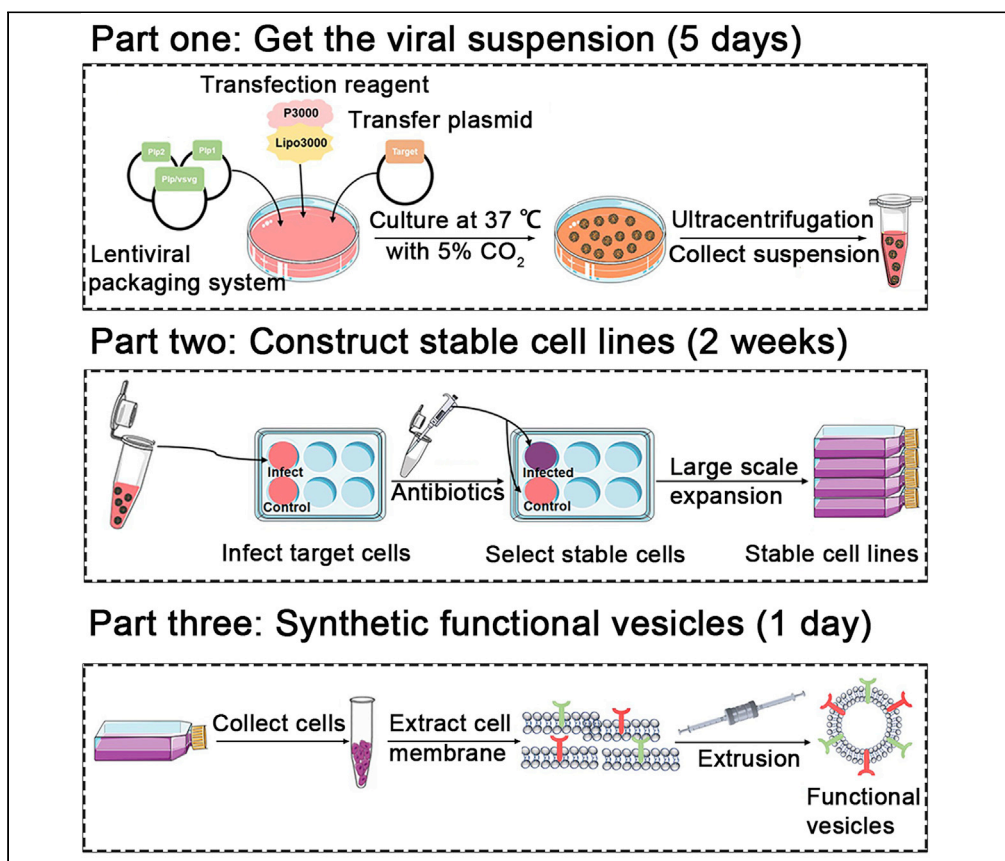


Protocol

Protocol to prepare functional cellular nanovesicles with PD1 and TRAIL to boost antitumor response



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HIGHLIGHTS

Established stable HEK293 FT cells expressing PD1 and TRAIL

Prepare functional cellular nanovesicles (FCNVs) from stable HEK293 FT cells

FCNVs can boost antitumor response by the cooperative roles of PD1 and TRAIL

Immunotherapy has achieved notable success in tumor treatment, but it is restricted to a small number of patients due to multiple immunosuppressive pathways in the tumor microenvironment. Here, we present a step-by-step protocol to prepare functional cellular nanovesicles from HEK293-FT cells displaying PD1 and TRAIL. TRAIL specifically induces immunogenic cancer cell death to initiate an immune response, and ectogenic PD1 blocks the PD1/PDL1 checkpoint signal to reactivate anergic tumor-specific CD8⁺ T cells.

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Protocol

Protocol to prepare functional cellular nanovesicles with PD1 and TRAIL to boost antitumor response

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SUMMARY

Immunotherapy has achieved notable success in tumor treatment, but it is restricted to a small number of patients due to multiple immunosuppressive pathways in the tumor microenvironment. Here, we present a step-by-step protocol to prepare functional cellular nanovesicles from HEK293-FT cells displaying PD1 and TRAIL. TRAIL specifically induces immunogenic cancer cell death to initiate an immune response, and ectogenic PD1 blocks the PD1/PDL1 checkpoint signal to reactivate anergic tumor-specific CD8⁺ T cells. For complete details on the use and execution of this protocol, please refer to Wu et al. (2020).

BEFORE YOU BEGIN

Design plasmids

⌚ Timing: 1 day

1. Download the target gene sequence from NCBI Gene database. For this protocol, we use house mouse PD1 (Programmed cell death 1, Pcd1) (Tang et al., 2018), TRAIL (Tumor necrosis factor superfamily member 10, Tnfsf10) (von Karstedt et al., 2017), and their Accession are NC_000067.7 and NC_000069.7, respectively. See Figure 1.
2. Integrate target gene sequence with Kozak sequence, linker sequence and fluorescent protein sequence (Figure 2).
 - a. Acquire Kozak sequence on the website (<https://baike.sogou.com/v7771534.htm>)
 - b. Determine the Linker sequence according to experimental needs (Chen et al., 2013). For this protocol, we choose the GS linker to connect the target gene sequence and the fluorescent protein sequence.

