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Use of cationic microbubbles targeted to P-selectin to improve ultrasound-mediated gene transfection of *hVEGF*$_{165}$ to the ischemic myocardium*

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Abstract: Gene therapies have been applied to the treatment of cardiovascular disease, but their use is limited by the need to deliver them to the right target. We have employed targeted contrast ultrasound-mediated gene transfection (TCUMGT) via ultrasound-targeted microbubble destruction (UTMD) to transfer therapeutic genes to specific anatomic and pathological targets. Phospholipid microbubbles (MBs) with pcDNA$_{3.1}$-human vascular endothelial growth factor 165 (pcDNA$_{3.1}$-hVEGF$_{165}$) plasmids targeted to P-selectin (MB+P+VEGFp) were created by conjugating monoclonal antibodies against P-selectin to the lipid shell. These microbubbles were divided into four groups: microbubble only (MB), microbubble+P-selectin (MB+P), microbubble+pcDNA$_{3.1}$-hVEGF$_{165}$ plasmid (MB+VEGFp), and microbubble+P-selectin+pcDNA$_{3.1}$-hVEGF$_{165}$ plasmid (MB+P+VEGFp). The reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) results showed that the *VEGF* gene was successfully transfected by TCUMGT and the efficiency is increased with P-selectin targeting moiety. UTMD-mediated delivery of *VEGF* increased myocardial vascular density and improved cardiac function, and MB+P+VEGFp delivery showed greater improvement than MB+VEGFp. This study drew support from TCUGMT technology and took advantage of targeted ultrasound contrast agent to identify ischemic myocardium, release pcDNA$_{3.1}$-hVEGF$_{165}$ recombinant plasmid, and improve the myocardial microenvironment, so promoting the restoration of myocardial function.

Key words: Vascular endothelial growth factor (VEGF); P-selectin; Targeted contrast ultrasound-mediated gene transfection; Heart function

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1 Introduction

New anti-myocardial ischemia medicines and the development of coronary artery bypass surgery have significantly improved the quality of life for patients suffering from coronary disease. However, these methods still cannot save dead myocardial cells, restore myocardium, or improve ventricular diastolic and systolic function. Gene therapy has been shown to reduce adverse cardiac remodeling and can improve recovery of ventricular function as well as enhance cell survival and angiogenesis (Yau et al., 2001; Bekeredjian et al., 2005), but the efficiency of gene delivery to target tissues still limits its application. In recent years, targeted contrast ultrasound-mediated gene transfection (TCUMGT) has shown promise for

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organ-specific gene and drug delivery (Bekeredjian et al., 2005). TCUMGT can transfer therapeutic genes via a microbubble contrast agent to specific anatomic and pathological targets (Sirsi and Borden, 2012). After being injected intravenously into target tissues, the agent will undergo cavitation because of the ultrasound energy (ultrasound-targeted microbubble destruction, UTMD) with increased gaps between the target organ endothelial cells and capillary permeability, and its encapsulated genes are released in a particular orientation to the extravascular matrix of target tissue, hence uplifting the partial concentration and increasing the cell ingestion of genes (Ferrara et al., 2007; Sirsi and Borden, 2012).

Vascular endothelial growth factor (VEGF) is a kind of highly specific and strong vascular endothelial cell mitogenic and angiogenic factor, which can regulate most links in angiogenesis, including endothelial extracellular matrix dissolution and migration. It can also act directly on vascular endothelial cells in a specific manner to trigger endothelial cell proliferation, hence playing the role of "triggering" in angiogenesis (Hoeben et al., 2004). Humans have at least five types of VEGF consisting of 121, 145, 165, 186, and 206 amino acids. As $VEGF_{165}$ has the greatest biological effect (Hoeben et al., 2004), its gene has been developed for cardiac function restoration after myocardial infarction. However, as VEGF concentration in blood is not maintained after injection (Douvaras et al., 2009), TCUMGT can be a powerful approach to deliver the organ-specific gene and drugs.

