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Generation of PD-L1-Deficient Cell Line by AAV-Mediated CRISPR Knockout for Ribosome Display Selection

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Abstract

Ribosome display is an effective technology for selection and evolution of antibodies against almost any protein antigen. This method allows omitting the immunization of animals and laborous production and screening of hybridoma clones. However, different approaches for panning of antibodies exist within the method. Conventionally, selection is carried out by immobilizing synthetic target antigen on polystyrene surface or magnetic beads. This approach is simple and straightforward, but it does not always lead to a high-quality final product. Non-specific antibodies that are able to bind broad range of targets can contaminate the final eluate, hindering selection of a highly specific antibody. Another option for panning is direct selection on a model cancer cell line. The advantage of this method is the preliminary counter-selection of the library on the same cell line with the knocked-out antigen. Although this approach may be more challenging in the process, it can yield antibodies with better selectivity and specificity. In the following report, we describe the process of obtaining a model breast cancer cell line with a knockout PD-L1 antigen for further use in antibody selection.

Keywords: AAV, CRISPR, PD-L1, saCas9, ribosome display, scFv selection

INTRODUCTION

Ribosome display is a powerful in vitro system allowing to screen peptide and protein libraries for specific ligands [1]. The main advantage of the technology is ability to construct libraries of much higher complexity in comparison with other methods, e.g. phage display [2]. Ribosome display is a strategy of choice for selection and *in vitro* evolution of antibodies against various antigens [3]. The method allows to obtain specific antibodies rapidly and in a cost-effective manner, therefore exceeding conventional selection strategies that require immunization of animals. These characteristics become especially valuable in the field of cancer adoptive immunotherapy and chimeric antigen receptor construction, where rapid development of therapeutics directly affects patient's survival.

Ribosome display relies on cell-free transcription and translation of DNA libraries coding proteins of interest with lack of stop-codon [4]. Absence of stop-codon prevents separation of translated mRNA as well as polypeptide from ribosome which results in forming of peptide-ribosome-mRNA complexes [5]. These complexes ensure that genotypes and phenotypes stay coupled [1]. Conventional techniques of ribosome display selection include three consequent steps: attachment home-made/commercial antigen to solid supports (polystyrene plates, magnetic beads); incubation with scFv or VHH library in the form of protein-ribosome-mRNA complexes; and iterative rounds of intensive washings to remove unbound or weakly bound complexes. Other possible selection strategy involves using cell line as a platform for antibody selection against the antigen of interest expressed on cells' surface. This *in vivo* selection strategy seems more convenient due to expressing target antigen in native form. Although a large number of recombinant proteins correspond to the sequence and folding structure of native counterparts, some recombinant proteins demonstrate major differences in structure and function. Moreover, *in vitro* methods rely on BSA as a blocking agent used to reduce nonspecific hydrophobic binding during selection. However, BSA may be not highly effective for counterselection: this technique does not involve exposition of antibodies to the natural repertoire of antigens on the cell surface, and many broad-specific molecules remain in the mix. This may lead to a selection of false-positive and sticky antibodies, thus decreasing the quality of final candidates. *In vivo* selection strategy seems to avoid this pitfall, due to counterselection and selection steps being carried out on model cancer cell line. The counterselection step leads to significant depletion of antibody library which then favors to selection. However, this advanced approach requires to obtain a pair of cancer cell lines that differ only in expression of antigen of interest which may be challenging by itself.

Here, we report on the development of model breast cancer cell line with PD-L1 knockout. CRISPR technology was used to turn off PD-L1 gene. In a perspective, this cell line may be applied for ribosome display panning in laboratory routine.

MATERIALS AND METHODS

Cell culture

Breast cancer cell line MDA-MB-231 was purchased from ATCC and cultured in RPMI 1640 medium (Paneco, Russia) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA), penicillin/streptomycin and 2 mM L-alanyl-L-glutamine (Paneco, Russia). Human embryonic kidney cell line HEK-293T was cultivated in DMEM/F12 (Paneco, Russia) with 2mM L-alanyl-L-glutamine, 10% FBS and penicillin/streptomycin. Both cell lines were grown at 37°C in 5% CO2.