Note: According to their functions in the construction of recombinant fusion proteins, peptide linkers are generally classified into three categories: flexible linkers, rigid linkers, and *in vivo* cleavable linkers. Among these linkers, flexible linkers (usually peptides rich in small or hydrophilic amino acids) could provide a certain degree of flexibility between the joined domains to allow interaction or increase spatial separation between the domains, while rigid linkers (such as helical structures and peptides rich in Prolines) could keep the distance between the joined domains to maintain the stability and bioactivity of the domains. And *in vivo* cleavable linkers



<p><input type="checkbox"/> Pcd1 ID: 18566</p>	<p>programmed cell death 1 [<i>Mus musculus</i> (house mouse)]</p>	<p>Chromosome 1, NC_000067.7 (93966027..93980278, complement)</p>	<p>Ly101, PD-PD-1 Pdc, Pdc1</p>
<p><input type="checkbox"/> Tnfrsf10 ID: 22035</p>	<p>tumor necrosis factor (ligand) superfamily, member 10 [<i>Mus musculus</i> (house mouse)]</p>	<p>Chromosome 3, NC_000069.7 (27371226..27393814)</p>	<p>A330042121Rik, AI448571, AP, APO-2L, Ly81, T, TL2, Tnlg6a, Trail</p>

Figure 1. The detailed target gene information from the NCBI database

are commonly used to generate commensurable proteins, which are physically separated from each other to prevent any potential interaction and steric hindrance. Particularly in this protocol, in order to make sure that the target protein and fluorescent protein are constantly fused to each other to facilitate the imaging, meanwhile to increase spatial separation to minimize steric hindrance, the most commonly used flexible linker consisting of Glycine and Serine (abbreviated as "GS" linker) is chosen as the peptide linker. In addition to act as a flexible linker used in fusion proteins, GS linker also can be used in single-chain variable fragment (scFv) to link the variable regions of heavy and light chain of the complete antibody, or optimized to improve the oral efficacy of recombinant granulocyte colony-stimulating factor and transferrin fusion protein.

- c. Download the fluorescent protein sequence as step 1 described from the NCBI Gene database. For this protocol, we select red fluorescent protein (mCherry, ADV78248.1) and green fluorescent protein (EGFP, NC_025025.1) as fluorescence tags.
- d. Kozak sequence, Linker sequence, target gene sequence and fluorescent protein sequence are connected in order as shown in [Figure 2](#), and the integrity of the integrated sequences is checked by Snapgene software.
- e. Entrust the company (Sangon Biotech (Shanghai) Co., Ltd. <https://www.sangon.com/>) to synthesize the integrated sequences and insert them into the lentivirus vector plasmid (pCDH-CMV-MCS-EF1-Puro and pCDH-CMV-MCS-EF1-Blasticidin) through linking at the end of the C-terminal of the plasmid ([Zhang et al., 2018a, 2018b](#)).

Alternatives: The pre-synthesized lentivirus vectors can also be homemade by the following steps. First, design the PCR primers to amplify the coding sequence of the target gene (remember to contain Kozak sequence, restriction enzyme cutting site and optimal bases for flanking the restriction site at the 5' end of the forward primer; for the reverse primer, stop codon should be removed to fuse the protein with GS linker and fluorescent protein). Second, the target gene fragment is generated by PCR amplification from cDNA template and purified by electrophoresis. Next, the PCR product and vector plasmid are respectively subjected to restriction enzyme digestion and purified by electrophoresis. The purified linear vector and the PCR fragment are ligated by T4 ligase to construct the interested plasmid, which is further imported into competent cells and dotted onto LB agar plates (plus antibiotic). The monoclonal colonies on the plate are selected and multiplied in LB medium for PCR identification, and the positive clones are sequenced. At last, the correct clone is expanded in large-scale and the plasmid is extracted with EndoFree Maxi Plasmid Kit following the manufacturer's instructions.

Plasmid amplification

⌚ Timing: 2 days

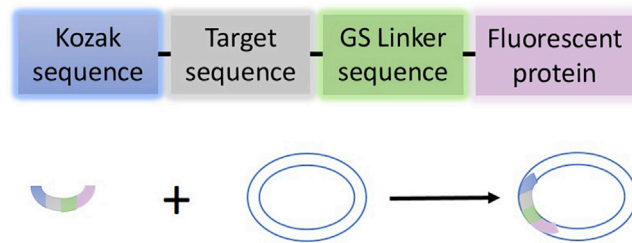


Figure 2. Schematic illustration of the integration and insertion of the sequence of interest

After obtaining the above mentioned two plasmids (pCDH-CMV-PD1-mCherry-Puro, pCDH-CMV-TRAIL-EGFP-Blasticidin), high-quality plasmids were obtained by purification with EndoFree Maxi Plasmid Kit (TIANGEN, DP117, <https://www.tiangen.com/?app.html>).

3. To prepare LB medium, dissolve 10 g tryptone, 5 g Yeast extract and 10 g NaCl in 1 L of ultrapure water (use magnetic stirrer, 500 rpm/min), aliquot to 200 mL and autoclave at 121°C for 15 min. After the medium is naturally cooled to 20°C–25°C, add antibiotics (containing ampicillin 100 µg/mL or kanamycin 50 µg/mL).
4. Take out the Competent Cell (TransGen, CD501-02, <https://www.transgen.com.cn/>) from the –80°C refrigerator and leave on the ice for 15 min.
5. Add 20 µL Competent Cell and 5 µL plasmids into a sterilized 1.5 mL Eppendorf (EP) tube, mix well, and place on ice for 0.5 h.
6. The mixture is then placed on 42°C water bath for 30 s, then immediately placed on ice for 2 min.
7. 500 µL free-antibiotic LB medium is added to EP tube and shaken at 37°C for 40–60 min to restore normal growth of competent cell, using bench type thermostatic oscillator.
8. The mixture is added to 200 mL LB medium containing the antibiotics and shaken at 37°C for 12–14 h to expand plasmids.

9. **Note:** Unless otherwise indicated, antibiotics are applied as: 100 µg/mL ampicillin and 50 µg/mL kanamycin. Plasmids are extracted from the microbial broth according to the instructions of the EndoFree Maxi Plasmid Kit (TIANGEN, DP117, <https://www.tiangen.com/?app.html>).

