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The specific binding of peptide ligands to cardiomyocytes derived from mouse embryonic stem cells

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Purification of pluripotent stem cell (PSC)-derived cardiomyocytes is critical for the application of cardiomyocytes both in clinical and basic research. Finding a specific cell marker is a promising method for purifying induced cells. The present study employed phage display technology to search for particular cell markers that could bind specifically to PSC-derived cardiomyocytes. After three rounds of biopanning, several peptides were obtained. The ELISA results show the no. 3 sequence peptide (QPFTTSLTPPAR), and other four sequences having a consensus motif [SS(Q)PPQ(S)], no. 9, 11, 14, and 10, have relatively high affinity and specificity to cardiomyocytes. Immunofluorescence confirmed that the selected peptides could bind specifically to the PSC-derived cardiomyocytes. Competition tests with chemically synthesized peptides revealed the binding ability was caused by the peptide itself. Western blot analysis proved the phages were both bound to two 17 kDa cardiomyocyte membrane proteins and the no. 9 sequence showed a 55 kDa protein that was not observed in the no. 3 sequence. These results suggest that the selected peptides specifically target receptors on PSC-derived cardiomyocyte membranes. The results will pave the way for further studies of cell surface markers and their applications, such as labeling, purification, and as vehicles for drug delivery. **Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.**

Supporting information can be found in the online version of this article

Keywords: mouse embryonic stem cell; embryoid body; in vitro differentiation; cardiomyocytes; phage display library; peptide

Introduction

Cardiomyocytes derived from pluripotent stem cells (PSCs) offer a large source of cardiomyocytes. When applying PSC-derived cardiomyocytes into clinical or even basic research, eliminating undifferentiated cells and getting highly pure cardiomyocytes are required. Existing differentiation methods have achieved efficiency, some of which reaching a high efficiency, but none of them can eliminate the non-cardiomyocytes to a satisfactory level. Moreover, discontinuous density gradient centrifugation could also be used to enrich the purity of embryonic stem cell (ESC)-derived cardiomyocytes but at a relatively low efficiency [1,2]. Using cardiomyocyte-specific reporters is another way to obtain highly pure ESC-derived cardiomyocytes [3–8], but this requires genetic modification of the cells. Transplantation of undifferentiated ESCs results in teratomas [9], and genetic modification carries risks such as possible tumor formation [10–12].

The marker-based technique is the ideal method for cardiomyocyte purification, but it currently lacks specific cell markers. Recently, a novel approach using the phage display library, which display peptides in their surface proteins, has been used to identify a number of cell-specific ligands [13–15].

When applying the phage display library to screen the specific binding ligands, using whole intact cells has some apparent advantages: the receptors of the ligands on the cell surface maintain a natural conformation with normal modifications; the information details of the receptor is unnecessary when processing the screening. A number of cell lines have been used as targets, such as human adipocytes [16], neuroblastoma cells [13], microglial cell lines [17], and breast cancer cells [18].

In the present study, the Ph.D.-12 Phage Display Peptide Library was applied, and cardiomyocytes derived from mouse embryonic stem cells (mESC) were used as the positive targets, whereas the mESC were used as the negative control to account for non-specific binding. After three rounds of screening, 14 peptide sequences were obtained. One sequence and one motif showed high proportion, and their binding abilities were checked. The approaches presented in the current study will aid purification of PSC-derived cardiomyocytes and promote their application in basic and clinical research.

Materials and Methods

Culture and Identification of Mouse Embryonic Stem Cells

The culture of feeder layer cells

Mouse embryonic fibroblasts (MEF) were chosen as the feeder layer for mESC. The MEF used in this experiment were derived from ICR mouse embryos (purchased from the Beijing Vital River Company, Beijing, China). MEF were cultured and propagated on

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Tissue Culture Flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, US) in Dulbecco's modified Eagle's medium (DMEM) (Gibco, BRL, Gaithersburg, MD, US), supplemented with 15% newborn calf serum (Gibco). At about 90% confluence, the MEF were treated with 10 μg/ml mitomycin C (Roche, Nutley, NJ, US) for 3 h to inhibit cell division, then trypsinized, and plated on 0.1% gelatin (Sigma, St. Louis, US) pre-coated culture plates overnight. Passages 3–5 MEF were used as the feeders for mESC.

Culture and propagation of mouse embryonic stem cells

The mESC (obtained from the Joint Lab of Stem Cell Research, Graduate School at Shenzhen, Tsinghua University, China) were cultured on MEF feeders prepared as previously described. Briefly, the mESC were cultured in Knockout DMEM (Gibco) containing 15% fetal bovine serum, 0.5% 200 mM L-glutamine solution (Gibco), 1% non-essential amino acids (100X solution, Gibco), 0.2% 55 mM 2-mercaptoethanol solution (Amresco, Solon, OH, US), 1% penicillin–streptomycin (Gibco 100X solution), and 1000 units/ml LIF (Chemicon, Billerica, MA, US) and were maintained at 37 °C with 5% CO_2 [19–21].

After 3 days of culture, the mESC and MEF were trypsinized with 0.25% trypsin from the culture plates, and dissociated into single cells by pipetting the cells' suspension vigorously. The cells suspension were transferred into a 100-mm tissue culture dish and incubated quiescently at 37 °C for about 45–60 min to let the MEF re-attach to the dish. The non-adherent cells (mostly mESC) were collected, centrifuged, and plated on a new culture plate/dish pre-coated with MEF feeders.

Identification of mouse embryonic stem cells

To identify the undifferentiated state of ES cells, alkaline phosphatase assay, reverse transcription polymerase chain reaction (RT-PCR), and embryoid body (EB) formation were performed.

Alkaline phosphatase assay. The mESC were cultured with MEF feeder cells in 24-well plates pre-coated with 0.1% gelatin. After the mESC colony formed, the plates were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for about 20 min. After discarding the paraformaldehyde, the plates were washed three times with PBS, and incubated with a color-developing agent [6.6 μl nitrotetrazolium blue chloride (5 mg NBT in 100 μl 70% dimethylsulfoxide (DMSO), 3.3 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP; 5 mg BCIP in 100 μl 100% DMSO), 1 ml Tris-HCl (100 mM Tris, 100 mM NaCl, 1 mM $MgCl_2$, pH 9.5)] at 37 °C for 1 h. Then the plates were washed with PBS for three times. An optical microscope was used to observe the result and recorded it with a camera.