Acute myocardial ischemia-reperfusion can lead to the up-regulation of leucocyte adhesion molecules in blood vessel endothelium until ischemia alleviation. Studies demonstrated that the expression of P-selectin occurs in blood vessel endothelium within several minutes of ischemia-reperfusion. After ischemia alleviation, adhesion molecules can stick to the vascular walls, leaving "marks" of ischemia (Zhao et al., 2003). Xie et al. (2012) reported that the transfection of complementary DNA (cDNA) using ultrasound to destroy P-selectin-targeted cDNA gene microbubble for located release at lower limb ischemic muscles of rats was effective. We therefore constructed P-selectin-targeted and $pcDNA_{3.1}$-$hVEGF_{165}$ plasmid-coupled cationic microbubbles (CMBs) to test the hypothesis that their use improves ultrasound-mediated gene transfection of $hVEGF_{165}$ to the ischemic myocardium.

2 Materials and methods

2.1 Preparation of cationic microbubbles

Biotinylated CMBs were prepared by adding disaturated phosphatidylcholine, polyethylene glycol (PEG) 40 stearate, and distearoylphosphatidylethanolamine-PEG2000-biotin (Avanti Polar Lipids, USA) to aqueous suspension. The CMB suspension was prepared by sonication with octafluoropropane (C_3F_8) gas (Shengtang Gas, China). The P-selectin-targeted microbubbles were prepared by conjugating biotin rat anti-mouse P-selectin to the surface of biotinylated microbubbles using a streptavidin link (Lindner et al., 2001). The plasmid (50 μg) was then coupled to the CMBs (1×10^8 microbubbles) by incubation for 15 min (Christiansen et al., 2003; Xie et al., 2012). The structure of the CMB is shown in Fig. 1.

2.2 CMB characterization

The mean diameter and zeta potential of the CMBs were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern, UK). The targeted microbubbles were subjected to fluorescent-targeted secondary antibodies against the targeting moiety. Plasmid and the antibody conjugation to P-selectin was observed using fluorescence microscopy (CKX41; Olympus, Japan).

2.3 Animal model

Adult male Sprague-Dawley rats (weight 200–225 g; n=4 per group) were obtained from Taconic Biosciences (Hudson, NY, USA). The left anterior coronary artery of the rat was occluded for 60 min under general anesthesia to generate ischemia/reperfusion (I/R) and followed by reperfusion (Fujii et al., 2011). The Institutional Animal Care and Use Committee at the Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China approved all animal procedures.

2.4 UTMD delivery

The UTMD delivery procedure was performed 5 d after I/R as previously reported (Sun et al., 2013). The microbubble solution (1.2 ml/h) was infused into the tail vein of the sedated rats (2% isoflurane). Simultaneously, ultrasound pulses were delivered for 20 min using a Vivid 7 system (GE Healthcare, USA) with an M3S transducer. The ultrasound system was