▮▮ Pause point: The microbial broth can be stocked in 25% glycerol at –80°C for 1 year.

10. The concentration of the extracted plasmids is detected by NanoDrop 2000/2000c Spectrophotometers (Thermo Fisher Scientific), and the sequence of plasmids is verified by biological company (BioSune (Shanghai) Co., Ltd. <http://www.biosune.com/>).

Note: A good quality and endotoxin free of plasmids are important for successful transfection in HEK293 FT cells.

Cell culture

⌚ **Timing: 2 weeks**

11. Prepare cell culture medium. Mix 500 mL of DMEM, 50 mL of FBS, 5 mL of Penicillin-Streptomycin solution (penicillin: 10,000 units/mL, streptomycin 10,000 mg/mL) in a sterilized environment.
12. Culture cells for transfection and infection
 - a. Thaw HEK293FT cells cryo-preserved in a liquid nitrogen freezer at 37°C for 5 min, add 10 mL of PBS to wash and centrifuge at 300 × g for 5 min. Aspirate the PBS.

- b. Resuspend the cell pellet in 1 mL of culture medium as prepared in step 11, transfer the cells into T25 cell culture flask and then supplement with 4 mL of culture medium.
- c. Place the cell culture flask in an incubator with 5% CO₂ at 37°C.

Note: After culture the cells in incubator for about 12 h, there are some dead cells floating in the medium, then the medium should be replaced by fresh one.

- d. When cells reach 80%–90% confluence, aspirate the culture medium from the cell culture flask and rinse the cells once with 5 mL of PBS using an electronic pipette.
- e. Add 0.5 mL trypsin-EDTA to the cells and place the cells into the incubator with 5% CO₂ at 37°C incubator for 3 min.
- f. Add 10 mL culture medium and detach the cells by slowly pipetting up and down, and count cell number under a microscope using a hemocytometer.
- g. Transfer the culture medium into a T75 cell culture flask using an electronic pipette.
- h. Place the cell culture flask in an incubator with 5% CO₂ at 37°C. When cells have reached 80%–90% confluence, split cells 1:4 into another T75 cell culture flask by repeating steps d–f.
- i. Continue to sub-culture cells, maintaining 80%–90% confluence, until ready for construction of stable cell lines expressing PD1 and TRAIL.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-PD1	Abcam	Cat# ab214421
Mouse monoclonal anti-TRAIL	Abcam	Cat# ab10516
Goat anti-rabbit IgG (H+L), HRP conjugate	TransGen Biotech	Cat# HS101-01
Goat anti-mouse IgG (H+L), HRP conjugate	TransGen Biotech	Cat# HS201-01
Bacterial and virus strains		
Trans1-T1 phage resistant chemically competent cell	TransGen Biotech	Cat# CD501-02
Chemicals, peptides, and recombinant proteins		
DMEM	Gibco	Cat# 11995500
Opti-MEM	Gibco	Cat# 31985-LSG
FBS	PAN	Cat# P30-3302
PBS	HyClone	Cat# SH30256.01
Penicillin-streptomycin	Gibco	Cat# 15140122
Ampicillin	Solarbio	Cat# YZ-1033000 Cas# 69-53-4
Kanamycin	Solarbio	Cat# YZ-1355006 Cas# 5965-95-7
Tryptone	OXOID	Cat# LP0042
Yeast extract	OXOID	Cat# LP0021
NaCl	Sinopharm	Cat# 73522260 Cas# 7647-14-5
DiO	Beyotime	Cat# C1038
Dil	Beyotime	Cat# C1036
DAPI	Sigma-Aldrich	Cat# D9542 Cas# 28718-90-3
PMSF	Beyotime	Cat# ST506
Protease inhibitor cocktail	Sigma-Aldrich	Cat# P8340
Polybrene	Santa Cruz	Cat# SC-134220
Paraformaldehyde, 4%	Solarbio	Cat# P1110
RIPA lysis buffer	Servicebio	Cat#G2002
Critical commercial assays		
EndoFree Maxi Plasmid Kit	TIANGEN BIOTECH (BEIJING)	Cat# DP117
Easy II Protein Quantitative Kit	TransGen	Cat# DQ111-01

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Membrane and Cytosol Protein Extraction Kit	Beyotime	Cat# P0033
Lipofectamine 3000 Transfection Kit	Thermo Fisher	Cat# L3000015
Mouse PD-1 Duo Set ELISA Kit	R&D Systems	Cat# DY1021
Mouse TRAIL/TNFSF10 Duo Set ELISA Kit	R&D Systems	Cat# DY1121
Experimental models: cell lines		
Human: HEK293FT	ATCC	N/A
Recombinant DNA		
Plasmid: pCDH-CMV-MCS-EF1-Puro	This paper	N/A
Plasmid: pCDH-CMV-MCS-EF1-Blasticidin	This paper	N/A
Plasmid: pCDH-CMV-PD1-mCherry-Puro	This paper	N/A
Plasmid: pCDH-CMV- TRAIL-EGFP-Blasticidin	This paper	N/A
Software and algorithms		
Flowjo	Tree Star	10.0
Zen	Carl Zeiss	2012 SP2(blue edition)
Other		
Confocal dish	NEST	Cat# 801005
6-well plate	Costar	Cat# 3335
0.22 μ m filter	Merck Millipore	Cat#PRO3683
15 mL centrifuge tubes	Corning	Cat# 430790
25 cm ² cell culture flasks	Corning	Cat# 430639
75 cm ² cell culture flasks	Corning	Cat# 430641
Cell scraper	Thermo Fisher	Cat# 179693PK
CO ₂ incubator	Thermo Scientific	HERAcell150i
Thermostat water bath	Jinghong	DK-8D
Clean bench	AIRTECH	SW-CJ-2FD
Autoclave sterilizer	ZEALWAY	GR85DR
Milli-Q gradient system	Milli-Q Reference	Milli-Q Integral 3
NanoDrop2000/2000c spectrophotometer	Thermo Scientific	ND2000LAPTOP
Ultracentrifuge	Beckman Coulter	Optima XPN-100
Centrifuge	Thermo Fisher	Sorvall ST 16R
Dry bath	ZHISUN	Mk-20
Avanti mini-syringe	Avanti	610017-1Ea
Confocal laser scanning microscope	Zeiss	LSM780
Flow cytometry	BD	FACSVerser
Fluorescence microscope	Zeiss	Axio Vert.A1