RT-PCR for specific genes in undifferentiated mouse embryonic stem cells. The total RNA of mESC was extracted by Trizol reagent (Invitrogen) for the RT-PCR assay. The RNA was reverse transcribed into cDNA with a ReverTra Ace® Kit (TOYOBO, FSK-101), and cDNA was amplified in a 25-μl reaction system(H_2O 18.35 μl; 10 × PCR buffer 2.5 μl; dNTP-mix 2 μl; cDNA 1 μl; F-primer 0.5 μl; R-primer 0.5 μl; r-Taq 0.15 μl) using the primers described in Table 1. The DNA primers for each gene were derived from different exons, which ensured that the PCR products represented relevant mRNA species and not the genomic DNA. The PCR conditions are as follows: 95 °C 5 min; 95 °C 30 s, 55–65 °C 25 s, 72 °C 25 s, repeat 30–35 cycles; and 72 °C 7 min; and then 4 °C forever. The PCR products were electrophoresed in 1.5% agarose gel containing 0.1 μl/ml ethidium bromide substitute agent (Applygen Co.).

Inducing embryoid body formation. The hanging drop (HD) method was used in the formation of EBs. The HD method has advantages such as: the initial number of cells in every drop is easily controlled by varying the cell number in suspension; gravity allows the cells to aggregate to the bottom of the drop and form EBs.

The 30 μl drops (~3×10^4 cells/ml) were plated on the lid of Petri dishes, which was inverted and covered onto a dish filled with 8 ml PBS to maintain the humidity. After 2 days of incubation, the ES cells aggregated into simple EBs.

Inducing Cardiomyocytes from Mouse Embryonic Stem Cells

The method of cardiomyocyte induction

For differentiation into cardiomyocytes, the mESCs were cultivated into embryoid bodies essentially as described. Briefly, ~700 cells in 30 μl cultivation medium (see above) containing 15% FCS were placed on the lids of Petri dishes filled with PBS and cultivated in hanging drops for 2 days. After a further 2 days of suspension cultivation in bacteriological plates, the retinoic acid (RA) (all-trans retinoic acid diluted in DMSO in the dark, stock solution of 10^{-3} M) was added into the culture medium. The EBs were cultivated in Petri dish for another 4 days. The 8-day-old EBs were then separately plated onto gelatin-coated (0.1%) tissue culture plates for morphological analysis.

Then, the EBs were cultivated with 10^{-8}–10^{-9} M RA during day 5–15 of EB development. The RA was diluted in DMSO, which also affected the differentiation of cardiomyocytes [22–24].

Isolation of cardiomyocytes

Under the microscope in a clean bench, a glass needle was used to isolate the cardiomyocytes. The beating area of the EBs was cut with a tiny sterilized glass needle and placed into a dish containing culture medium. After all the beating area was cut off, the medium was discarded, and the cell aggregates (beating area) were washed three times. These were then trypsinized with 0.05% trypsin, and dissociated into single cells by gently pipetting the cell suspension so as to not harm the cells.

Tiny glass needles were used to cut and low-concentration trypsin was used to trypsinize the cells to isolate cardiomyocytes with relatively high purity, which were dissociated into the maximum number of single cells while ensuring minimal harm to the cells.

Identification of cardiomyocytes

Optical microscope observation. After the 8-day-old EBs were separately plated onto gelatin-coated (0.1%) tissue culture plates, the EBs were observed everyday to determine if spontaneous contraction occurs.

RT-PCR for specific genes in cardiomyocytes. The total RNA was extracted from the cells using Trizol reagent (Invitrogen). The cDNA was synthesized with the ReverTra Ace® Kit (TOYOBO, FSK-101). PCR was carried out with the primers depicted in Table 1. The PCR products were electrophoresed and photographed via a gel imaging system.

Immunofluorescence to identify cardiomyocytes. As described previously, the 8-day-old EBs were separately plated onto gelatin-coated (0.1%) 24-well plates. After the EBs adhered and beating cardiomyocytes occurred, the plates were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, then washed three

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Table 1. Primers for RT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size (bp)
Primers for RT-PCR of the specific genes in undifferentiated mESCs			
Oct 4	AAAGCGAACTAGCATTGAGA	AGCAGTGACGGGAACAGA	358
C-myc	AAGGGAAGACGATGACGG	TGAGCGGGTAGGGAAAGA	353
Sox2	TCTTCCTCCCACTCCAGG	TAGTCGGCATCACGGTTT	380
Klf4	TGCCTTGCTGATTGTCTATT	GCAGTGTCTTCTCCCTTCC	345
Primers for RT-PCR of the specific gene in cardiomyocytes			
α-actin	CTCGATTCTGGCGATGGTGTA	CGGACAATTTCACGTTCAGCA	173
GATA4	CCTCTCCCAGGAACATCAAA	AGCCATAGTCACCAAGGCTG	185
Nkx2.5	ACCCAGCCAAAGACCCTC	GACAGGTACCGCTGTTGCTT	190
Mef2C	GAGGATAATGGATGAGCGTAA	AGTGCTGGCGTACTGGAA	154
Irx4	CCAAGGTCCAACGGGTGT	GGCCTGGTTCAAAGTGCTGT	169
Ncx1	GGCGACTTGAGCACCACT	ATGCCAACTCTGATTTCCTTCT	137
cTN1	ACGCTCGGGTGGACAAAG	TGGGCCGCTTAAACTTGC	126

times with PBS, and incubated in blocking buffer (1% bovine serum albumin, BSA) for 2 h at 37 °C. Then, the plates were incubated overnight with primary antibodies at 4 °C, washed three times with PBS, and incubated with secondary antibodies for 1.5 h at 37 °C. The primary and secondary antibodies were diluted with 1% BSA–PBS. After the final wash, the cells were mounted in PBS and detected with an Eclipse TE2000 fluorescence microscope (Nikon, Tokyo, Japan).

Phage Peptide Library Selection

Performing the phage peptide library selection

A 12-mer M13 phage display peptide library (Ph.D.-12 Phage Display Peptide, New England Biolabs, E8110S) was applied for ligand screening. The areas with induced beating cardiomyocytes were selected and trypsinized into single cells for the positive screen; undifferentiated mESC were used as the negative screen. The cells for screening were pre-suspended in PBS containing 3% BSA.

In each round of panning, 1.5×10^{11} plaque forming unit (pfu) phages were first added into the negative screen cells. After 1 h of gentle agitation at room temperature, the cells and phages were centrifuged at 3000 rpm for 5 min, and the unbounded phages were in the suspension. Then, the negative screen was repeated, and the suspension was added into the positive screen cells, gently agitated for 1.5 h at room temperature. The sample was then centrifuged, and the cells were washed twice with PBS-T (0.1%), and twice with PBS. The bounded phages were then eluted with 1.6 ml 0.2 M glycine-HCl (pH 2.2). After gently shaking the tube for about 10 min, 0.3 ml 1 M Tris-HCl was added into the tube to neutralize the eluent. A tiny amount of eluted phages were titrated, and the rest were amplified using *Escherichia coli* strain ER2738 according to the manufacturer's instruction [25].