Guide RNA selection and synthesis

RNA-guided endonucleases against PD-L1 were searched with Cas-designer tool (rgenome.net). Next oligo pairs encoding the selected guide sequences were commercially synthesized (Evrogen, Russia) (table 1):

Table 1: Commercially Synthesized Oligo pairs

Oligo sequences 5'-3'	Cas endonucleases origin
TGAACATGAACTGACATGTC	SpCas
GATGGCTCCCAGAATTACCAA	SaCas

Cloning into the vector

Oligo pairs were treated with PNK and annealed in Tris-containing buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA) at 95°C for 5 minutes. Annealed sequences for SpCas endonuclease were cloned into pCas-guide vector (OriGene, USA) via BsmB I and BamH I sites. pX601-AAV-CMV::NLS-SaCas9-NLS-3xHA-bGHpA;U6::BsaI-sgRNA vector (Addgene, USA) was digested by BsaI sites, where then the oligo pair for SaCas was cloned. Correct insertions were verified by diagnostic restriction and target sequencing.

Cell culture transfection with pCas-guide

One day before transfection, the MDA-MB-231 cells were seeded at $3.5x10^4$ cells/well in 96-well plate. The next day cells were washed once with PBS and serum-starved for two hours in serum- and antibiotics-free RPMI 1640. Then MDA-MB-231 cell line was transfected by using TurboFect reagent (Thermo Fisher Scientific, USA), Lipofectamine LTX or Lipofectamine 3000 (Thermo Fisher Scientific, USA) according to manufacturer's instructions. Since pCas-guide plasmid didn't contain any fluorescent reporter protein or antibiotic-resistant gene, control plasmid pLCMV-tagRFP-puro was used to monitor transfection efficiency.

Virus preparation and purification

AAV particles were generated in HEK-293T by co-transfecting the cell line with pX601 and adenovirus-independent helper plasmid pDP1rs (PlasmidFactory, Germany). For small-scale assay, cells were plated at $2x10^5$ cells/well in 12-well plates. Next day cells were serum-starved for 2 hours in non-supplemented DMEM/F12, then treated with pX601 and pDP1rs in equimolar amounts (3 mkg total DNA per well). TurboFect was used as a transfection reagent. For medium-scale preparation, $2.5x10^6$ cells were seeded in each of two 10-cm dishes and transfected the next day with 20 ug of plasmid mixture. After overnight incubation, the medium was substituted with fresh DMEM/F12 containing 10% FBS. Transfected HEK-293T cells were grown for 72 hours, then washed three times in PBS and harvested by trypsinization. After centrifugation, pelleted cells were resuspended in 1 ml of PBS and lysed by 5-8 freeze-thaw cycles in liquid nitrogen and a 37°C water bath, followed by sonication. To disaggregate lysates, DNase I was added to the final concentration of 0.05 U/ul and incubated for 30 minutes 37 °C. Finally, the lysates were centrifuged for 20 minutes 3700 g and the supernatants were collected for virus purification.

Virus purification was conducted using iodixanol gradient that was produced as described elsewhere [6, 7]. Briefly, 15%, 25%, 40% solutions of iodixanol in PBS-MK (1xPBS, 1 mM MgCl2, 2.5 mM KCl) were prepared from 60% stock solution (OptiPrep, Alere technologies, Norway). NaCl was added to the 15% phase at 1M final concentration to destabilize ionic interactions between AAV particles and proteins. 25% and 60% iodixanol solutions were supplemented with phenol red to visualize the boundaries between dense phases. Diluted iodixanol was underlaid in next order: 900 ul 15%, 600 ul 25%, 500 ul 40% and 460 ul 60% iodixanol at the bottom of 3-ml ultracentrifuge tubes. Clarified lysates were overlaid on the gradient and centrifuged for 90 minutes at 362440 g in TL-100 ultracentrifuge (Beckman Coulter, USA). About 2/3 volume of 40% phase with the concentrated virus was collected, transferred to clean tubes and frozen.