MATERIALS AND EQUIPMENT

Preparation of 2\times loading buffer	
Reagent	Amount
1 M Tris-HCl (pH 6.8)	1.25 mL
10% SDS	3.0 mL
Bromophenol Blue	1.0 mg
Glycerin	1 mL
β -Mercaptoethanol	0.5 mL

STEP-BY-STEP METHOD DETAILS

Construction of stable cell lines expressing PD1 and TRAIL

⌚ Timing: 3 weeks

Table 1. The detailed description of transfection reagent

	Amount
Reagent	
Opti-MEM	2 mL
Lipo 3000	40 μ L
P3000	40 μ L
Plasmid	
Plp1	7.5 μ g
Plp2	3.0 μ g
Plp/vsvg	4.0 μ g
Target gene plasmid (PD1 or TRAIL)	10.0 μ g

When the plasmids containing PD1 or TRAIL sequence and HEK293FT cells are ready, the genetic engineered cells are constructed according to the overexpression protocol which involves plasmid transfection, antibiotic selection to construct stable cell lines (Elegheert et al., 2018). The procedure includes two parts. In the first part (steps 1–9), the HEK293FT cell lines are transfected using Lipofectamine 3000 kit (Thermo Fisher, L3000015), and lentivirus supernatant are collected. In the second part, according to the resistance gene in the target gene plasmid, the corresponding antibiotic is added to screen the infected cells, then large-scale expanded (steps 10–13) (Chaudhary et al., 2012).

1. Inoculate the parent HEK293FT cells in T75 culture flask in advance, and proceed to the next step after the cell density reaches 70%–80%.
2. Prepare reagents for gene transfection. Prepare a 15 mL centrifuge tube (numbered A) and a 1.5 mL EP tube (numbered B). Transfer 1 mL Opti-MEM medium (Gibco, 31985-LSG, <https://www.thermofisher.com/us/en/home/brands/gibco.html?CID=cn-ebz-sem-sgk-gene-Gibco-aBrand-pc-subb-082920-abcd-00000009FD973428>) and 40 μ L Lipo 3000 in Tube A, mix them by using gentle pipette motion. Tube B contains target gene plasmid (10 μ g), lentivirus packaging systems (plp1 plasmid: 7.5 μ g; plp2 plasmid: 3 μ g; plp/vsvg plasmid: 4 μ g) and P3000 (40 μ L), which are dissolved in 1 mL Opti-MEM medium (Gibco, 31985-LSG, <https://www.thermofisher.com/us/en/home/brands/gibco.html?CID=cn-ebz-sem-sgk-gene-Gibco-aBrand-pc-subb-082920-abcd-00000009FD973428>). See Table 1 for the description of all reagent for transfection. Then, set tube A and B aside for 5 min.
3. Transfer the solution in Tube B to Tube A, mix them by using gentle pipette motion, and let the mixture stand for 20 min.
4. Add the above mixture to the cell culture flask, and lentiviral supernatant are collected after 24 h to 72 h of incubation.

Note: mCherry tag and EGFP tag inserted in the target gene plasmid provide the fluorescent signals to evaluate the gene transfection efficiency. Under fluorescence microscope, when the percent of fluorescence cells reach above 60% (Figure 3), and the cell maintain normal morphology, it is suitable to collect the supernatant. If the transfection efficiency is low, See [Troubleshooting: Problem 1](#).

△ CRITICAL: Collect the supernatant and add fresh culture medium. Try to operate along the side wall of the culture flask, gently and slowly, and avoid direct contact with the cells.

5. Filter the supernatant containing lentivirus by passing it through a 0.22 μ m filter to remove large cell fragments.

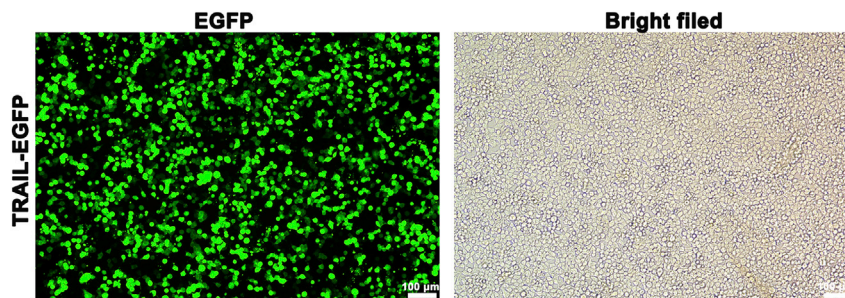


Figure 3. Fluorescence images of HEK293 FT cells after transfection for 24 h

Scale bar, 100 μm .

