Proceedings of ICBioMed 2025 Symposium: Interdisciplinary Frontiers in Pharmaceutical Sciences
DOI: 10.54254/2753-8818/2025.SH26480

Search for Cardiomyocyte-Specific Targeting Sequences and Their Application in the Treatment of Myocardial Infarction

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Abstract. To find cardiomyocyte-specific targeting sequences, transform existing cardiac targeting peptides (CTPs) to the gene level, use this as a basis to improve cardiomyocyte-specific delivery systems, and explore new promising directions for the treatment of myocardial infarction (MI). Based on the CTP selected by the literature, pEGFP-C1 was used as the skeleton to construct a secretory protein expression vector. After the positive clone was identified correctly, the recombinant plasmid was transfected into rat umbilical vein endothelial cells (RUVECs) to express and secrete fusion proteins, and their targeting efficiency was evaluated with rat cardiomyocytes. Compared with the control group, the recombinant plasmid can normally express and secrete fusion proteins in RUVECs. The secretory proteins show strong and dense fluorescence signals in rat cardiomyocytes, and the targeting efficiency increases with time, indicating that the secretory proteins mediated by WLSEAGPVVTVRALRGTG can specifically bind to cardiomyocytes, verifying its efficient targeting and low off-target effect. This study confirmed the effectiveness of WLSEAGPVVTVRALRGTG as a CTP. The secretory proteins mediated by it have efficient and specific cardiomyocyte targeting capabilities, providing an experimental basis for the development of drug delivery systems in the treatment of MI.

Keywords: MI, specific targeting sequence, CTP, cardiomyocyte, secretory protein

1. Introduction

The prevalence of cardiovascular disease (CVD) in China is in a continuous increasing, .with an estimated 330 million CVD patients, including 11.39 million with coronary heart disease (CHD) [1]. CVD ranks first in the proportion of disease deaths among urban and rural residents, accounting for 48.98% and 47.35% of rural and urban deaths in 2021, respectively. The "China PEACE Millions Persons Project" (2020-2022) showed that the prevalence of CHD among residents aged ≥18 years was 758 per 100,000, with a crude incidence of acute myocardial infarction (AMI) of 79.7 per 100,000, which increases rapidly with age [1]. Myocardial infarction (MI) occurs when coronary blood supply fails to meet myocardial metabolic demands, causing persistent severe ischemia-hypoxia and myocardial necrosis. Acute coronary syndrome (ACS), encompassing unstable angina (UA), non-ST-segment elevation MI (NSTEMI), and ST-segment elevation MI (STEMI), arises from acute myocardial ischemia, often secondary to coronary atherosclerosis with thrombosis or spasm.

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AMI is widespread in Europe and the United States, with rising incidence in China's urban and rural areas. From 2002 to 2021, AMI mortality showed an upward trend, accelerating after 2005. Half of AMI-related deaths occur before hospital admission, mostly sudden, with peak mortality in the first week, particularly in patients with severe arrhythmia, shock or heart failure.

Current MI treatment emphasizes early reperfusion to salvage endangered myocardium, with coronary revascularization (CRV) as the mainstay, including percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG). In 2022, the PCI：CABG ratio was 28.8：1.0, with in-hospital mortality (IHMR) and non-routine discharge composite (NRDC) rates of 0.7%/2.7% for PCI and 1.4%/2.9% for CABG [1]. However, PCI shows no significant reduction in MI incidence or mortality compared to medical therapy alone, while CABG is more invasive, with higher perioperative mortality.

Reperfusion of acutely ischemic myocardium can induce myocardial ischemia-reperfusion injury (MIRI), leading to additional cardiomyocyte deaths [2], necessitating adjunctive cardiac protection [3]. Despite preclinical progress, clinical translation of cardioprotective strategies for MIRI remains limited [4], with many interventions failing in trials [5], highlighting the need for innovative approaches.

Systemic drug administration for MI causes fluctuating concentrations, poor dose control, and systemic side effects. Nanodrugs, while advantageous, lack specificity [6], making specific targeting sequences a promising solution. These sequences (nucleic acid or polypeptide) selectively recognize target molecules, enabling precise regulation in gene editing, drug delivery, etc.

Cardiomyocytes are central to cardiac diseases. Specific targeting sequences, especially CTPs, are increasingly linked to exosomes. Mesenchymal stem cell (MSC)-derived exosomes with surface-targeting sequences exhibit tissue/cell specificity, e.g., CTP-expressing small extracellular vesicles (sEVs) target cardiomyocytes [7, 8]. In 2004, McGuire et al. identified CTP WLSEAGPVVTVRALRGTGSW with high cardiomyocyte affinity [9]. In 2024, Li et al. optimized CTPs, with WLSEAGPVVTVRALRGTG showing superior targeting [10]. This study aims to identify cardiomyocyte-specific sequences, transform CTPs to the gene level, improve delivery systems, and explore new MI treatments.