Virus infection and immunocytochemistry

MDA-MB-231 cells were seeded at about 70% confluence in a 96-well plate. After cells adhered, AVV particles were added to the wells to the final dilutions of 0, 1:10, 1:100, 1:1000 or 1:10000. On next day the culture medium was changed for a fresh one with 2% FBS. After 42 hours, cells were washed 3 times in PBS, fixed by 3.7% paraformaldehyde solution, permeabilized with 0.5% Triton X-100 in PBS for 8 minutes and blocked by 3% BSA for 1 hour. Then the cell culture was incubated with primary antibodies against HA (Roche, Switzerland) at the final concentration 200ng/ul overnight. Next day, cells were washed, incubated with the secondary antibodies for 90 minutes and stained with DAPI for 5 minutes. After final washing steps, cells were visualized under fluorescent microscope (Zoe, Bio-Rad, USA).

Single cell cloning by serial dilutions

Isolation of clonal cell populations from infected cells was performed 48 hours after virus transduction. Transfected MDA-MB-231 cell line was detached from plates and filtered through cell strainer to avoid clumping. Cells were counted and diluted to 60 cells in 12 ml of RPMI 1640 medium for 96-well plate according to recommendations [8]. Then each 100 ul of diluted cell suspension was transferred to each well of a 96-well plate. After one week cells were examined for clonality potential.

When cells became 80% confluent, 30% of cell population from each well was plated into the replica plate. When cells reached 80-90% confluence again, they were dissociated, seeded into 24-well plate and after expansion harvested for western-blot.

Western-blotting

Lysates for western-blot were prepared in RIPA buffer. MDA-MB-231 cells were washed three times in PBS, incubated with 300 ul of ice-cold RIPA for 10 minutes on ice, harvested with cell scraper and centrifuged for 10 minutes at 13000 rpm, 4°C. Supernatants were collected and frozen. Western-blot was conducted according to general protocol for western-blotting by Bio-Rad. Detection of the PD-L1 protein level was accomplished by using primary anti-PD-L1 antibodies ([28-8], Abcam, England).

RESULTS AND DISCUSSION

Specific and reliable method is needed to obtain pure fractions of highly-specific antibodies against the antigen of interest. False-positive clones, as well as antibodies with nonspecific cross-reaction, are frequently selected during in vitro panning. Selection strategy using live cells as antigen carriers allows to avoid these pitfalls and obtain scFv pools with much higher specificity towards desired target. CRISPR-Cas9 technology, a novel tool for genome editing, enables production of cell lines with precise and complete knockout of antigen of interest. Resulting knockout cell line serves as a perfect match for counterselection step allowing to minimize the presence of non-specific high-affinity clones in selection mix.

We used MDA-MB-231 as a convenient breast cancer cell line for generating antibodies against PD-L1. According to scientific reports, MDA-MB-231 is characterized by overexpression of PD-L1 among other breast cancer cell lines [9-11]. Knockout of PD-L1 was performed by CRISPR technology using transient plasmid transfection and AAV infection.

Unfortunately, efficiency of both gene delivery methods was quite low. Although MDA-MB-231 is frequently used breast cancer cell line in laboratory routine, its direct transfection was mainly unsuccessful in our practice. Using different transfection reagents (turboFect, Lipofectamine LTX, Lipofectamine 3000) as well as various DNA/reagent ratios didn't lead to satisfactory transfection level with control plasmid. Viral transduction with AAV vector was more efficient compared to plasmid transfection, although it also yielded moderate amounts of transduced cells (fig.1). Nevertheless, we conducted screening of single cell clones to find cell population with complete PD-L1 loss. Over 100 clones were analyzed by western-blotting, and as a result we established one cell clone with complete loss of PD-L1 expression (figure 1).