6. Transfer the sample to a sterilized ultracentrifugation tube. Place the centrifugation tube into a pre-cooled centrifuge. Counterbalance when needed.
7. Program the ultracentrifuge run using the following settings:
 - a. Rotor type: SW 32.1 Ti Rotor
 - b. Speed: 76,800 $\times g$
 - c. Time: 90 min
 - d. Temperature: 4°C
 - e. Acceleration: 0 (MAX)
 - f. Deceleration: 0 (MAX)
8. Start the ultracentrifuge.
9. After centrifugation, the supernatant is gently discarded, and 1 mL of fresh culture medium is added to resuspend the virus, then aliquot into 3–5 tubes (virus titer: 10^6 – 10^7 IFU/mL), and stored at -80°C for future use.
10. Seed 1 mL of the cell suspension of HEK293FT cells (containing 1×10^5 cells) in a 6-well cell culture plate, place the plate in an incubator with 5% CO_2 at 37°C overnight.

Note: Incubation time can be adjusted and terminated when 70%–80% cell confluence reach.

11. The cell culture is replaced by fresh medium supplemented with a tube of virus solution (containing 10^6 – 10^7 IFU) and 5 $\mu\text{g}/\text{mL}$ polybrene reagent to infect the cells. If the infection efficiency not high, see [Troubleshooting: Problem 2](#). After 2 or 3 days of culture, add puromycin or blasticidin to the cell for selecting PD-1 or TRAIL stably expression cells, respectively. The parent cells without lentiviral infection are used as control.

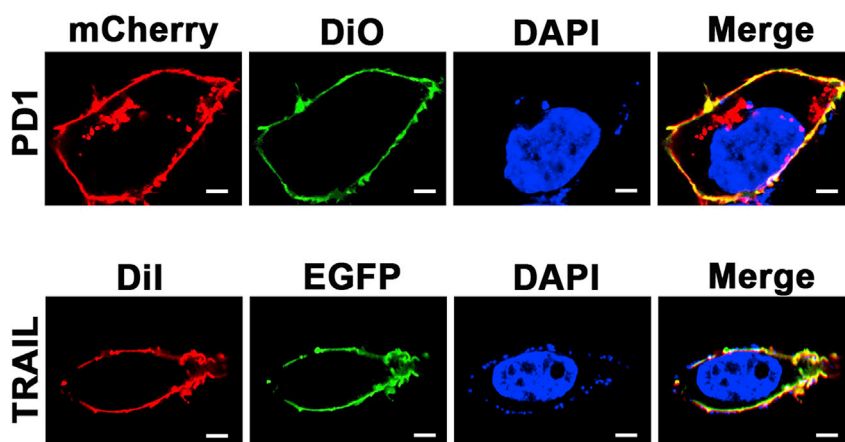


Figure 4. Confocal images of HEK293 FT cells stably expressing mouse PD1

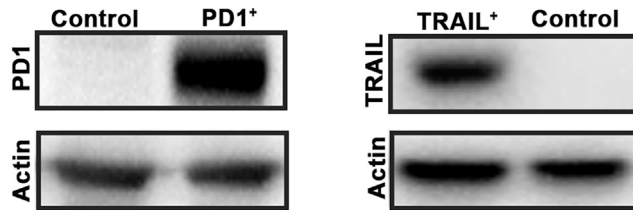


Figure 5. Western blot assay to confirm the expression of PD1 (indicated as PD1⁺) and TRAIL (indicated as TRAIL⁺) in 293FT cells

Actin was used as an internal reference.

Note: The concentration of puromycin or blasticidin should be adjusted above the lethal doses that kill nearly all the parent cells. If the high dose antibiotics also fail to screen, see [Troubleshooting: Problem 3](#).

12. Cells in the infected group are screened for 1–2 weeks until the percent of fluorescence cells reached above 95%, proved that the stably expression cells are successfully screened.

△ **CRITICAL:** Different cells have different tolerance to antibiotics, so the concentration of drugs needs to be optimized.

Note: Formation of puromycin or blasticidin resistance cells suggests the successful infection of lentivirus.

13. The stably expression cells are large-scale expanded.

Note: When cells have reached 80%–90% confluence, split cells 1:3 onto another 6-well plate with fresh culture medium.

Characterization of stable cell lines

⌚ **Timing:** 5 days

The constructed stable cell lines are characterized by confocal laser scanning microscopy observation ([Dong et al., 2019](#)), western blot assay ([Ohno et al., 2013](#)) and flow cytometry assay ([Koh et al., 2017](#)) to confirm the target protein is well expressed and localized onto the cell membrane.

14. Confocal microscopy observation
 - a. Cells are seeded in a confocal dish at a density of 1×10^5 per well. Place the dish in an incubator with 5% CO₂ at 37°C until they completely adhered.
 - b. Wash cells three times with PBS, and fix them with 4% paraformaldehyde for 10–15 min
 - c. Cell nucleus is stained with DAPI for 10 min, washed three times with PBS.
 - d. Cell membrane is stained with DiO or DiI for 15 min, washed three times with PBS, and observed under a confocal laser scanning microscope (Zeiss LSM780). See [Figure 4](#).
15. Western blot assay
 - a. Collect cells into a 1.5 mL EP tube by using cell scraper (Thermo Fisher Scientific), when the cells reach 70%–80% confluence in T75 cell culture flask.
 - b. The cells are lysed on ice with RIPA lysis buffer for 30 min. After lysis, the cells are centrifuged at 4°C for 30 min ($13,000 \times g$), and the supernatant is collected for BCA protein quantification, using EasyII Protein Quantitative Kit (TransGen, DQ111-01, <https://www.transgen.com.cn/>).
 - c. According to the BCA protein quantification result, the sample is diluted to 4 mg/mL and mixed evenly with 2× loading buffer (volume ratio 1:1), and the mixed sample is boiled at