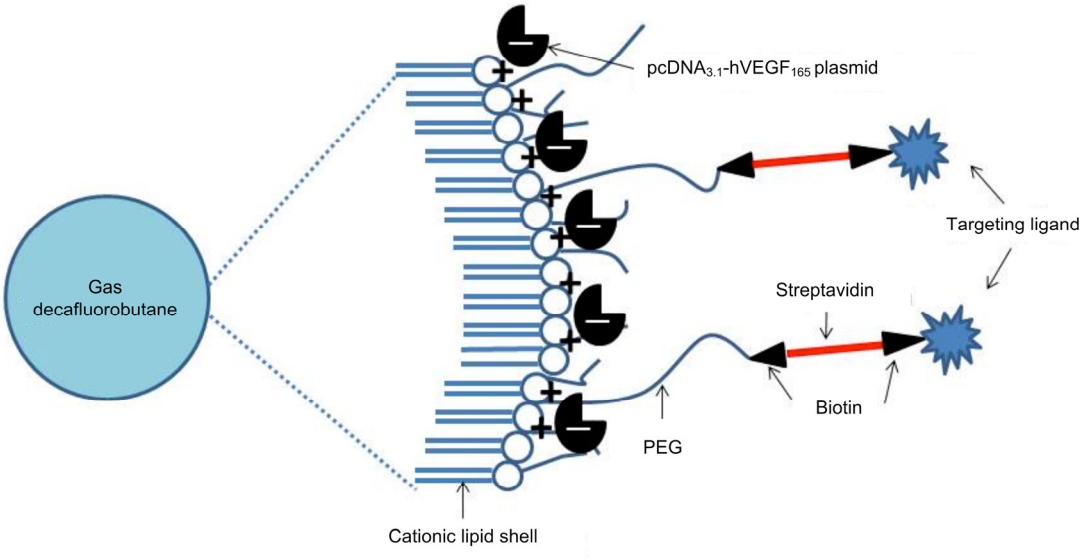

Fig. 1 Structure of P-selectin-targeted and pcDNA$_{3.1}$-hVEGF$_{165}$ plasmid-coupled cationic microbubble
PEG: polyethylene glycol

operated in the second harmonic mode with an electrocardiograph trigger. Pulsing interval was adjusted to allow for complete myocardium replenishment of microbubbles between bursts. After each treatment, rats were recovered.

2.5 ELISA

Seven days after TCUMGT treatment, peripheral blood was collected and plasma was harvested after centrifugation. Enzyme-linked immunosorbent assay (ELISA) was performed to detect the content of VEGF according to the manufacturer's instructions.

2.6 RT-PCR

Seven days after TCUMGT treatment, infarcted myocardium cells were collected and washed with phosphate-buffered saline (PBS), and RNA was extracted using RNeasy Plus Micro Kit (QIAGEN, USA). Then, cDNA was synthesized with Revert AIDTM First Strand cDNA Synthesis (TaKaRa, Dalian, China). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed with Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, USA) under the following conditions: 95 °C for 30 s, 95 °C for 5 s and 60 °C for 34 s for a total of 45 cycles. The messenger RNA (mRNA) expression of VEGF was detected by PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. Primers were synthesized in TaKaRa (Dalian, China). All data are displayed as the mean±standard deviation (SD) of three independent experiments.

2.7 Preparation of sections of frozen heart tissue

The hearts were perfused with normal saline and removed from euthanized animals. They were then snap-frozen in liquid nitrogen and stored at −80 °C. The 10-μm slides were sectioned from the frozen tissue and cells were confirmed by green fluorescent protein (GFP) expression under fluorescent microscopy.

2.8 Vascular density measurement

Sections of the infarct border region were obtained on Day 21 after I/R and double immunofluorescent staining for α-smooth muscle actin (α-SMA) and factor VIII (Kelly et al., 2015). The primary antibodies used for immunofluorescence analysis were as follows: Cy3-conjugated mouse anti-α-SMA (Sigma-Aldrich, USA) and mouse anti-human factor VIII (Ray Biotech, USA). The appropriate secondary antibodies for α-SMA and factor VIII were obtained from Invitrogen (USA). 4'-6-Diamidino-2-phenylindole (DAPI) was used to counterstain the myocardium tissue. Capillaries and arterioles were quantified in six fields per slide under a fluorescent microscope (200×)

and all data are displayed as the mean±SD. The mean number of vessels per 0.4 mm^2 was taken to be the blood vessel density.

2.9 Myocardial function measurement

On the day of I/R, and then 5 and 21 d later, end-systolic and end-diastolic diameters and volumes of the left ventricle were evaluated using echocardiography. The left ventricular structural parameters were used in the calculation of left ventricle ejection fraction (LVEF) and left ventricle fractional shortening (LVFS).

2.10 Statistical analysis

Data were presented as mean±SD. All experiments were repeated at least three times. Statistics were performed using a statistical software package (SPSS 13.0; SPSS, USA). Student's *t*-test was used to compare the data between two groups; for multiple group comparison, an analysis of variance (ANOVA) test was performed. $P<0.05$ was considered statistically significant.

