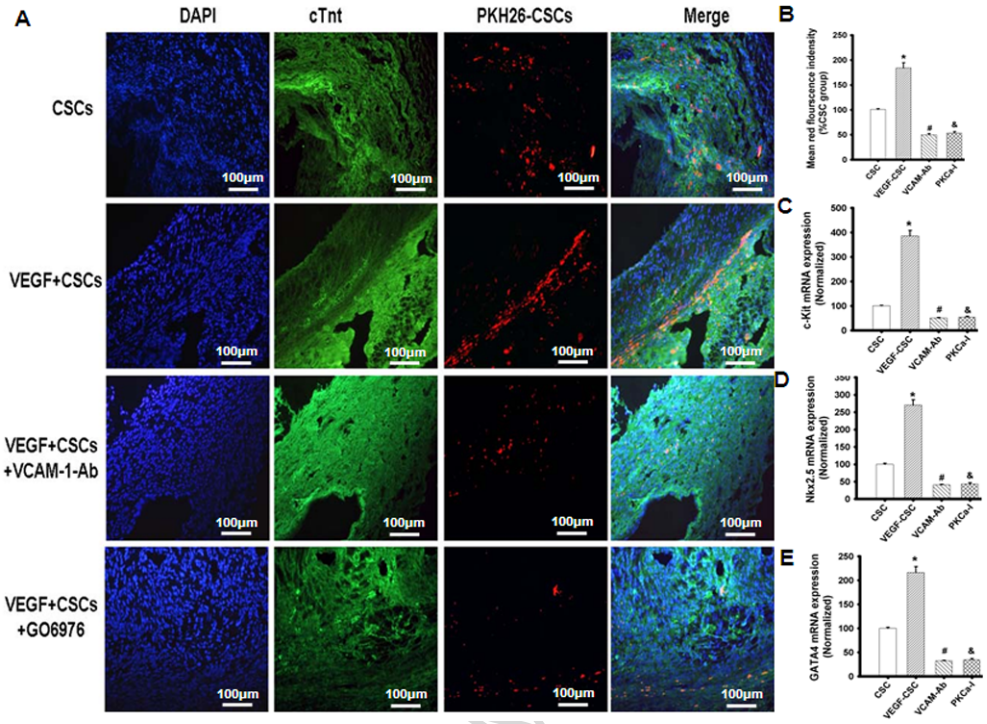



Fig. 6

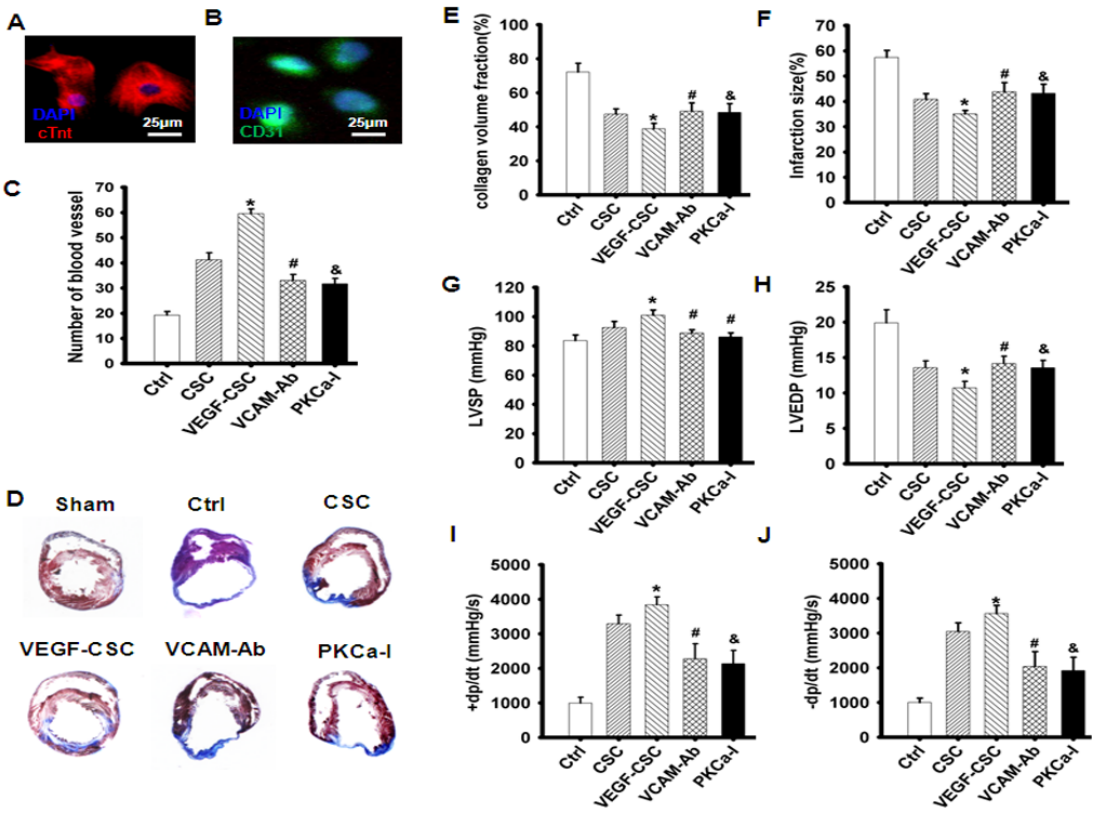
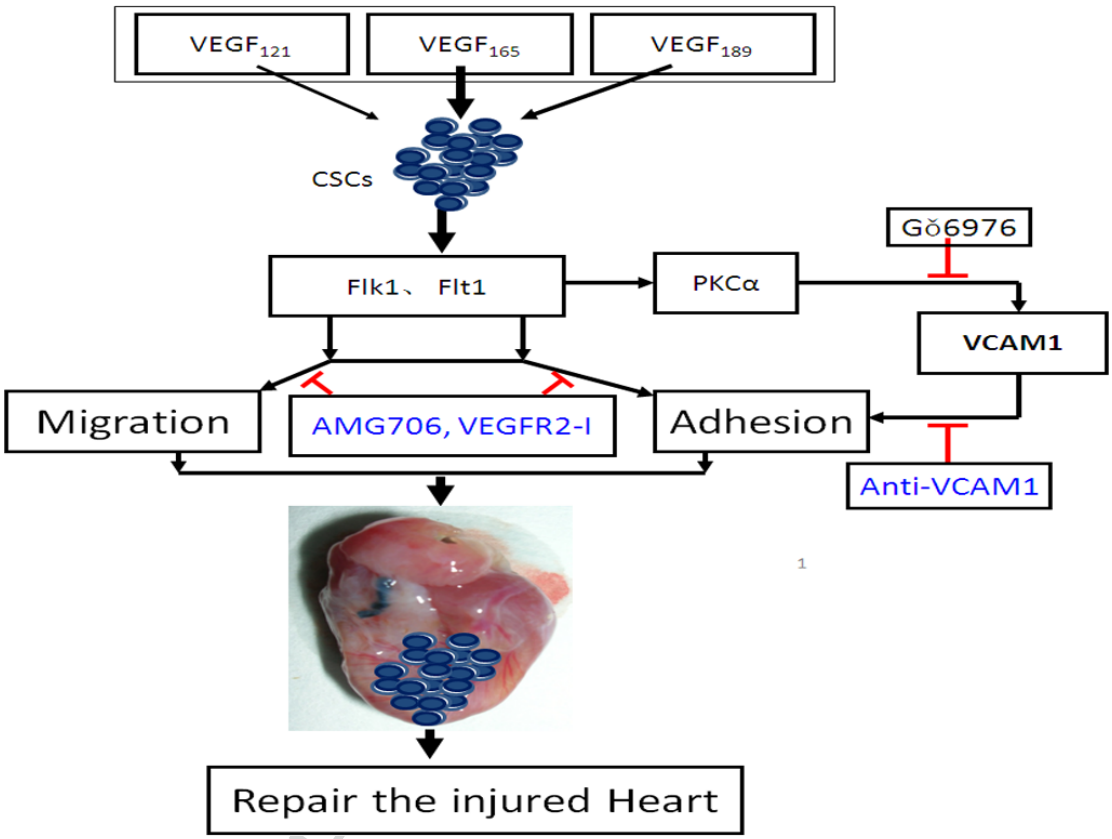



Fig. 7

Table 1: PCR conditions.

| Gene | Fragment size(bp) | Forward (5'-3') | Reverse (5'-3') | Annealing (℃/s) |
|---|---|---|---|---|
| Nkx2.5 | 163 | ACAATTCACTCCCAGCATCC | GGCTTGGACACCTTGTGTTT | 59 |
| GATA4 | 165 | CTGTGCCAACTGCCAGACTA | AGATTCTTGGGCTTCCGTTT | 59 |
| c-Kit | 171 | TTCCTGTGACAGCTCAAACG | AGCAAATCATCCAGGTCCAG | 58 |
| GAPDH | 207 | AGACAGCCGCATCTTCTTGT | CTTGCCGTGGGTAGAGTCAT | 59 |

Graphical abstract



Highlights

VEGF-A treatment enhanced CSC adhesion to ECs and the sites of infarcted heart through induction of VCAM-1, not ICAM-1, which required PKCα signaling.

CSCs activated *in vitro* with VEGF-A amplified CSC engraftment, increased angiomyogenesis in myocardium, and improved cardiac function of infarcted heart.