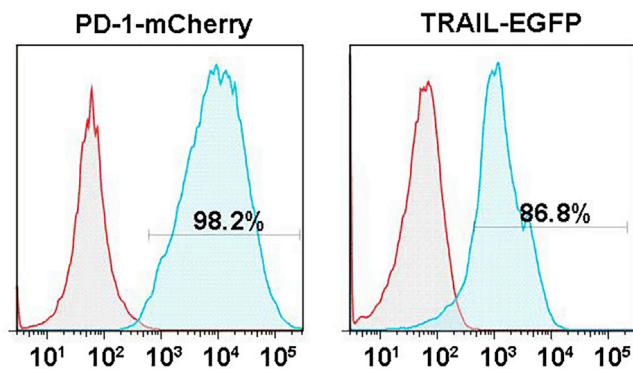


Figure 6. The representative flow cytometric analysis of HEK293 FT cells before and after transfected with PD 1 and TRAIL, respectively

100°C for 10 min. For details on preparation of 2× loading buffer, see the [Materials and equipment](#).

- d. The prepared sample is subjected to polyacrylamide gel electrophoresis for protein separation. Select β -actin protein as the internal reference protein for comparison. And then, transfer to the NC membrane by membrane transfer. Afterwards, the NC membrane is placed in 5% milk and blocked at 20°C–25°C for 2 h, and washed with TBST buffer (100 mM Tris-HCl (pH=8.0), 1.5 mM NaCl, 0.1% Tween 20) for 3 times, 10 min per time. After that, add the primary antibody (dilution rate, 1:1,000), See [Key resources table](#), and incubate for 12–16 h at 4°C.
- e. The next day, recycle the primary antibody and wash the membrane with TBST buffer for 3 times with 15 min per time. Afterwards, add the secondary antibody and incubate for 1 h at 20°C–25°C. Then recycle the secondary antibody (dilution rate, 1:5,000), See [Key resources table](#), and wash the membrane with TBST buffer for 3 times with 10 min per time. Preparation the ECL and imaging on the gel imager (ChemiDoc MP) ([Figure 5](#)).

16. Flow cytometer assay

Above prepared $10^5 \sim 5 \times 10^5$ cells are collected, centrifuged at 20°C–25°C for 5 min ($300 \times g$) and resuspended in PBS. The fluorescent protein signal was analyzed by flow cytometry (BD, FACSVerse) ([Figure 6](#)).

Cell membrane extraction

⌚ Timing: 2.5 h

17. When cells have reached 80%–90% confluence, remove medium and add 1 mL trypsin to digest cells. Harvest cells from plate and centrifuge cells at $600 \times g$ for 5 min ([Kim et al., 2017](#)).
18. Discard the supernatant, collect the cell precipitation and extract the cell membrane with a cell membrane extraction kit according to the manufacture’s instruction (Beyotime, P0033, <https://www.beyotime.com/index.htm>).
19. Determine the protein content in the extracted membrane by BCA kit, and then store the membrane in -80°C refrigerator for future use.

Note: The storage time of the extracted membrane should be no more than 1 month in -80°C refrigerator.

⚠ CRITICAL: In order to maintain the biological function, the extracted membrane cannot be lyophilized for storage.



Figure 7. The Avanti mini-syringe extruder

Synthesize the functional vesicles

⌚ Timing: 2 h

An Avanti mini-syringe extruder (Figures 7 and 8) is used to prepare cell membrane vesicles using the mechanical force imposed by the extruding process to disrupt the membrane structure and enable it to reform into nano-scale vesicles (Fang et al., 2014).

20. Assemble the mini-syringe extruder
 - a. Place the 2 Internal Membrane Supports on a flat test bed with the O-rings vertically upward
 - b. Pre-wet 2 Filter Supports with DI water, or buffer, and place over orifice of Internal Membrane Supports. The Filter Supports should adhere to the Teflon orifice inside the O-ring inner scope.
 - c. Insert the Internal Membrane Support into the Extruder Outer.
 - d. Place 1 Polycarbonate Membrane in the Extruder Outer Casing over the Filter Support and O-ring.

Note: Do not install the blue paper disks which separate the polycarbonate membranes in packing box.

- e. Pre-wet a second pair of Filter Supports with DI water, or buffer, and place over orifice of remaining Internal Membrane Support.
- f. Carefully place the second Internal Membrane Support into the casing (O-ring facing down).
- g. Place the Retainer Nut on the threaded end of the Extruder Outer Casing and tighten. Tighten the Retainer Nut by hand just until it is finger tight; do not use a wrench.

Note: Autoclaving the Teflon inserts is not recommended as slight distortion may occur (~0.002 mm). The use of ethylene oxide or UV irradiation is suggested as an alternative. The syringes should be sterilized with ethanol.

△ CRITICAL: See Figure 9 and Methods Video S1 for detailed operate instructions of step 20.

21. Add the cell membrane solution into one of the gas-tight syringes and carefully place into one end of the mini-extruder (Figure 10).

Note: To reduce the dead volume and facilitate extrusion, pre-wet the extruder parts by passing a syringe full of buffer through the extruder.

22. Place the empty gas-tight syringe into the other end of the mini-extruder. Make sure the empty syringe plunger is set to zero; the syringe will fill automatically as the cell membrane is extruded through the polycarbonate membranes.
23. Gently push the plunger of the filled syringe until the cell membrane solution is completely transferred to the alternate syringe (Figure 11).
24. Gently push the plunger of the alternate syringe to transfer the solution back to the original syringe.