3 Results

3.1 Characterization of CMBs

The newly prepared CMBs were characterized by DLS. The results showed that they had a Zeta-potential of (23.25±1.58) mV and an average diameter of (4.12±0.92) μm (Fig. 2a). To confirm the conjugation of the P-selectin antibody and plasmid to the CMBs, the plasmid was labeled with the nucleotide-avid fluorophore YOYO-1 (green fluorescence, excitation/emission: 491/509 nm; Thermo Fisher Scientific, USA) and the P-selectin antibody was labeled with rhodamine mouse anti-human immunoglobulin (Ig) G (red fluorescence, excitation/emission: 550/570 nm; Thermo Fisher Scientific, USA). The plasmid and P-selectin antibody were observed under a fluorescence microscope. CMBs subjected to fluorescent-targeted secondary antibodies were bright green or red under fluorescence microscopy, suggesting that the CMBs were conjugated with the plasmids or P-selectin antibody with high efficiency (Fig. 2b). The merged view of CMBs fluoresced yellow, confirming that both plasmids and P-selectin are colocalized to the same CMB.

3.2 Effect of TCUMGT of $pcDNA_{3.1}$-$hVEGF_{165}$ on myocardial levels of $VEGF_{165}$

The TCUMGT was performed 5 d after I/R. We used the pAcGFP1 vector (TaKaRa, Dalian, China) to study the transfection efficiency of microbubbles with P-selectin targeting, and prepared CMBs into four groups: microbubble only (MB), microbubble+P-selectin (MB+P), microbubble+pAcGFP1 vector (MB+GFPp), and microbubble+P-selectin+pAcGFP1 vector (MB+P+GFPp). The CMBs in the four groups were delivered 5 d after I/R and released with UTMD.

(a)

Zeta-potential (mV)	Diameter (μm)	Concentration ($\times10^9$)
23.25±1.58	4.12±0.92	4.84±0.32

(b)

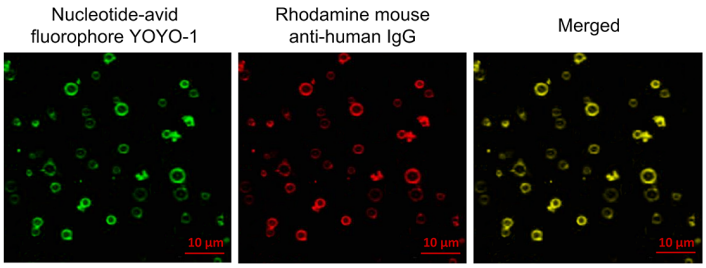

Fig. 2 Characterization of cationic lipid-shelled decafluorobutane microbubbles

(a) Dynamic light scattering measurements of the mean diameter and Zeta-potential of the cationic microbubbles (CMBs). (b) P-selectin antibody and $pcDNA_{3.1}$-$hVEGF_{165}$ plasmid targeted microbubbles as observed under a fluorescent microscope (×100 magnification) to show the nucleotide-avid fluorophore YOYO-1 and rhodamine mouse anti-human immunoglobulin (Ig) G labeled microbubbles. The merged view was shown

Frozen sections were prepared and GFP expression within the myocardium was measured under fluorescent microscopy 7 d after gene delivery (Figs. 3a–3d). The intensity of GFP-positive cells in heart tissue was significantly greater with MB+P+GFPp than with MB+GFPp delivery (Fig. 3e; $P<0.01$). To evaluate delivery of the therapeutic pcDNA$_{3.1}$-hVEGF$_{165}$ gene into the myocardium, the microbubble groups were set as microbubble only (MB), microbubble+P-selectin (MB+P), microbubble+pcDNA$_{3.1}$-hVEGF$_{165}$ plasmid (MB+VEGFp), and microbubble+P-selectin+pcDNA$_{3.1}$-hVEGF$_{165}$ plasmid (MB+P+VEGFp). Each group was divided into two subgroups (n=4 per subgroup): one for *VEGF*$_{165}$ expression studies and one for myocardial vascular density and cardiac function studies. The expression levels of VEGF$_{165}$ protein in the infarcted myocardium and peripheral blood were determined by RT-PCR (Fig. 3f) and ELISA (Fig. 3g) 7 d following UTMD-mediated plasmid delivery. As shown in Figs. 3c and 3d, VEGF$_{165}$ protein levels were significantly higher with MB+P+VEGFp than with MB+VEGFp delivery ($P<0.01$), suggesting that *VEGF* gene transfection by UTMD was successful and that the efficiency is increased with P-selectin targeting moiety.

