Targeting Nanoparticle Probes to Differentiating Stem Cells

J. Cabral-Teixeira*, K. Talsky**, L. Zhang*, J. Maurer*, M. Tsuda*, Y. S. Chao**, N. Prigozhina*, J. Price*, E. Snyder*, M. Mercola*, E. Ruoslahti*, M. D. West** and D. Larocca**

*Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, CA, jteixeira@burnham.org

**Advanced Cell Technology, 1201 Harbor Bay Parkway, Alameda, CA 90025, dlarocca@advancedcell.com

ABSTRACT

The stability of bacteriophage particles and the ability to genetically target them to specific cells make bacteriophage a promising nanobiotechnology tool for labeling and tracking pluripotent stem cells and their progeny. We are using phage display to identify progenitor cell-binding peptides and have isolated several families of peptides from a T7 phage display library that bind and internalize into embryonic progenitor (EP) cells derived from murine and human hES cells. One selected family contained the RXXR minimal binding recognition site for the furin family of proprotein convertases. Peptide targeted phage were used to target quantum dots to specific populations of differentiating embryonic stem cells. We anticipate that cell-targeted nanoparticles will be generally applicable for tracking, and isolating progenitors derived from hES cells as well as other types of pluripotent or multipotent stem cells including iPS and adult stem cells.

Keywords: embryonic stem cells, phage display, quantum dots, cell targeting, imaging

1 INTRODUCTION

Embryonic stem cells have the potential to provide a renewable source of cells for cell replacement therapy provided that the appropriate therapeutic cells can be identified and prepared in sufficient quantities for the development of regenerative cell therapies (1). Human embryonic stem (hES) cells can be propagated indefinitely in the undifferentiated state and therefore provide a potentially unlimited source of cells for therapy (2). Recently, alternative sources of pluripotent stem cells have been reported involving blastomere sampling (3) and reprogramming of human somatic cells (induced pluripotent stem (iPS) cells) (4, 5). Reprogramming can be performed with cells taken from cheek scrapings but currently requires engineering of the cells with lentiviral gene delivery vectors and are therefore not yet clinically suitable but offer immediate research opportunities. Regardless of their source, the ability of pluripotent stem cells to differentiate into any cell type has generated great excitement about their potential for regenerative medicine for a wide variety of degenerative diseases such as heart disease, macular degeneration, diabetes, Parkinson's, Alzheimer's, multiple sclerosis, to name a few.

With the promise of more cost effective and plentiful pluripotent stem cell lines on horizon there will be an increasing need for reagents for stem cell research and development. It will be even more important to face the challenge of how to develop conditions for directing differentiation along a desired lineage as well as ways of isolating well characterized cell populations for preclinical research and clinical applications (6). We are using phage display technology to identify reagents for identifying, tracking and isolating hESC derived progenitors of therapeutic value. Much is known about the changes in intracellular markers on cells as they differentiate but relatively little is known about changes in specific extracellular membrane markers that appear on early progenitors of differentiated cells (7). Identification of such surface markers would allow live sorting of progenitor cell populations and subsequent expansion of the marker-defined populations. Here we describe the use of phage display to develop tools to target these receptors for cell identification, tracking and isolation.

2 MATERIALS AND METHODS

2.1 Cell line and phage library.

The mouse embryonic stem cell line, CGR8-MHCα-eGFP, was obtained from Dr Tomosaburo Takahashi, Harvard University, Boston, Mass, and maintained on gelatin-coated tissue culture dishes under conditions promoting maintenance of pluripotency (DMEM supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.1 mM βME and 1000U/mL leukocyte inhibitory factor. Mouse embryonic stem cells were allowed to aggregate on low-attachment bacterial plates and form embryoid bodies under conditions permissive to differentiation by removal of LIF from culture medium (day 0). The T7 phage peptide display library was constructed in the T7Select415-1b vector (Novagen). The NNK-encoded CX_7C peptide library was constructed and amplified as previously described (8).