2. Materials and methods

2.1. Materials

2.1.1. Cell lines

RUVEC line, rat cardiomyocyte line H9c2 (National Collection of Authenticated Cell Cultures)

2.1.2. Strains

Escherichia coli DH5α

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2.1.3. Other materials

Table 1. Other materials

Name	Brand	Country
Clean Bench	AIRTECH	Germany
Biosafety Cabinet	AIRTECH	Germany
Benchtop Centrifuge	ThermoFisher	USA
Microvolume Spectrophotometer	ThermoFisher	USA
Inverted Fluorescence Microscope	ZEISS	Germany
Horizontal Electrophoresis System	BIORAD	USA
Single-Button Pipettes	Eppendorf	Germany
6-Well TCT Plates	BEAVER	China
pEGFP-C1	YouBio	China
EcoR I	NEB	USA
Sal I	NEB	USA
T4 DNA Ligase	Takara	China
Trypsin-EDTA (0.25%)	Gibco	USA
DMEM	Gibco	USA
FBS	Gibco	USA
TIANgel Purification Kit	TIANGEN	China
EndoFree Mini Plasmid Kit II	TIANGEN	China
DH5α Chemically Competent Cell	TransGen	China
Lipo2000	Invitrogen	USA

2.2. Methods

2.2.1. Design and synthesize genes

1). Rat cells were selected as templates, so the codons were optimized according to the commonly used selections of rats.

2). EcoR I (GAATTC) and Sal I (GTCGAC) are used to avoid identifying the target gene.

3). Introduce the Kozak sequence (CCACC), a core regulatory element for efficient translation of eukaryotic organisms.

4). The Igκ light chain signal peptide was selected as the signal peptide, with an amino acid sequence of METDTLLLWVLLLWVPGSTG

5). Send it to Sangon Biotech for further optimization and synthesis of genes.

2.2.2. Construct secretory protein expression vectors

1). Dual enzyme cleavage reaction (20 μL system): 1μg DNA, 2μL 10× Buffer, 1μL EcoR I, 1μL Sal I, ddH_2O is supplemented to 20 μL, incubate at 37℃ for 4 h, and inactivate at 65℃ for 10 min.

2). Purification of enzyme cleavage product: Separately run 1% agarose gel electrophoresis and cut the gel using TIANgel Purification Kit for gel recovery (vector about 4.7 kb, target gene 130 bp).

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3). Ligation reaction (10 μL system): 50 ng vector linearized fragments, synthetic gene fragments (molar ratio 3∶1), 1 μL T4 DNA ligase, 1 μL 10× Buffer, ddH_2O is supplemented to 10 μL, mix well and ligate overnight at 16℃.

4). Transformation: In the biosafety cabinet, add the ligation product to DH5α competent cells, ice bath for 30 min, heat shock at 42℃ for 90 s, and then immediately ice bath for 2 min.

5). Resuscitation: Add 800 μL of fresh anti-resistant liquid LB medium to each of the above tubes, and resuscitate them on a shaker at 37℃ for 1 h.

6). Spread plates: Centrifuge at 4℃ and 8000 rpm for 30 s, discard 800 μL of supernatant in the biosafety cabinet, mix the remaining mixture well, spread the bacterial solution to the LB plate containing kanamycin in one direction, and place it in an incubator and incubate at 37℃ overnight.

7). Positive cloning identification: Pick a single colony in the biosafety cabinet, add 500 μL of liquid LB medium containing kanamycin, shake the bacteria for 4~6 h, take 200 μL for double enzyme cleavage verification, and store the remaining.

8). Shake bacteria: Add the remaining bacterial solution to 5 mL of liquid LB medium containing kanamycin and shake bacteria overnight.

9). The constructed recombinant plasmid was extracted using EndoFree Mini Plasmid Kit II.

2.2.3. Assess the targeting efficiency of secretory proteins on cardiomyocytes

1). Resuscite RUVECs and rat cardiomyocytes, and passage them when the cell confluence reaches 80%~90%.

2). The no-loaded plasmids and recombinant plasmids were transiently transfected into RUVECs.

3). The no-loaded plasmids and recombinant plasmids were secondary transfected into RUVECs.

4). The culture medium of RUVECs after the secondary transfection was taken and added to a six-well plate, whose wells were respectively covered with RUVECs and rat cardiomyocytes. The fluorescence results were observed after 24 h and 48 h.