3.3 Effect of TCUMGT of pcDNA$_{3.1}$-hVEGF$_{165}$ on myocardial vascular density and cardiac function

At 21 d after I/R (16 d after TCUMGT), we stained the infarct border region with factor VIII and α-SMA immunofluorescent staining to identify the small (mainly capillaries) and larger (arterioles) vascular structures, respectively. As shown in Fig. 4, the densities of both types of vessel were significantly increased in the MB+P+VEGFp compared with the MB+VEGFp delivery groups ($P<0.01$). These results further proved that the *VEGF* gene was transfected by UTMD, the efficiency increasing with the P-selectin targeting moiety.

Cardiac function was evaluated by echocardiography (Figs. 4d and 4e). Before I/R (Day 0), the ejection fraction (Fig. 4d) was at approximately 63% in all groups. Prior to gene delivery, it decreased to approximately 52% at 5 d after I/R. However, the

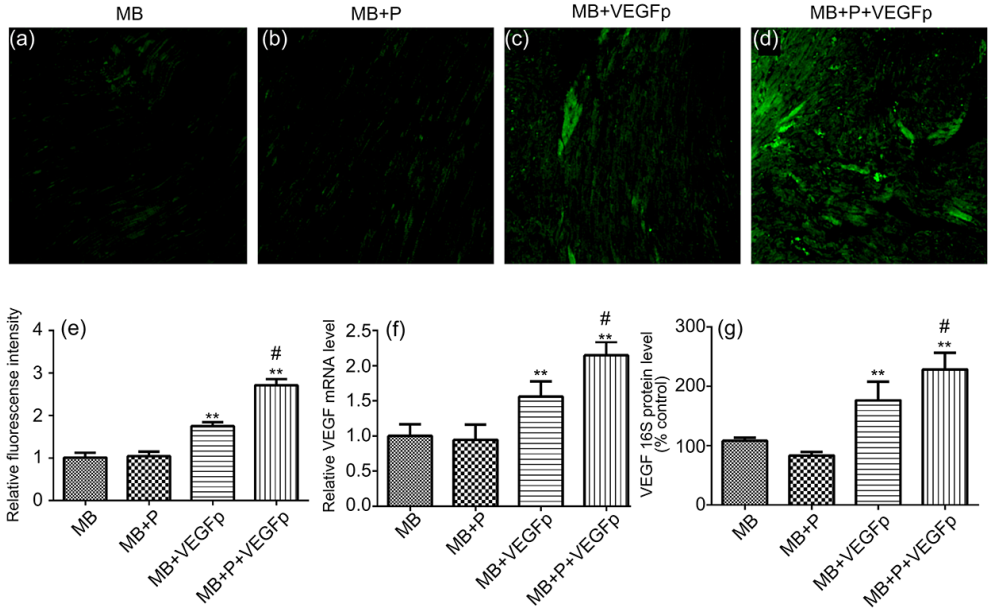

Fig. 3 Effect of TCUMGT of pcDNA$_{3.1}$-hVEGF$_{165}$ on myocardial levels of VEGF$_{165}$

(a–d) Presence of GFP-positive cells in injured myocardium. GFP-positive spots were detected under fluorescent microscopy (×200 magnification). (e) Relative fluorescence intensity of GFP-positive cells in different groups. (f) The mRNA expression levels of *VEGF*$_{165}$ in the infarcted myocardium and (g) the protein expression levels of VEGF$_{165}$ in the peripheral blood determined by RT-PCR and ELISA, respectively, 7 d following UTMD-mediated plasmid delivery. MB: microbubble only (control); MB+P: microbubble+P-selectin; MB+VEGFp: microbubble+pcDNA$_{3.1}$-hVEGF$_{165}$ plasmid; MB+P+VEGFp: microbubble+P-selectin+pcDNA$_{3.1}$-hVEGF$_{165}$ plasmid. Data are expressed as mean±SD (n=3). $^{**}P<0.01$ vs. MB group; $^{\#}P<0.05$ vs. MB+VEGFp group