After three rounds of panning under the same conditions, the phage plaques obtained from the final round were picked up, amplified, and purified. DNA was extracted from these plaques and analyzed using DNA sequencing.

Sequences of the phage DNA

The DNA extracted from the selected plaques was sequenced by the Invitrogen Company (Guangzhou, China). The corresponding peptide sequences were analyzed with GENE RUNNER software.

Positive Phage Analyses

To determine the binding properties of the positive phages to the target cells, affinity analyses were carried out.

Whole-cell ELISA

Undifferentiated mESCs and the picked beating cardiomyocytes were plated into a 0.1% gelatin pre-coated 96-well microtitration plate (Falcon). After about 12 h, the plate was washed with PBS, and the cells were fixed with 4% paraformaldehyde (in PBS) for 15 min. After the fixation, the plate was washed thrice with PBS. The cells were incubated with blocking buffer (1% BSA–PBS) for 2 h in 37 °C, and then washed twice with PBS. Next, 2×10^9 phages diluted with blocking buffer were added to every well and incubated for 1.5 h at room temperature. The plates were then washed thrice with 0.3% PBS-T, and thrice with PBS. Horse Radish Peroxidase (HRP)-conjugated mouse anti-M13 phage antibodies (1:1000 diluted by blocking buffer) were added to the plate and incubated for 1.5 h at 37 °C, then washed thrice with PBS. Finally, a chromogenic reaction was performed by adding the substrate solution (citrate acid·H_2O 0.5585 g, Na_2HPO_4 0.7298 g/100 ml, pH 5.0; before use, 4 mg *o*-phenylenediamine and 0.5% ambroxol hydrochloride was added to 10 ml of the above solution) to the plate for 15 min in the dark, stopped with 2 M sulfuric acid, and then read at 492 nm in an ELISA reader.

Immunocytochemistry

Cardiomyocytes induced from mouse embryonic were trypsinized into single cells and plated into 24-well plates coated with 0.1% gelatin. At the same time, MEF cells and undifferentiated mESC were also plated into 24-well plates. After 12 h, the cells were fixed with 4% paraformaldehyde for about 20 min, and then the plates were washed thrice with PBS. Blocking buffer (2% BSA in PBS) was then added into the cells for 1 h at 37 °C, and then incubated with positive phage (~2×10^9 pfu) for 2 h, and HRP-conjugated mouse anti-M13 phage antibodies for 90 min at 37 °C. Afterward, the rabbit-anti-mouse fluorescein isothiocyanate (FITC) -IgG antibody was added to the cells and incubated in the dark for 1 h. Finally, the cells were incubated with Hoechst 33258 (5 μg/mL, Sigma) for 15 min.

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Determination of sequence binding ability by free peptide competition test

The sequences QPFTTSLTPPAR (no. 3) and NNWSSPPQMISR (no. 9) were used in the experiment. Their corresponding control sequences LAPTRPTFPTSQ (LQ-12) and SNRQPSNIMWPS (SS-12), which were synthesized by the Terabio Biotechnology Company, Guangzhou, China, and have the same peptide compositions but not in the same order, were also used.

The synthetic peptides were added to the cells at concentrations varying from 10 nM to 10 μM before the phages were added into the screening process. The binding ratio of the phage content in the test sequences was calculated using the output titer of phages panning with the synthesized free peptides normalized to the output titer of phages panning without adding the synthesized free peptides.

Western blot

The membrane proteins of induced cardiomyocytes, mESC, and MEF were extracted using the Membrane Protein Extraction Kit (Keygene Co.) and were resolved through 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then electrophoretically transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 3% BSA in PBS for 2 h and then incubated with 3×10^9 phages diluted in PBS with 3% BSA for 1.5 h. Then, the HRP-conjugated anti-M13 phage antibodies (1:5000 dilution in PBS, 2% BSA, GE Co.) were added and incubated for 1 h. The membrane was further developed using a BeyoECL plus Kit (Beyotime), and the blot was detected with electrochemical luminescence by exposing the membrane to an X-ray film (Kodak, Tokyo, Japan). Molecular weight was determined with a pre-stained protein molecular weight marker (Fermentas).

Database search

The obtained peptide sequences were subjected to multiple sequence alignments and motifs identification with the CLUSTAL (version 2.1) multiple sequence alignment tool (EMBL-EBI, http://www.ebi.ac.uk/Tools/msa/clustalw2). The results are shown in Table 2. The GenBank search adopted the National Center of Biotechnology Information (NCBI) protein–protein blast BLASTP 2.2.25. (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 3).

Results

Identification of Mouse Embryonic Stem Cells

Alkaline phosphatase assay

To confirm that the mESCs maintained an undifferentiated state, the undifferentiated state and undifferentiated makers were first

Table 2. Exogenous amino acid sequences (deduced from DNA sequences)

Phage Clones	Sequence Number	Amino Acid Sequences	Alignment result
C8	1	APLPYDHHSAGS	9 NNWSSPPQMISR-- 12
C2	2	HHQNPPLFLGTS	10 -NFSQPPSKHTRS- 12
C1,C10,C14	3	QPFTTSLTPPAR	11 ATFS-PPQQSLMM- 12
C4	4	ASLTDRPTLTPV	14 ISTS-PPQGSTSS- 12
C5	5	SLNETQHFAFHH	5 -SLNETQHFAFHH- 12
C13,C15	6	LKGPMTDVRESA	13 HPLFHHQTATAT-- 12
C6	7	TVYSMNSAAPRP	12 NSIQALDSTNG--- 11
C7	8	SAHGTSTGVPWP	8 -SAHGTSTGVPWP- 12
C19	9	NNW*SSPPQ*MISR	6 --LKGPMTDVRESA 12
C18	10	NF*SQPPS*KHTRS	2 -HHQNPPLFLGTS- 12
C11	11	ATF*SPPQQS*LMM	3 QPFTTSLTPPAR-- 12
C16	12	NSIQALDSTNG*	4 ASLTDRPTLTPV-- 12
C20	13	HPLFHHQTATAT	7 -TVYSMNSAAPRP- 12
C9	14	IST*SPPQGS*TSS	1 APLPYDHHSAGS-- 12

The amino acids underlined represented the potential consensus peptide motifs.
'Alignment result' was the alignment result of the 17 sequences in the left column. The number before the sequences is their 'sequence number' in the left column, and the same type of amino acid was shown in same color.