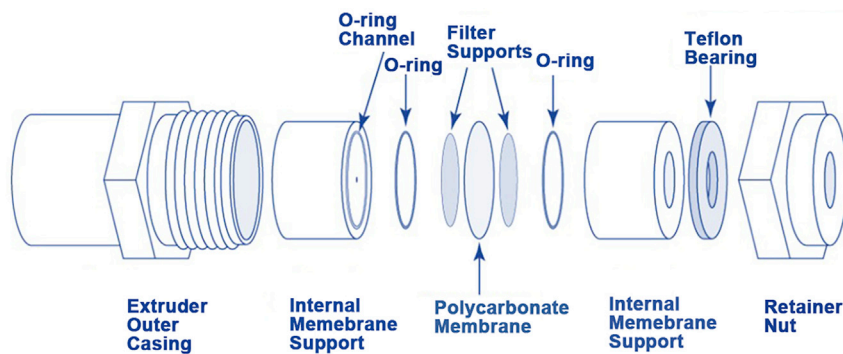


Figure 8. Schematic illustration of the parts of the Avanti mini-syringe extruder

Repeat above steps 23 and 24. Total of 11 passes through the 1,000 nm, 400 nm, 200 nm, and 100 nm polycarbonate membranes, respectively. If the FCNVs fail to maintain their biological functions, see [Troubleshooting: Problem 4](#).

Note: The final extrusion should fill the alternate syringe. This is to reduce the chances of contamination with larger particles or foreign material.

△ CRITICAL: After being pushed through the syringes for enough times, the diameter of final product will be around 100 nm with a polydispersity index (PDI) less than 0.2. When the diameter and/or PDI are larger than these values, increasing the pass times appropriately.

25. After the final extrusion, inject the obtained solution into a clean sample vial.

△ CRITICAL: When removing syringes, pull the syringe straight out of the extruder. See [Methods Video S2](#) for detailed operate instructions of steps 21–25.

The characterization of FCNVs

26. The size distribution of FCNVs is measured by dynamic light scattering (DLS) method through Zetasizer NanoZS system (Malvern Instruments, Southborough, MA, USA). Therein, the size measurement is performed in Size module with 200 s equilibration time using DTS0012 cuvette. The ultrastructure, integrity and morphology of FCNVs are evaluated by transmission electron microscopy (TEM). Therein, the sample is dropped onto a Cu-grid that covered with carbon film, and dried for 2 h. Subsequently, the sample is stained by 50 μ L of tungstophosphoric acid for 5 min, and the redundant solvent was removed blotters. After drying again, the morphology was observed and photographed using JEM-1400Plus transmission electron microscopy (JEOL, Japan). The specific ratio and content of TRAIL and PD1 in FCNVs are determined by ELISA (R&D Systems, DY1121 for TRAIL and DY1021 for PD1, <https://www.rndsystems.com/cn>).

Assessment of immunotherapy of FCNVs

The TRAIL component on FCNVs could initiate the immune responses by specifically inducing immunogenic cancer cell death, which could be further strengthened by collaborating with presented ectogenic PD1 proteins as the checkpoint blockade to reactivate the anergic tumor-specific CTLs. The corresponding function of FCNVs can be evaluated both *in vitro* and *in vivo*.

27. *In vitro* immune checkpoint inhibition of FCNVs: naive mouse CD8⁺ T cells (CTLs) are first isolated from the spleen of BALB/c mice, followed by CD3/CD28 antibody stimulation to obtain activated CD8⁺ T cells. Subsequently, cancer cells with various treatments, such as blocking PDL1 on cell membranes by our FCNVs or PDL1 antibody, are added to the activated CD8⁺ T cells to check the cytotoxic activity by using Annexin V-FITC and PI cell apoptosis assay.

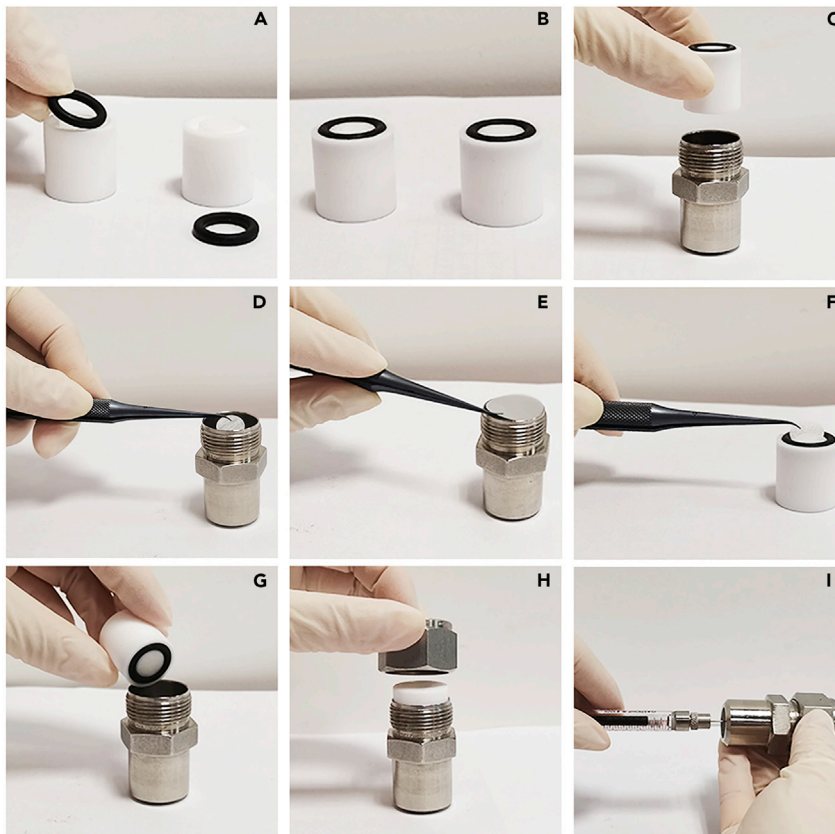


Figure 9. Installation process for the Avanti mini-syringe extruder

- (A and B) Place two Internal Membrane Support on a flat test bed with the O-rings vertically upward.
 (C) Insert one Internal Membrane Support into the Extruder Outer Casing.
 (D) Place one pre-wetted Filter Support over orifice of the Internal Membrane Support in the Extruder Outer Casing.
 (E) Place one Polycarbonate Membrane over the Filter Support Support and O-ring in the Extruder Outer Casing.
 (F) Place the second pair of pre-wetted Filter Support over orifice of the other Internal Membrane Support.
 (G) Carefully place the second Internal Membrane Support into the Extruder Outer Casing (O-ring facing down).
 (H) Place the Retainer Nut on the threaded end of the Extruder Outer Casing and tighten.
 (I) Place the empty gas-tight syringe (with plunger is set to zero) into the one end of the mini-extruder.