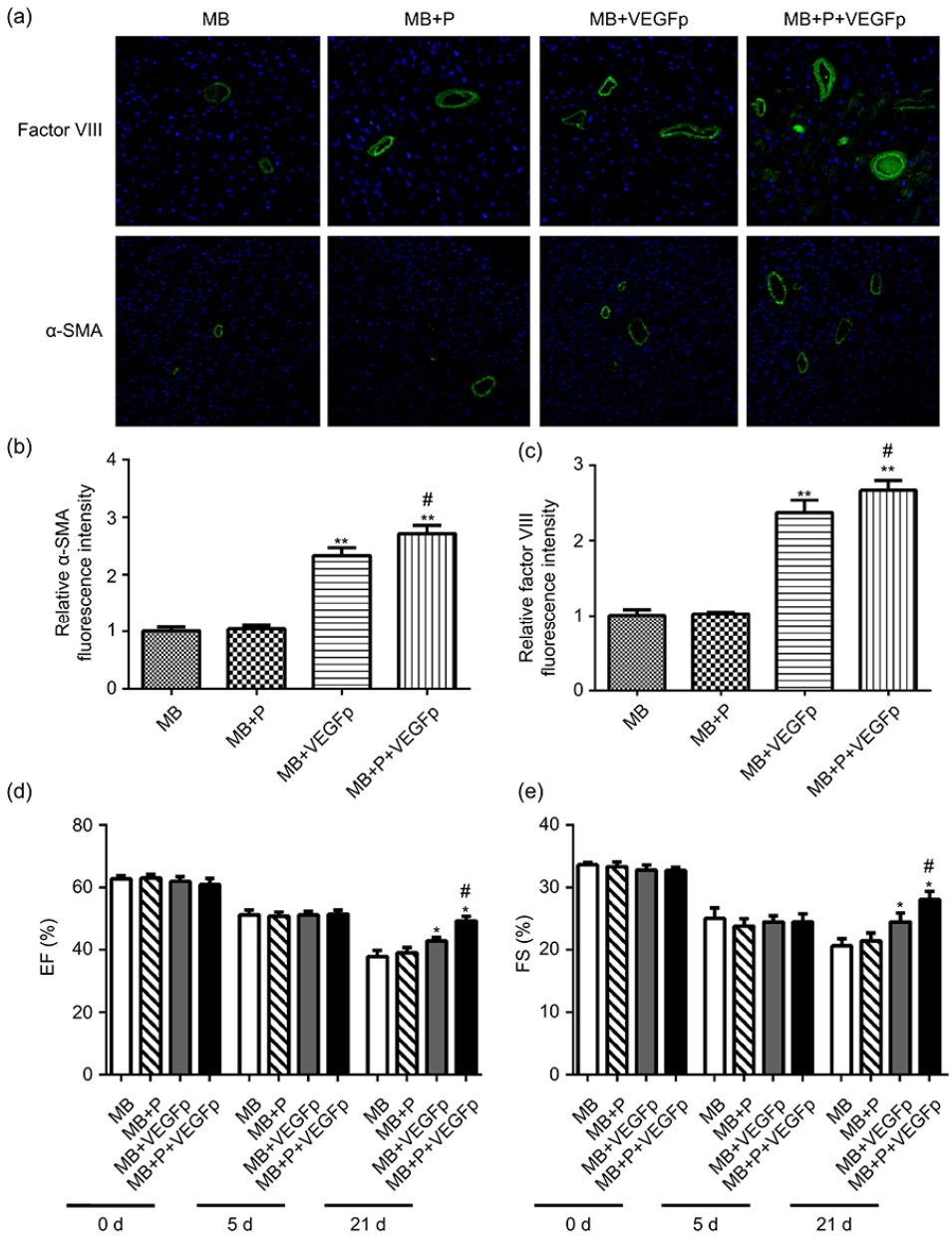

Fig. 4 Blood vessel density and myocardial function following UTMD-mediated pcDNA$_{3.1}$-hVEGF$_{165}$ delivery
(a) The infarct border region was immunofluorescent-labeled with α-SMA or factor VIII (×200 magnification); (b, c) Relative fluorescence intensities of α-SMA (b) and factor VIII (c) were detected. The densities of arteriolar (b) and capillary (c) vessels within the border region of the infarct were significantly increased with MB+P+VEGFp compared with the MB+VEGFp delivery. Ejection fraction (d) and fractional shortening (e) were determined by echocardiographic examination. Data are expressed as mean±SD (n=3). $^{*}P$<0.05, $^{**}P$<0.01 vs. MB group; $^{\#}P$<0.05 vs. MB+VEGFp group. EF, ejection fraction; FS, fractional shortening

ejection fraction was significantly increased in the MB+P+VEGFp and MB+VEGFp delivery groups compared with the MB or MB+P controls at 21 d after I/R (P<0.05). The greatest improvement was seen in the MB+P+VEGFp delivery group (P<0.05 vs. MB+VEGFp). A similar pattern was seen in the fractional shortening results (Fig. 4e). At Day 0, fractional shortening of about 34% was exhibited in all groups, which decreased to approximately 24% at 5 d after I/R. At 21 d after I/R, MB+P+VEGFp delivery showed the greatest improvement in fractional shortening (P<0.05 vs. MB+VEGFp).

4 Discussion

Following myocardial infarction (MI), ventricular dysfunction and heart failure are the major causes of morbidity and mortality (Sutton et al., 1997; Sutton and Sharpe, 2000; Sun et al., 2013). In recent years, intravascular gene therapy has been applied to prevent adverse ventricular remodeling after MI with promising results (Jessup et al., 2011; Sun et al., 2013). However, the efficiency of gene delivery is a major limiting factor. In this study, biotinylated CMBs were constructed, which carry a positive surface charge and can combine with negatively charged cDNA through electrostatic interaction, and can also combine with target-substrate antibodies through biotin-avidin interaction via PEG on the microbubble surface (Xie et al., 2012; Unger et al., 2014). Xie et al. (2012) reported the effective transfection of cDNA using ultrasound to cavitate P-selectin-targeted cDNA gene microbubbles for located release of lower limb ischemic muscles in rats. In our study, we took advantage of the microbubbles, conjugating biotin rat anti-mouse P-selectin to the