2.2 Phage library selection.

Mouse ES cells were differentiated by removal of leukocyte inhibitory factor (LIF) from the culture medium. Embryoid bodies (EB) were grown from differentiating mES cells on non-adherent plates. At day 4 after the initiation of differentiation, the EBs were transferred to gelatin coated tissue culture 6 well plates and grown for 36 hours. The EBs were placed on ice and washed 1x gently with cold DMEM (4°C) before adding the library at 2 x 10^{10} pfu/ml in PBS with Mg^{++} and Ca^{++} (Invitrogen) containing 10% fetal bovine serum (1ml/well). The library was incubated with the cells with gentle rocking for 2 hours at 4°C. The cells were washed 6x with PBS with Mg^{++} and Ca^{++} to remove unbound phage particles and removed from the plate by incubation with 0.536 mM EDTA at room temperature for 10 minutes. Cells were washed at 4°C with 3x 14 ml of PBS/1%BSA after removal from the plate. Cell associated phage were rescued by lysing the cells on ice in 0.1ml of PBS/1% NP40 for 5 minutes. The rescued phage particles were amplified by adding 0.9 ml of BL21 host bacteria (Novagen) at O.D. of 0.6 and rotating the tube for 5 minutes at room temperature. The titer of the rescued phage was obtained from 1/10 the rescued particles and the remaining phage amplified in BL21 (grown at 37°C for 2-3 hours with vigorous shaking until lysis occurred). The selected library was prepared by pelleting the cell debris for 10 minutes at 8,000 x g and dialysing the bacterial lysate against PBS. The selected phage library solution was filter sterilized by passing through a 0.2 micron filter (Millipore) and stored at 4°C. Each selected library was used as input for the next round of selection.

The displayed peptide sequence of individual phage clones was determined by PCR amplification and subsequent sequencing of the peptide encoding DNA inserts using T7up and T7down sequencing primers (Novagen).

2.3 Cell binding assay.

Mouse embryonic cells were differentiated to EBs and plated and peptide phage binding measured at day 5.5 using the same method used for library selection. Binding of individual phage clones was determined by measuring the ratio of peptide phage recovery (pfu) compared to control phage (no displayed peptide) recovery (pfu). The titer of recovered phage in the cell lysate from phage treated cells was normalized to total protein to control for variations in the number of cells plated. Assays were performed in 6 well plates using 2 duplicate wells per sample. Cells were washed once in DMEM and incubated with 10^8 pfu of phage/well in 2 ml for 2 hours at 4°C with gentle rocking. Cells were washed 5x in PBS/1%BSA, 1x in PBS, and lysed in 0.2 ml of PBS/1% NP40/well. Phage were titered as described for library selection and total protein was measured using BCA assay (Pierce).

2.4 Immunocytochemical detection of cell-targeted phage.

The differentiated mouse EBs at d5.5 were incubated with peptide phage or control (no peptide displayed) for 2 hours at 37 C. Unbound phage particles were removed by washing 6x with PBS. The cells were fixed, permeabilized, and stained for phage particle binding as previously described (9). Phage were stained using a mouse anti T7 tail fiber antibody (Novagen) and an Alexa Fluor 568 conjugated anti-mouse secondary antibody. Nuclei were stained with DAPI. Phage staining was analysed using an epifluorescence inverted microscope (Olympus) and digital camera. Pseudocolor was added using Metamorph software.

2.5 Targeted quantum dots.

Peptide phage were purified by 2 rounds of PEG precipitation (9) and biotinylated by reacting with Sulfo-NHS-LC-Biotin (Pierce) for 2 hours on ice. Phage titer was not significantly affected by biotinylation. Streptavidin (SA) conjugated quantum dots (655nm; InVitrogen) were complexed with biotinylated peptide phage by incubating 2 x 10^9 pfu of biotinylated phage particles with 4nM quantum dots in 50 ul of PBS for 15 minutes at room temperature. Synthetic peptides (New England Peptide) were biotinylated at the N terminus, conjugated to SA-quantum dots, and free peptide removed by separation on a Centricon 100 filter (Millipore).

3 RESULTS

3.1 Selection of cell-targeting peptides on embryonic progenitor (EP) cells.

A cysteine-constrained 7mer random peptide library (CX7C) in the T7 select-415 vector (Novagen) was subjected to reiterative selection against CGR8-MHCα-eGFP cells that were differentiated to EBs and grown on adherent plates for 36 hours (d5.5 of differentiation). An increase in the output ratio (output pfu/input pfu) of about 10-fold after 3 rounds of selection indicated successful enrichment of the library for phage displayed peptides that bind cells in the differentiating mouse EP cell population.