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Table 3. Homological search results identified by NCBI protein–protein blast

Protein	Accession number	Homology sequence/position
QPFTTSLTPPAR		
Inositol 1,4,5-triphosphate receptor	EFW46868.1	1238 PFTTSLAPPPR 1248
Transcription factor [Mus musculus]	CAA64216.1	184 PFSVSLTPPA 193
Periplasmic solute binding protein	YP_003696852	47 FTVTSLTPPGR 57
Integral membrane sensor signal transduction histidine kinase	YP_004087640	498 QPFSASLTP 506
Holin protein	YP_003719036	137 QPAFKTSSTPPAR 149
Lipoprotein	ZP_07035075.1	272 QPFTTSLGKQVTP 284
ABC superfamily ATP binding cassette transporter, membrane protein	ZP_07869180.1	194 TVSLTPPAR 202
NNWSSPPQMISR		
Respiratory nitrate reductase alpha subunit apoprotein	ZP_08022542.1	618 DWSRPPRQMIS 628
Membrane bound nitrate reductase	ABF20973.1	21 NWSRPPRQMI 30
Potential chitinase	XP_002181971.1	2 SSPPQMI 8
		667 SSPPQSI 673
Na+ −glucose cotransporter type 1	AAI70304.1	541 WTEPPSKQMISR 552
Leucine rich repeat family protein	XP_001027878.1	328 NSSSPSQMIS 337
Alpha/beta hydrolase	YP_521619.1	228 DNWS-PPQM 235
Peptidylprolyl isomerase	ZP_07684313.1	5 WSSPPEM 11

checked. The ESCs formed round, compact, and clearly defined colonies, as shown in Figure 1A. Compared with differentiated cells, the embryonic cells had positive alkaline phosphatase activity. The color revealed by the mESCs in the alkaline phosphatase stain test is much darker (Figure 1B).

Specific genes analysis in undifferentiated mouse embryonic stem cells

The ES cells expressed totipotency genes, such as Oct4, C-myc, Sox-2, and Klf-4. As shown in Figure 3A, the mESCs expressed all these four maker genes.

Embryoid body formation experiment

The undifferentiated embryonic cell was totipotent and believed to have the ability to produce all the three-germ layer cells. The EBs were formed using the hanging drop method. After 2 days in a hanging drop culture, a tiny white dot was formed in every single drop hanging on the lid of the Petri dish. The dots were collected and cultured for another several days of suspension culture. During the suspension culture, a series of changes in the EBs were found. At about the fifth day, a distinct three-germ layer structure can be seen, complete with an endoderm, a mesoderm, and an ectoderm (as indicated by the arrows: red-ectoderm; black-mesoderm; white-endoderm); thus, the EB was called a 'simple embryonic body'. Later, the cystic structure can be found in the EB (as in Figure 2), and the EB this time was called a 'cystic embryonic body' (as indicated by arrows: the structures similar to the 'inner cell mass' and 'blastopore'). These results suggest the totipotency of the mESCs used in this study.

Inducing Cardiomyocytes from Mouse Embryonic Stem Cells

Optical microscope observation

Spontaneous contractions can be observed in some embryoid bodies when the differentiation process was in 10 ± 1 days (8 days of EB culture, and 1–3 days plated onto the tissue culture plate) (as shown in Supporting Information, video record 1). The frequency of the spontaneous contractions was 40–150 times per minute.

RT-PCR for the specific genes in cardiomyocytes

Cardiomyocytes expressed some maker genes such as: α-actinin (sarcomeric), GATA4, Nkx2.5, Mef2C, Irx4, Ncx1, and cTN1 (Figure 3B).

As shown in Figure 3B, these genes were all expressed in the induced cardiomyocytes.

Immunofluorescence to identify cardiomyocytes

After the immunofluorescence staining of the EBs, the beating EBs were positive for α-actinin, GATA4, and Nkx2.5, as shown in Figure 4.

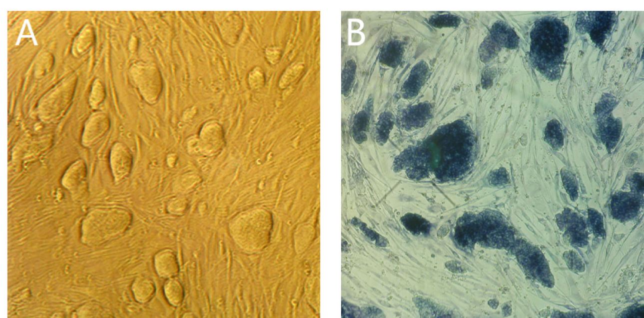

Figure 1. Identification of mouse embryonic stem cells. (A). Mouse embryonic stem cells under optical microscope. (B) Alkaline phosphatase stains of mouse embryonic cells.

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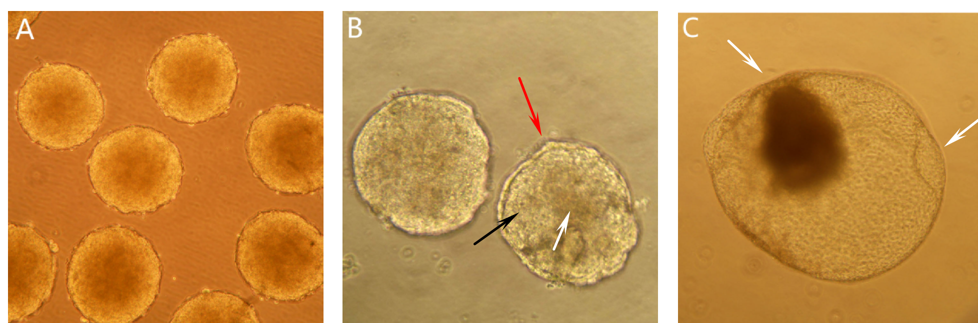

Figure 2. Embryoid body (EB) formation. (A) Embryonic body after 2 days of hanging drop culture. (B) Simple embryonic body. (C) Cystic embryonic body.