surface of biotinylated microbubbles using a streptavidin link to construct P-selectin-targeted microbubbles, and coupling functional plasmids to the CMBs (Fig. 1). The CMBs prepared in this study exhibit a positively charged surface ((23.25±1.58) mV) and an average diameter of (4.12±0.92) μm, which is suitable for the delivery system. Also, both plasmids and P-selectin can be properly colocalized to the same CMB in order to take full advantage of the targeted delivery property of the microbubbles.

Acute myocardial ischemia-reperfusion can lead to the up-regulation of adhesion molecules in blood vessel endothelium until ischemia alleviation. Many studies have shown that the ischemic area can be identified earlier by connecting antibodies of specific adhesion molecules to the microbubble surface enabling them to reach tissues or organs of interest by associating selectively with corresponding adhesion molecules (Leung, 2004; Liu et al., 2012; Xie et al., 2012). Lindner et al. (2001) and Hu et al. (2016) compared P-selectin monoclonal antibody with the control group and concluded that the stay time of microbubbles inside ischemic area could be significantly increased, with enhanced ultrasound signal strength. Among the various adhesion molecules, P-selectin is distinguished by quick expression on the surface of blood vessel endothelium even in the case of slight ischemia, and continuous up-regulation for several hours (Liu et al., 2012). It has been demonstrated that P-selectin monoclonal antibody contrast agent selectively adheres to blood vessel endothelium in ischemic tissue and generates signals in heart, brain, and kidney ischemia reperfusion injury models (Lindner et al., 2001; Takalkar et al., 2004; Ferrante et al., 2009). These studies have provided the foundation for studying the identification of ischemic myocardium with the goal of targeting therapy at an early stage. Therefore, in this study, we prepared CMB bearing a P-selectin targeting moiety. To investigate the transfection efficiency of microbubbles with P-selectin targeting, a GFP-containing plasmid, pAcGFP1, was coupled to the CMBs. Our results showed that the fluorescence intensity of GFP-positive cells in heart tissue 12 d after I/R was significantly greater with the MB+P+GFPp than with MB+GFPp delivery. This indicates that microbubbles with P-selectin targeting can efficiently target P-selectin on vascular walls induced by ischemia-reperfusion.