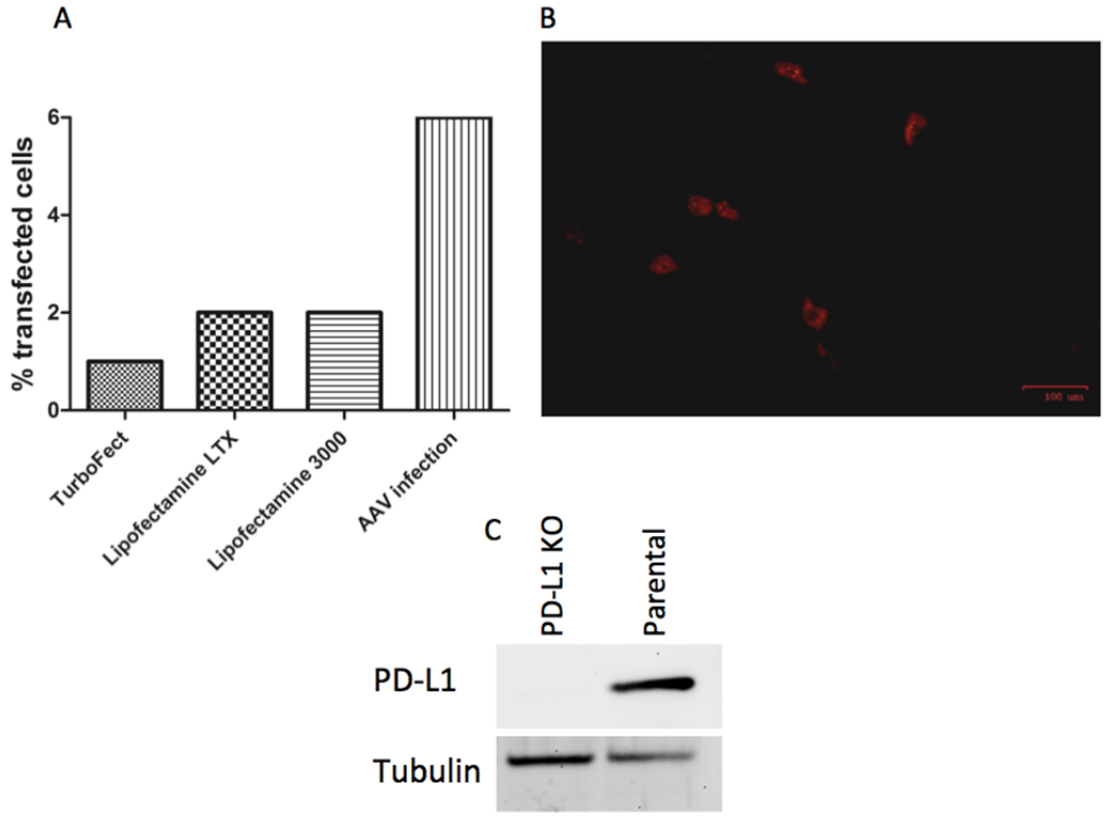

Figure 1. A. Transduction efficiency of different transfection reagents and AAV vector on MDA-MB-231 cell line. B. Immunostaining of saCas9 endonuclease fused with three tandem HA epitope tags. Here MDA-MB-231 is infected with AAV (1:1000 dilution) carrying hybrid expression cassette with both saCas9 and gRNA sequence. C. Western-blot showing complete PD-L1 knockout in MDA-MB-231 cell line clone.

CONCLUSION

We obtained a model breast cancer cell line with PD-L1 knockout which may be used for in vivo ribosome display selection. MDA-MB-231 with loss of PD-L1 represents a convenient cell platform for counterselection step. Incubation of mixtures of antibody-ribosome-mRNA complexes with knockout cell line leads to efficient elimination of most high-affinity non-specific antibody fragments in a cell-specific manner. Consequent steps of panning with depleted library could result in selection and evolution of high-quality scFv candidates against PD-L1.

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