3.2 Binding of selected peptide phages to mouse EP cells.

Sequencing of a sample of 42 phage clones from round 3, and 12 from round 4 and 5 revealed that the complexity of the library had collapsed to several families of related peptides. A high percentage of the sampled sequences shared a common RXXR motif and most of these contained a C-terminal arginine residue. The increase in binding of the selected library correlated with the increase in

prevalence of R/KXXR containing sequences. Individual peptide phage were prepared and tested for binding to d5.5 mouse EP cells. As shown in Figure 1A, RXXR containing peptide phage had significant binding to mouse EP cells relative to the control phage. Linear RXXR peptide phage bound significantly greater than cyclic peptide phage containing the same or similar RXXR sequences and greater than a linear sequence that lacked an R/KXXR motif but terminated with 3 positive amino acids (CASRRK).

We used immunocytochemistry to analyse the binding of RXXR phage to mouse EP cells (Figure 1B). Peptide phage particles were readily detected in subpopulations of EP cells derived from differentiating mouse ES cells. Typically, the degree of phage staining correlated with the relative binding efficiency of a particular peptide phage. The staining appeared punctate indicating endosomal uptake of peptide phage particles. Relatively little staining was observed when equivalent amounts of control (no displayed peptide) phage were used.

3.3 Peptide targeted internalization of quantum dots into mouse EP cells.

We conjugated 655nm quantum dots to synthetic CRPPR peptide to direct quantum dot internalization to mouse EP cells. Confocal fluorescence microscopy revealed internalized quantum dots in mouse EP cells incubated with the CRPPR quantum dot conjugates (Figure 1C). Similar results were obtained with CRSPR peptide quantum dot conjugates (not shown). The peptide phage particles themselves were also capable of targeting quantum