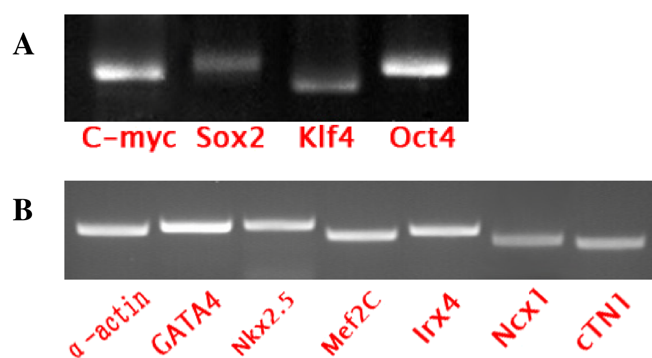

Figure 3. RT-PCR analyses of mESCs and cardiomyocyte. (A) Mouse embryonic stem cell marker genes: C-myc, Sox2, klf4, and Oct4. (B) Cardiomyocyte marker genes: α-actin, GATA4, Nkx2.5, Mef2C, Irx4, Ncx1, and cTN1.

The efficiency of inducing cardiomyocytes from mouse embryonic stem cells

The percentage of EBs containing beating cardiomyocytes was determined in independent experiments, and the mean values were evaluated. The significance was tested with a Student's *t*-test at a significance level of 0.05 (Table 4).

Isolation of cardiomyocytes

After the isolation of the beating area of the EBs (as shown in Supporting Information, video records 2 and 3), the cells were dissociated into single cells, which were again plated onto culture plates. The next day, the single beating cells could be observed under the microscope. The contraction was much more apparent in the area where the cells were not completely dissociated into single cells, and formed aggregates (as shown in Supporting Information, video record 3).

Phage Peptide Library Selection

Specific enrichment of positive phages

Too many rounds of panning may cause the enrichment of phages with amplification advantages [41]. Consequently, on one hand, the enrichment of phages needs to have specific binding ability to cardiomyocytes. On the other hand, the differences caused by the amplification ability should be avoided. Thus, three rounds of selection were performed.

When the numbers of input phages were the same, the results show that the efficiency of binding with cardiomyocytes increased from 1.19×10^6 to 5.32×10^7, and the phage yield rate increased from 7.93×10^{-6} to 3.55×10^{-4} (about 45-fold), as shown in Table 2. These indicate an effective enrichment of the phages that specifically bind to cardiomyocytes Table 5.

Analyses of the DNA sequence of selected phages

After three rounds of panning, 20 clones were randomly selected and their DNA were extracted and sequenced. Because two phages lost the insert DNA segment encoding the displayed peptides and one phage DNA failed sequencing because of low extracted DNA content, we only got 17 sequences result. After translating the DNA sequences to the peptides, the results showed a consensus sequence QPFTTSLTPPAR and a consensus motif SS(Q)PPQ(S). All the sequences are shown in Table 2.

Positive Phage Analyses

Whole-cell ELISA

The binding ability of the selected phages was examined by whole-cell ELISA. Phage clones binding to the cardiomyocytes were detected, whereas mESCs and MEF were chosen as the negative control. The A492 of cardiomyocytes were about 0.3, but the mESCs were about 0.1 compared with the blank value 0.05 (Figure 5). The results indicate that the selected phages have higher binding efficiency with cardiomyocytes and lower binding efficiency with MEF and mESCs ($p < 0.01$). Based on the results, the sequence QPFTTSLTPPAR (no. 3) and NNWSSPPQMISR (no. 9), which share the same motif with ATFSPPQQSLMM (no. 11) and ISTSPPQGSTSS (no. 14), also have relatively stronger binding abilities, and were chosen as the candidate for the next study.

Immunocytochemistry with the selected peptide

The phages carrying the peptide sequence QPFTTSLTPPAR (no. 3) and NNWSSPPQMISR (no. 9) were used in the immunocytochemistry to confirm their binding properties. Figure 6 shows that HRP-conjugated anti-M13 phage antibodies could bind to the cardiomyocytes (A and B in Figure 6). In contrast, mESCs (C and D in Figure 6) and MEF (E and F in Figure 6) showed no obvious fluorescence signals. At the same time, no obvious fluorescence signal was observed when the wild-type M13 phages were used.

The results demonstrate that the peptide sequence QPFTTSLTPPAR and NNWSSPPQMISR can specifically bind to cardiomyocytes.

Competition with free peptides

A chemically synthetic peptide containing the sequence QPFTTSLTPPAR (QR-12) and sequence NNWSSPPQMISR (NR-12)

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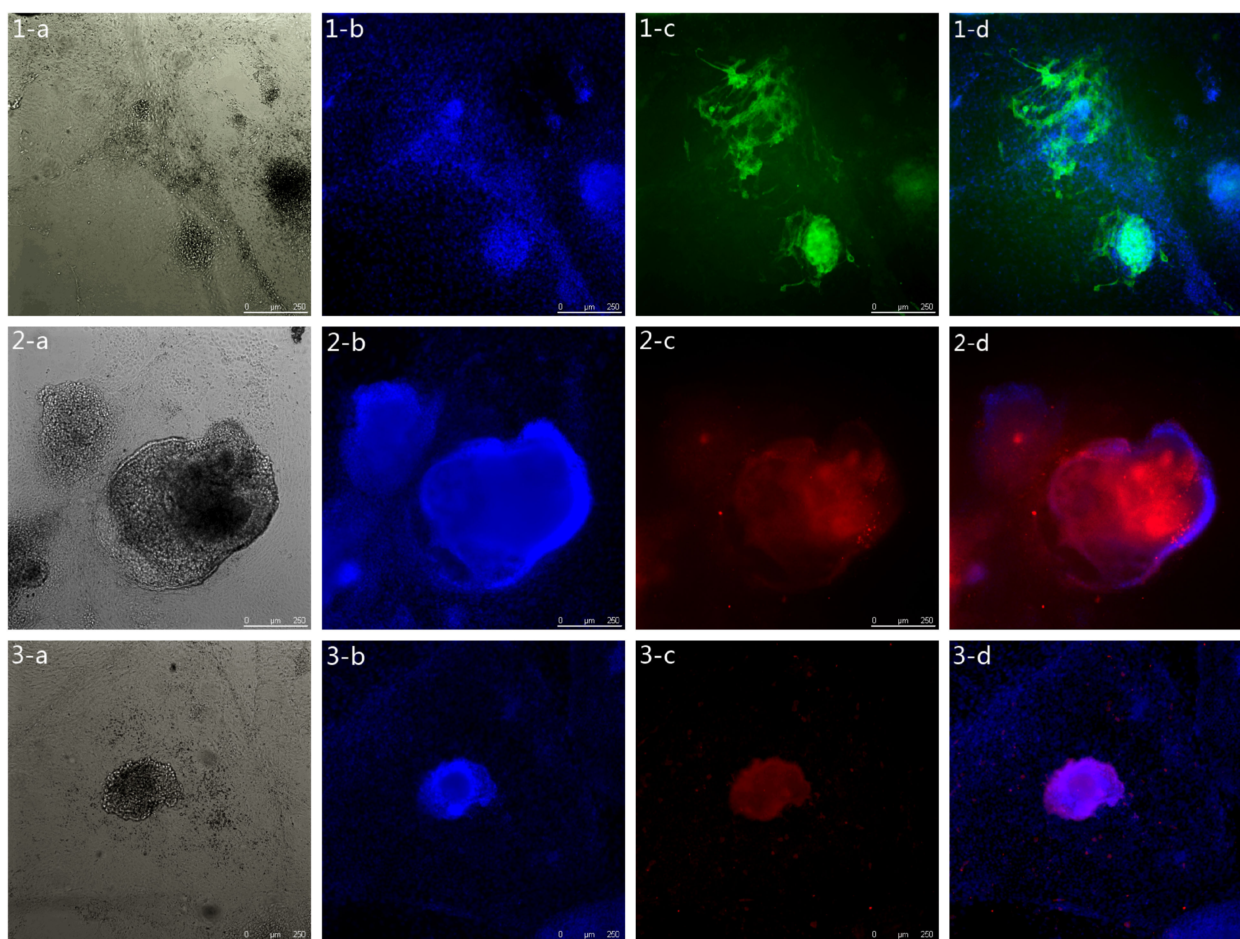