28. *In vitro* immunogenic cancer cell death (ICD) induced by FCNVs: Anticancer effect of TRAIL proteins on FCNVs can be assessed by Annexin V-FITC and PI cell apoptosis assay, and the danger-associated molecular patterns (DAMPs) like CRT proteins released from dead cancer cells are checked by immunofluorescent staining. Finally, the release of DAMPs together with tumor-associated antigens (TAAs) from dead cells are co-incubated with borrow-derived dendritic cells (BMDCs) to check whether they can promote the maturation of BMDCs though dual staining for CD80 and CD86, the typical markers on the surface of matured DCs.
29. *In vivo* antitumor effect of FCNVs: The comprehensive immuno-modulating ability and robust anti-tumor immunity of our FCNVs can be demonstrated through evaluating tumor growth regression, metastasis prevention, distal and immune-memory effects, and long-term survival benefits.

All of the above corresponding results can be found in our recent iScience publication (Wu et al., 2020).

EXPECTED OUTCOMES

Following this protocol, we prepared functional cellular nanovesicles (FCNVs) with surface presenting PD1 and TRAIL. We advise to perform the particle and protein characterization to qualitatively



Figure 10. Schematic illustration of drawing the sample by syringe

and quantitatively assess the FCNVs, respectively. ELISA determination of PD1 and TRAIL content on FCNVs is necessary. Immunoprecipitation (IP) assay verifies the orientation of functional proteins on FCNVs. The size distribution is measured by dynamic light scattering (DLS) methods. FCNVs ultrastructure, integrity and morphology are evaluated by transmission electron microscopy (TEM). For detailed instructions on how to perform FCNVs characterization and the corresponding results can be found in our recent iScience publication (Wu et al., 2020).

We demonstrated the ability of this protocol to prepare FCNVs with high purity, which is a prerequisite to obtain reliable data for antitumor immune responses. For full details, we refer to (Wu et al., 2020). Theoretically, in addition to PD1 and TRAIL, other functional proteins decorated cellular nanovesicles can be also obtained by our protocols. Furthermore, the inner core of FCNVs can be used to encapsulate other functional components, for example, catalase is loaded into nanovesicles to overcome tumor hypoxia after producing O_2 , in turn to increase infiltration of effector T cells while deplete immunosuppressive cells in tumor (Wu et al., 2020).



Figure 11. Schematic illustration of extrusion operation

LIMITATIONS

While this protocol was developed to obtain the functional cellular nanovesicles with multiple immuno-modulating ability to elicit robust and systematic antitumor immunity, the procedure is labor intensive and low-throughput, thus keep the same quality FCNVs from batch to batch and large-scale production might be an obstacle for pharmaceutical translation. Moreover, some functional proteins on FCNVs have right orientation (outwards), but some presented inwards which certainly can not play the right function in immunotherapy after co-extrusion, but in our current protocol it is very difficult to precisely control the orientation of functional proteins on the surface of FCNVs.

TROUBLESHOOTING

Problem 1

Why the efficiency of gene transfection is not high?

Potential solution

There are many factors affecting the transfection efficiency, such as the state of the cells, the quality of the transfection reagent and the purity of the plasmid. Therefore, the cells should be handled in a good condition, the transfection reagent free of quality problems, and the plasmid has high purity and the sequence has been verified before transfection.

Problem 2

Why the efficiency of infected target cells is not high?

Potential solution

We found that the transfection efficiency and the viral titer may directly influent the infection efficiency. Make sure the transfect efficiency more than 80% after 48 h of transfection. And the viral titer should reach above 10^6 IFU/mL to ensure better infection efficiency. Meanwhile, the storage time of collected virus supernatant at 4°C should not exceed 1 week. Also, if the efficiency of infection is still very low and you may choose secondary infection.

Problem 3

Why the antibiotics fail to screen even at high dose?

Potential solution

Make sure the resistant drugs are within its warranty and properly stored. Then, determine whether the target cells already have the corresponding resistance gene. Alternatively, flow cytometry can also be used to select stably expression cells instead of drug screening.

Problem 4

How to obtain homogeneous FCNVs with uniform morphology while maintain their biological functions?

Potential solution

We advise to mechanically harvest stable cells from cell culture flask by cell scraper rather than trypsin digestion to avoid potential harms to the functional proteins on the cell membranes including

PD1 and TRAIL. Another suggestion to maintain the biological function is that the extracted membrane should not be lyophilized for storage. To improve the homogeneity of FCNVs, the repeats of extruding process in steps 23 and 24 can be appropriately increased.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Xiaolong Liu (xiaoloong.liu@gmail.com).

Materials availability

This study did not generate new unique materials.

Data and code availability

This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2021.100324>.

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AUTHOR CONTRIBUTIONS

P.Y. and D.Z. wrote and prepared the manuscript. C.Z., M.W., and X.L. edited and helped in section organization.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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