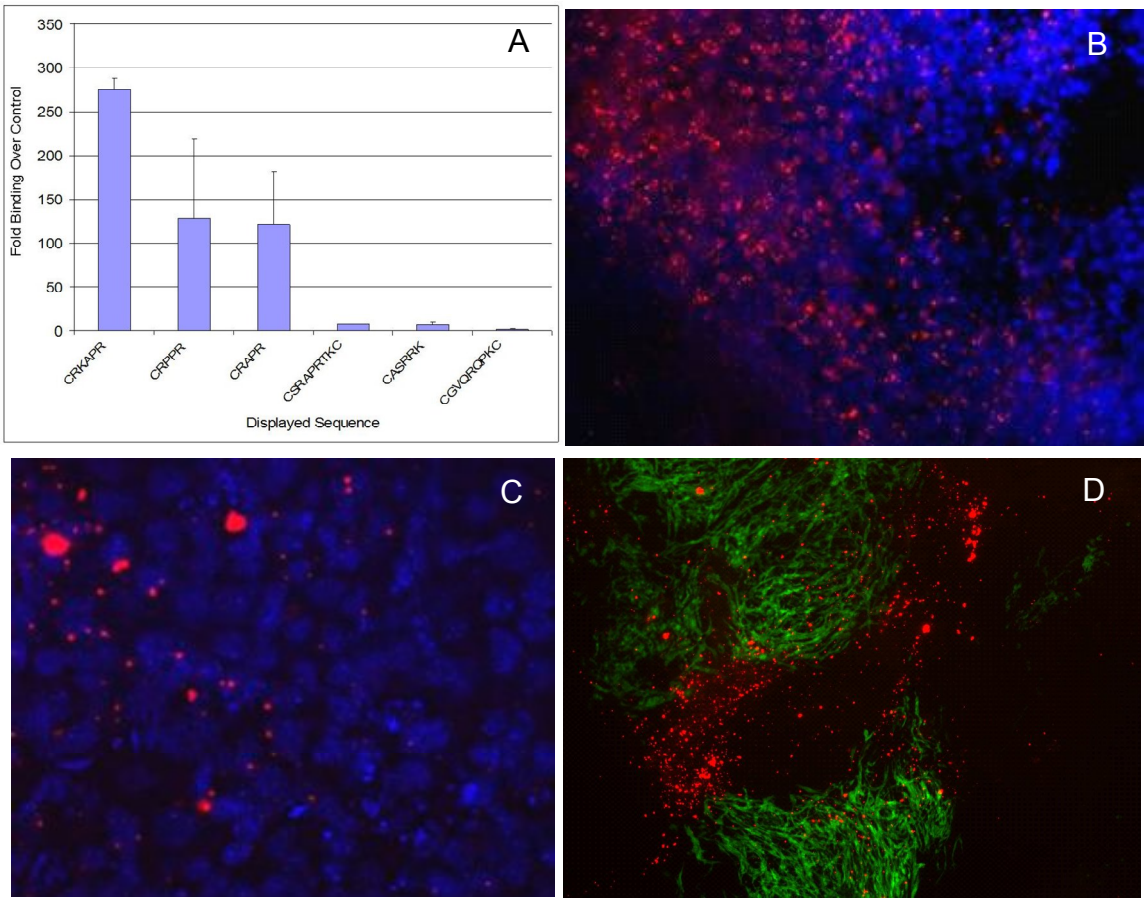

Figure 1: Selection of Embryonic Progenitor Targeting Peptides from a Phage Display Library. A. Selected K/R-X-X-K/R Peptide-Phage Bind Mouse EPs. Peptide phage with terminal K/R-X-X-K/R sequence have strongest binding relative to cyclic K/R-X-X-K/R peptide phage. B. Immunolocalization of CRKAPR-phage on mEPs. Anti T7 antibody (red) staining of CRKAPR-phage treated mEP cells. Nuclei are DAPI stained (blue). Magnification = 200x. C. Peptide targeted quantum dots (655nm) are internalized by mEPs. Confocal image shows internalization of quantum dots (red) in cells exposed to CRPPR labeled with quantum dots (red). Nuclei are DAPI stained (blue). Magnification = 600x. D. Tracking of peptide targeted embryonic cells using Q-dot labeled peptide phage. Biotinylated CRKAPR-display phage were conjugated to streptavidin-quantum dots (655nm) and added to mEPs (MHCα-GFP-CGR8 at d5.5). Free phage particles were washed away and the cells cultured for an additional 30 days. Internalized quantum dots (655nm) are shown in red. Green cells are differentiated cardiomyocytes expressing GFP regulated by the myosin heavy chain α promoter. Magnification = 100x.

dots to mouse EP cells. The cell-targeted quantum dots were shown to be stable following targeting to cells and could be detected as long as 30 days following exposure of the cells to peptide phage targeted quantum dots (Figure 1D). The peptide targeted cell population was distinct from but closely associated with GFP positive differentiated cardiomyocytes.

4 DISCUSSION

We have selected mouse embryonic progenitor cell internalizing peptides from a highly diverse library of random peptides displayed on T7 phage particles. Phage display is a powerful selection technology for identifying protein binding partners which to our knowledge has not previously been applied to the selection of targeting peptides on differentiating ES cells and their progeny. Although we selected from a library of cyclic 7-mer peptides, the strongest binding peptides were linear RXXR peptides that contained a terminal arginine. We have also selected the same library on human EP cells and found that the selected sequences also share similar linear RXXR sequence motifs (not shown). The RXXR motif is the minimal binding motif for the furin family of preprotein convertases which are involved in processing a variety of peptide hormones as well as bacterial toxins and viral coat proteins. Such proteins would be expected to play an important role in embryogenesis. Indeed, the RXXR peptides that we selected share homology with a variety of peptide growth factors. Thus, these peptides may bind to cell surface proteases that are present on early differentiating stem cells.

Progenitor cell targeting peptides will be useful tools for pluripotent stem cell studies and regenerative medicine. The synthetic peptides or the peptide phage themselves were capable of directing quantum dots into targeted progenitor cells and the quantum dot signal was stable for at least 30 days. These data demonstrate the feasibility of using phage display to identify targeting peptides that can be used to label specific progenitor cells in cultures of differentiating pluripotent cells. The long-lived quantum dot signal will allow tracking of targeted progenitor cell populations over time to determine their developmental fate and their interaction with surrounding cells. We are currently investigating the use of time lapse photomicroscopy to perform ES cell derived progenitor cell tracking studies. The targeting peptides will also be useful for isolating populations of progenitor cells to provide well defined progenitor populations from which differentiated cells can be grown for preclinical and clinical studies. Fluorescently tagged cell-targeting peptides will also be useful reagents in high throughput assays for differentiation agents.

In addition to T7 library selection we are currently selecting EP cell targeting peptides from M13 libraries. Selection of peptides that are displayed as N terminal coat protein fusions on a different phage particle should allow selection of additional types of targeting peptides. Indeed, our initial selections do not contain RXXR peptide motifs. We are currently developing new methods to enrich for specific progenitor cell targeting peptides and have begun to select endothelial and keratinocyte progenitor targeting peptides.

We have identified EP cell targeting peptides from phage display libraries and shown that the peptides or the phage particles themselves can be used to label populations of early differentiating embryonic cells. The specific targeting of quantum dots to early progenitor cells will be a useful tool for identifying, tracking and isolating populations of progenitor cells for characterization and assessment of their therapeutic potential.

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