Figure 4. Immunofluorescence of cardiomyocyte markers. (1) α-Actinin, (2) GATA4, and (3) Nkx2.5. (a) Cells under the optical microscope. (b) Cell nuclei stained with Hoechst 33258 (blue). (c) Immunofluorescence signal of the marker. (d) Overlap of pictures b and c.

Table 4. Cardiomyocytes differentiation efficiency

	control	Group 1	Group 2	Group 3
The percentage of beating EBs	5	45	27	31
	12	34	43	44
	4	56	46	36
Average	7	45	38.7	37
t-test		0.04881*	0.031771*	0.004415*

Control group is the group without adding the cardiomyocytes differentiation medium. Groups 1–3 are the experimental group added the cardiomyocytes differentiation medium and are derived from three individual experiments with three replicates each.

Table 5. Recovery of the screened phage

Rounds	Input phages (pfu)[a]	Output phages (pfu)[b]	Yield rate (output/input)[c]
1	1.5×10^{11}	1.19×10^{6}	7.93×10^{-6}
2	1.5×10^{11}	1.292×10^{6}	8.61×10^{-6}
3	1.5×10^{11}	5.32×10^{7}	3.55×10^{-4}

[a]Number of phages put into the selection process.
[b]Number of phages in the eluate.
[c]Phage yield rate = output phage/input phage.

competes with the phages containing the same sequence for the binding of cardiomyocytes. As shown in Figure 7, this synthetic peptide was able to compete with the phages that share the same peptide sequence. The starting concentration of the synthetic peptide in the competition test was 1 nM, and other concentrations used in this experiment were 10, 100, and 1000 nM. The control peptide [LAPTRPTFPTSQ (LQ-12) and SNRQPSNIMWPS (SS-12)] failed to compete with the phages in the competition test. Thus, the binding ability of the phages to cardiomyocytes is caused by the displayed peptide [QPFTTSLTPPAR (QR-12) and NNWSSPPQMISR(NR-12)].

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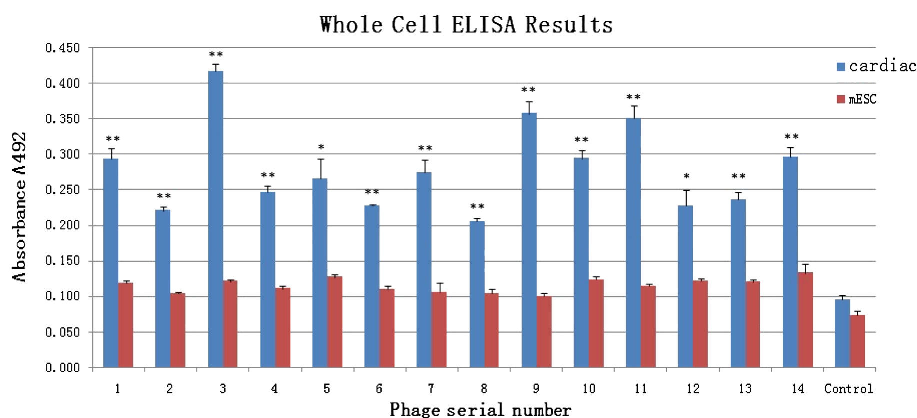

Figure 5. Whole-cell ELISA results for selected phage candidates. The phage with displayed peptides no. 1 (APLPYDHHSAGS), no. 2 (HHQNPPLFLGTS), no. 3 (QPFTTSLTPPAR), no. 4 (ASLTDRPTLTPV), no. 5 (SLNETQHFAFHH), no. 6 (LKGPMTDVRESA), no. 7 (TVYSMNSAAPRP), no. 8 (SAHGTSTGVPWP), no. 9 (NNWSSPPQMISR), no. 10 (NFSQPPSKHTRS), no. 11 (ATFSPPQQSLMM), no. 12 (NSIQALDSTNG*), no. 13 (HPLFHHQTATAT), and no. 14 (ISTSPPQGSTSS) were incubated with cardiomyocytes (blue bars) and mESCs (red bars). Wild-type M13 phages were used as control. Average values from three independent experiments are shown.

Western blot analysis

Western blot analysis of the phages containing the peptide sequence PFTTSLTPPAR (no. 3) showed two distinct bands at around 17 kDa among the cardiomyocyte membrane proteins. At the same time, the mESC membrane proteins showed no obvious stain, but the mESC cytoplasmic proteins showed very weak bands (Figure 8A). As shown in the figure, one band at around 17 kDa can be spotted, and other bands whose weights ranged from about 43–130 kDa were observed, too.

Under the same conditions, phages containing peptide NNWSSPPQMISR (no. 9) were also tested in the western blot. In Figure 8B, two distinct bands around 17 kDa were seen among the cardiomyocyte membrane proteins, similar to the former sequence (no. 3 PFTTSLTPPAR). Notably, one band at around 55 kDa was also observed. However, unlike the no. 3 sequence, only one band was found around 17 kDa among the mESC cytoplasmic proteins. Wild-type M13 phages were used as the control (data not shown). The result demonstrates that the peptide specifically bind to the cardiomyocytes through protein–protein interaction.

Database search results

A GenBank search using the NCBI protein–protein blast BLASTP 2.2.25 revealed several sequences with varying degrees of homology (Table 3).