Recently, VEGF protein has received great attention in cardiac function restoration after MI. VEGF is involved in the induction of vascular endothelial cell proliferation and angiogenesis (Hoeben et al., 2004; Gerhardt, 2008). Yu et al. (2015) showed that combined myocardial mesenchymal stem cells (MSCs) and recombinant human $VEGF_{165}$ plasmid injection could improve cardiac function. However, the direct injection method is associated with large myocardial damage and low bone mesenchymal stem cell (BMSC) survival rate, even causing death and arrhythmia, so it can only be conducted in coronary artery bypass grafting, limiting clinical application (Everaert et al., 2012). We chose TCUMGT as a safer and more effective means to deliver P-selectin-targeted $pcDNA_{3.1}$-$hVEGF_{165}$ genes to the ischemic myocardium, promoting angiogenesis. The results showed that the *VEGF* gene was successfully transfected by UTMD and the efficiency was increased with P-selectin targeting moiety. Also, UTMD-mediated delivery of *VEGF* increased myocardial vascular density and improved cardiac function. This study drew support from TCUGMT technology and took advantage of targeted ultrasound contrast agent to identify ischemic myocardium, release $pcDNA_{3.1}$-$hVEGF_{165}$

recombinant plasmid, and improve the myocardial microenvironment, so promoting the restoration of myocardial function.

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Compliance with ethics guidelines

Wei-hui SHENTU, Cao-xin YAN, Chun-mei LIU, Rui-xiang QI, Yao WANG, Zhao-xu HUANG, Li-ming ZHOU, and Xiang-dong YOU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：超声靶向击碎微泡技术介导 P-选择素靶向阳离子超声微泡改善人血管内皮生长因子 165 基因转染缺血心肌的实验性研究

目　的：构建一种靶向阳离子微泡，探讨其提高超声靶向击碎微泡技术（TCUMGT）介导的体内基因转染效率及治疗效果。

创新点：提出运用 TCUMGT 介导的基因转染技术，利用微泡的携基因和靶向定位释放双项功能来上调缺血区的人血管内皮生长因子 165（$hVEGF_{165}$）的表达水平，发挥其成血管作用，从而改变缺血心肌的存活性。

方　法：通过聚乙二醇 40 硬脂酸酯、二硬脂酰基磷脂酰乙醇胺-聚乙二醇 2000、$pcDNA_{3.1}$-$hVEGF_{165}$ 和抗 P-选择素单克隆抗体等制备 P-选择素靶向阳离子微泡。微泡分成四组：（1）仅微泡（MB）；（2）微泡+P-选择素（MB+P）；（3）微泡+$pcDNA_{3.1}$-$hVEGF_{165}$ 质粒（MB+VEGFp）；（4）微泡+P-选择素+$pcDNA_{3.1}$-$hVEGF_{165}$ 质粒（MB+P+VEGFp）。逆转录聚合酶链反应（RT-PCR）和酶联免疫吸附试验（ELISA）结果显示：TCUMGT 成功转染 $hVEGF_{165}$ 基因，并且通过 P-选择素为靶点可以提高转染效率。另外与其他组相比，MB+P+VEGFp 组的心肌血管密度增加和心功改善最为明显。

结　论：本研究表明，通过 TCUMGT 技术，靶向超声微泡可以有效识别缺血心肌，释放 $pcDNA_{3.1}$-$hVEGF_{165}$ 重组质粒，提高心肌微环境，促进心肌功能的恢复。

关键词：血管内皮生长因子；P-选择素；靶向超声微泡介导的基因转染；心功能