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3. Results

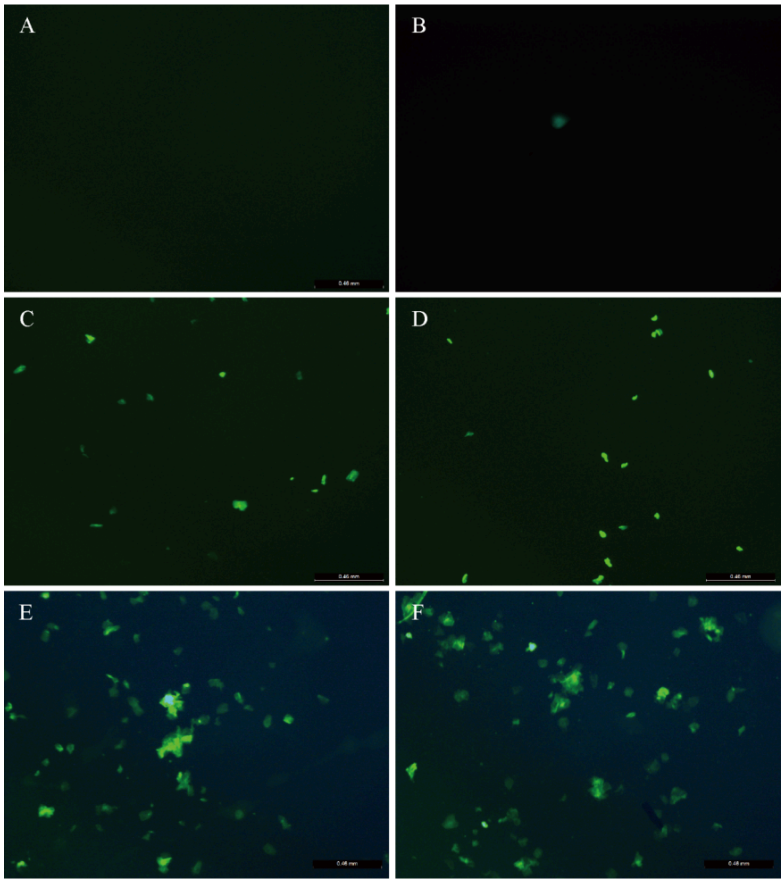


Figure 1. Secretory proteins targeting at cardiomyocytes (observation by fluorescence microscopy). (A) Blank control: No fluorescence in H9c2 wells with culture medium of RUVECs transfected with pEGFP-C1,indicating EGFPs from no-loaded pEGFP-C1 lack secretion ability. (B) Negative control: Faint, dilute fluorescence in RUVEC wells with culture medium of RUVECs transfected with recombinant plasmid, indicating secretory proteins hardly target RUVECs. (C/D) Experimental group (24 h): Strong, dense fluorescence in H9c2 wells with culture medium of RUVECs transfected with recombinant plasmid, showing efficient targeting; C (culture medium retained) and D (culture medium removed) indicate proteins attach to or are absorbed by cardiomyocytes. (E/F) Experimental group (48 h): More intense, dense fluorescence in H9c2 wells with culture medium of RUVECs transfected with recombinant plasmid, indicating increased targeting efficiency over time. n = 3. Scale bar = 0.46 mm

4. Discussion

Specific targeting sequences are highly promising in precision medicine for their ability to precisely identify specific genes, molecules, or cell types, enabling targeted therapies. In cardiac therapy, existing strategies such as viral vector-mediated gene delivery, nanoparticle drug delivery, and stem cell transplantation face challenges like immune rejection, insufficient specificity, or unproven safety. Thus, a safer and more specific targeted delivery approach is needed, with specific targeting sequences being ideal candidates due to their low immunogenicity and high targeting efficiency.

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5. Conclusion

This study constructed a secretory protein expression vector based on the CTP WLSEAGPVVTVRALRGTG. Evaluations showed that recombinant plasmids were properly expressed in RUVECs, and the resulting secretory proteins exhibited strong, time-dependent fluorescence signals in rat cardiomyocytes, with minimal fluorescence in RUVECs. This confirms the specific and efficient cardiomyocyte-targeting ability of CTP-mediated proteins, along with low off-target effects. In summary, WLSEAGPVVTVRALRGTG is validated as an effective CTP, with its mediated proteins showing efficient and specific cardiomyocyte targeting. This provides an experimental basis for developing MI treatment drug delivery systems.. Future work will focus on constructing expression vectors of protective protein-CTP fusions and conducting in vivo experiments to assess targeting efficiency and cardioprotection.

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