Discussion

To find a useful PSC-derived cardiomyocyte surface marker, the Ph.D.-12 Phage Display Peptide Library was applied in the present study. First, cardiomyocytes were induced from mouse embryonic cells using all-trans RA through the hanging drop method, which is good for the development of embryoid bodies and helpful for cardiomyocyte formation. The RA concentration that most benefitted the induction of the cardiomyocytes was 10^{-8}–10^{-9} M. The RA should be diluted in DMSO, which also affects cardiomyocyte differentiation.

Next, we used some marker genes to identify the induced cardiomyocytes. α-Actinin (sarcomeric) is a microfilament protein, and is an F-actin cross-linking protein thought to anchor actin to a variety of intracellular structures [26]. The actin-binding domain of α-actinin seems to reside in the first 250 residues of the protein. A similar actin-binding domain has been found in the N-terminal region of many different actin-binding proteins [27,28], such as the beta chain of spectrin (or fodrin), dystrophin, the slime mold gelation factor (or ABP-120), filamin, and fimbrin. GATA4 (GATA-binding factor 4) regulates embryonic development and cardiomyocyte-related gene expression [29,30]. Missing or mutated GATA4 will cause cardiomyocyte differentiation defects. Nkx2.5 (NK2 transcription factor related, locus 5) regulates tissue-specific gene expression and organ differentiation, and plays an important role in the formation of the heart in mice and humans [31,32]. Ncx1 [Slc8a1 solute carrier family 8 (sodium/calcium exchanger), member 1] is a Na^+/Ca^{2+} exchanger, and maintains the calcium concentration of the sarcoplasmic reticulum in myocardial cells [33]. cTN1 (*Mus musculus* troponin I, cardiac 3) (Tnni3) also has important effects on heart development [34,35]. Irx4 (Iroquois-class homeodomain protein) has many functions in pattern formation in vertebrates. It affects heart development through DNA-dependent transcription regulation [36,37]. Mef2C is myocyte enhancer factor 2C [38–40].

The cardiomyocytes applied in the phage display screen were isolated using the tiny glass needle cut method, and was dissociated into single cells using a low concentration of trypsin (0.05%). Only the beating area was cut to obtain a relatively high purity cardiomyocytes. The trypsin dissociation procedure was thought to cause the change of the cell surface protein. To avoid these changes, a low trypsin concentration was employed to minimize the damage to cell surface markers.

In the screening process, the liquid phase screen method was carried out on suspension cells. As expected, the cells attached to the plate were used as the target. However, in this method, some activity sites were lost because of cell attachment. The liquid phase screen method prevented the activity site loss, thereby facilitating the selection of all possible kinds of cell surface markers.

Theoretically, the more rounds of selection performed, the greater the likelihood of the phage carrying the desired peptide would be found. In practice, however, the multiplication capacity

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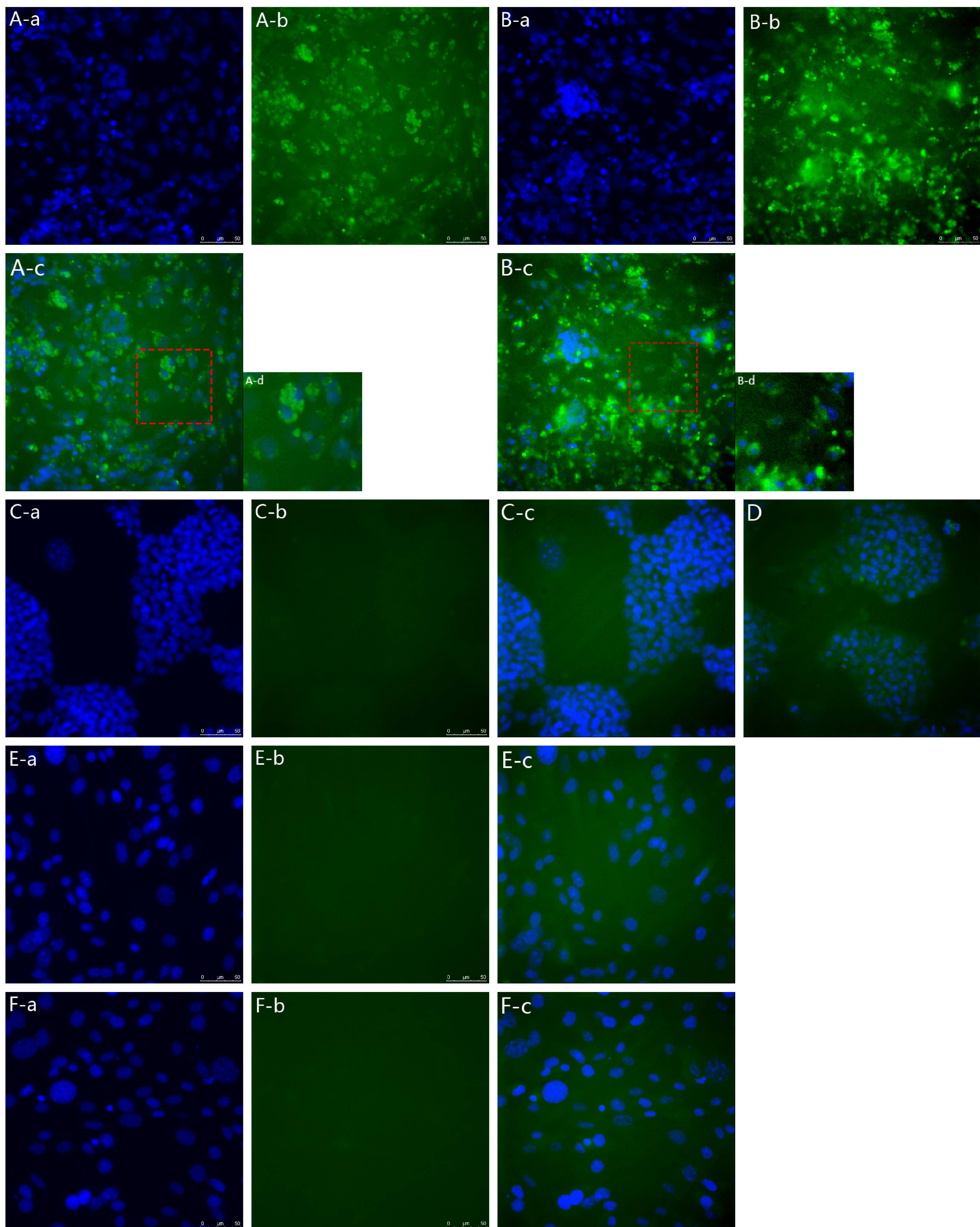

Figure 6. Immunofluorescence microscopy of phages no. 3 and no. 9 bound to cardiomyocytes, mESC, and MEF. (A) Binding of phage no. 3 (QPFTTSLTPPAR) to cardiomyocytes. (B) Binding of phage no. 9 (NNWSSPPQMISR) to cardiomyocytes. (C) binding of phage no. 3 (QPFTTSLTPPAR) to mESCs. (D) Binding of phage no. 9 (NNWSSPPQMISR) to mESCs. (E) Binding of phage no. 3 (QPFTTSLTPPAR) to MEF. (F) Binding of phage no. 9 (NNWSSPPQMISR) to MEF. (a) Cell nuclei stained with Hoechst 33258 (blue). (b) Immunofluorescence signal of M13 phage (green). (c) Overlap of pictures a and b. (d) Magnification of the corresponding areas in picture c.

diversity caused the enrichment of phages with faster reproduction ability [41], which could impact on our study. So, three rounds of selection were performed.

Mouse embryonic stem cells were used as the negative target of subtraction in the panning procedure, whereas mESC-derived cardiomyocytes were used as the positive targets. These were

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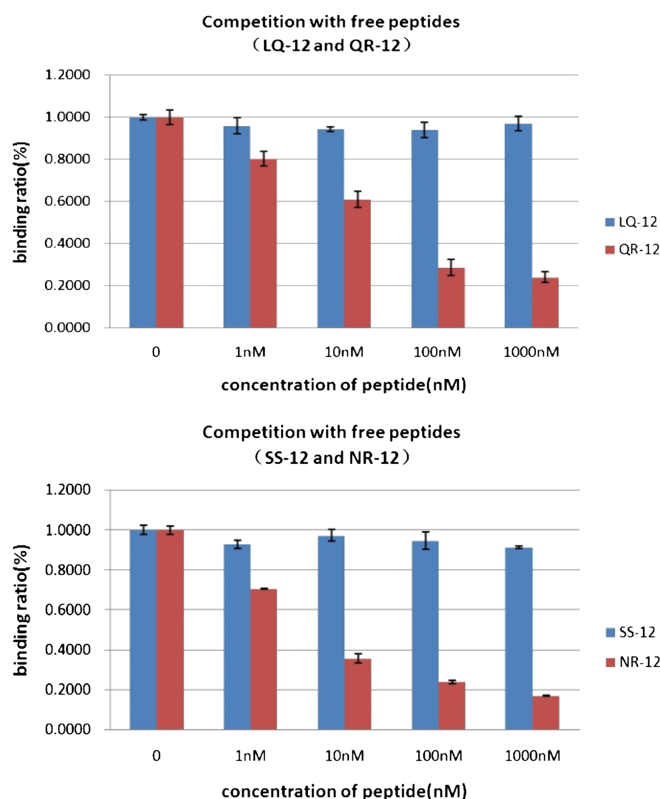

Figure 7. Competition with free peptides. Competitive binding of the phages and chemically synthesized peptides onto cardiomyocytes. Chemically synthesized peptides QR-12 (QPFTTSLTPPAR), NR-12 (NNWSSPPQMISR), and free peptide LQ-12 (LAPTRPTFPTSQ), and SS-12(SNRQPSNIMWPS) were added to compete with the no. 3 (QPFTTSLTPPAR) or no. 9 (NNWSSPPQMISR) phage. Upon addition of 1×10^{11} phages, peptides with increased concentration from 1 nM to 1 μM were added to 2×10^{6} of mESCs. The binding ratio was determined using the output titer of the phages, and with the addition of peptides normalized without the peptides. Each experiment was repeated three times. Values are shown as mean ± standard derivation.

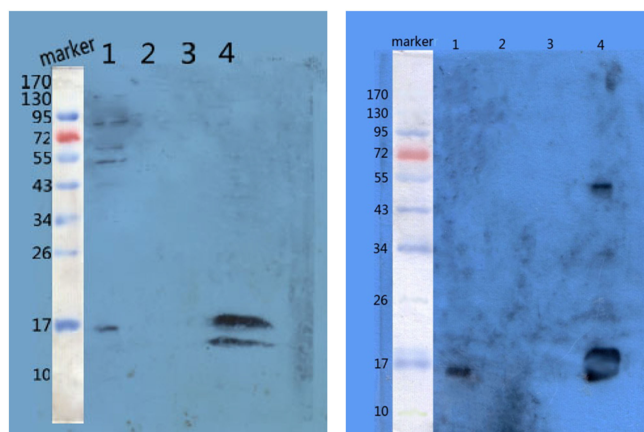

Figure 8. Western blot results. Western blot analysis of the binding ability to cardiomyocytes of no. 3 (A) and no. 9 (B) peptides. Lane 1: mESc cytoplasmic proteins; Lane 2: mESc membrane proteins. Lane 3: cardiomyocyte cytoplasmic proteins. Lane 4: cardiomyocyte membrane proteins.

done to exclude the phages bound to undifferentiated cells, and enrich the phages bound to cardiomyocytes.

After three rounds of biopanning, several clones were selected, and the peptide display region was sequenced. The DNA sequence was translated into their peptides, from which a consensus sequence, QPFTTSLTPPAR, and a consensus motif, SS (Q) PPQ(S), were found. Data sequences were compared in the multiple sequence alignment software CLUSTALW. According to the ELISA results, sequences no. 3 (QPFTTSLTPPAR), no. 9, no. 11, no. 14, and no. 10 [sharing the motif SS (Q) PPQ(S)] were all shown to possess strong binding to cardiomyocytes compared with the mESC. Immunofluorescence also verified their binding ability to cardiomyocytes.

The Western blot results indicate that peptide no. 3 (QPFTTSLTPPAR) binds to cardiomyocytes using two 17 kDa proteins. The no. 9 peptide (NNWSSPPQMISR) also showed these two bands. At the same time, the no. 9 peptide showed a 55 kDa protein that was not observed in no. 3. Interestingly, some bands were also spotted in mESC cytoplasmic proteins. These are possibly other

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proteins that could interact with these peptides, and they may be the same protein located in the mESC cytoplasm, with the high molecular weight bands their polymer.

In conclusion, the phage display library is a very useful tool for finding cell surface markers. Its drawbacks include the difference caused by the multiplication capacity diversity. The peptides selected in the study could specifically bind to the PSC-derived cardiomyocytes, and they may be used as cell markers for the purification of cardiomyocytes and as drug- or gene-delivery vehicles. The results of the present study could pave the way for basic and clinical research.

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Supporting information

Supporting information can be found in the online version of this article

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