Isolation, Culture and KSHV infection of Oral Mesenchymal Stem Cell

YuanLab Protocol

1. Criteria for healthy human isolated teeth: 5-25 years old (Orthodontic reduction of teeth), no caries damage, Dental integrity, No history of systemic disease, No recent history of medication;

2. Preparation before teeth extraction

(1) Hospital: Basic media with 2% penicillin-streptomycin, ice pack, 15ml centrifuge tubes (Each tube corresponds to one tooth, 3-4ml culture medium, indicating the patient's age, gender, and cause of tooth loss information)

(2) Lab: Sterilized tooth extraction tool box (needle holder, tweezers, surgical knife handle), No.11 blade, 10X Collagenase I, 10X dispase I, complete culture medium (1% penicillin-streptomycin and 10% FBS), 6 cm dishes and 10cm dishs, T25 flsak;

(3) Return the tooth to the laboratory within 2 hours after receipt.

3. Oral MSCs extraction (In units of 1 tooth)

(1) Take two 6cm dishes, add 2ml basic culture medium to the cover and bottom respectively, pick up the teeth at the neck with a needle holder/bending forceps, and flush the teeth from crown to root, rinse the tooth surface 3-4 times to remove blood and impurities (change a new dish and replace the gun tips each time), and rinse until there is no blood;

(2) With a 10cm dish, No.11 blade quickly scraped 1/3 of the root, keeping the root moist during operation;

(3) Rinse the 10cm dish with basic culture medium, transfer to 15ml centrifuge tube, centrifuge 1200rpm, 3min, RT;

(4) Discard the supernatant. 2ml basic culture medium suspension, plus 200ul 10×

collagenase I and 200ul 10×dispaseI;

(5) Water bath at 37°C for 1h, oscillating every 5 minutes;

(6) 4ml complete culture medium was added to terminate digestion and centrifuged at 1200rpm for 3min and RT;

(7) Discard the supernatant. 1ml complete culture medium was re-suspended into 1.5mL EP tube, and then waited for natural precipitation after elasticity. Supernatant was absorbed into T25 flask;

(8) Add 800ul to EP tube again, mix evenly, precipitate naturally, and then transfer supernatant to T25 flask. Repeat this for 3-4 times;

(9) Finally, complete culture medium was added up to 5ml in T25 flask, and let the culture flask in the incubator for more than 24 hours;

(10) The culture medium was replaced after the cells crawled out on day 3-4, and the cell could be passed/frozen when the density reached 70-80%.

4. KSHV infection of Oral MSCs

(1) Oral MSCs were seeded in 6-well plates;

(2) Cells were infected with KSHV in the presence of polybrene (4 μg/ml) at an MOI = 50 (viral genome copy equivalent) when the cell density reached 60-70%. After centrifugation at 2500 rpm for 60 min at room temperature, the cells were incubated at 37°C with 5% CO2 for 2 hours. Then, the inoculum was removed by changing culture medium.

Tube Assay Experimental Protocol

Preparation before experiment:

(1) Take out the aliquoted Matrigel (Corning, store at -80℃ after aliquoting) in advance and place it on ice (Note: only on ice. Matrigel is solidified at 4℃ or room temperature and cannot be Thaw). This process may take several hours. If you experiment the next morning, you can take the Matrigel out of -80°C the night in advance and thaw it on ice.

(2) Serum-free medium

experiment process:

(1) Dilute the completely melted Matrigel 1:1 with serum-free culture medium (operate gently and try not to produce bubbles), and then spread it into a 96-well plate at 50uL/well. It is best to set up three replicates. . Work as quickly as possible, or place 1:1 diluted Matrigel on an ice brick. A 1:1 dilution of Matrigel readily becomes semi-solid at room temperature. Place the 96-well plate covered with Matrigel in a 37°C cell culture incubator for about 1 hour to allow it to solidify. (Note: Bubbles should not appear when plating Matrigel, otherwise it will directly lead to uneven cell plating and subsequent photography.)

(2) After the Matrigel in the well plate solidifies, resuspend the target cells in serum-free medium, 100uL per well, and inoculate 50,000 cells/well (the cell volume can be adjusted according to the specific cell type). The cell suspension is added vertically without shaking the plate (to prevent cells from gathering in the center of the well plate). Place in a 37°C cell culture incubator for 4-8 hours.

(3) Take pictures and images with an inverted microscope. Since tube formation time will vary depending on the type of cells, tube lumen formation can be observed under a microscope 4 hours after cell plating. The specific time needs to be determined according to the experimental conditions.

(4) Calculate the number of tubes. The captured pictures were analyzed and calculated using Image J software. The results can be processed in the following ways:

nodes; junctions; meshes; branches; number of branches; length; branching length; segments length. Tot. length should be the total pipe length. Total Length=Total branching length + Isolated branches length; Total branching length=total segment length + total branches length In the end, I chose total segment length, but I think there is no absolute standard for this. Maybe it depends on which data can better explain what I want. solved problem. (Sourced from the Internet, for reference only)

Immunohistochemistry

Materials and reagents:

Xylene, absolute ethanol, 90% alcohol, 80% alcohol, PBS, PBST (0.1% Tween 20), citrate repair solution, 3% H2O2 (prepared with ddH2O), 0.1% TritonX-100 (prepared with PBS) antibody Diluent (or 2.5% BSA), neutral resin, and special pen for immunohistochemistry.

Procedure:

Bake in 1.60% oven for 2 hours.

2. Dewaxing and hydration: Dewax the sections in xylene while hot, xylene I for 10 min, xylene II for 10 min, xylene III for 10 min, absolute ethanol for 10 min, 90% ethanol for 5 min, and 80% ethanol for 3 min.

3. Wash three times with PBS, 3 minutes each time

4. Antigen retrieval: high-pressure repair, place the slices on a rack, immerse them in citric acid repair solution (1.2-1.4L), make them soft, and use soup mode for 26-30 minutes.

5. Cool the pressure cooker with running water (or naturally cool it). After the temperature of the repair solution is lower than 50°C, soak the slices in PBS and wash them three times, each time for 3 minutes.

6. Use an immunohistochemistry pen to draw a circle, confine the tissue within the circle, drop 50ul of 3% H2O2 and incubate for 10 minutes to remove endogenous catalase. Wash three times with PBS, 3 minutes each time.

7. Prepare the primary antibody, pass the sections through PBST, add 50ul of antibody dropwise, cover the tissue, and incubate at 4°C overnight (or incubate at room temperature for 4 hours).

8. Wash three times with PBS, 3 minutes each time. Prepare secondary antibody (HRP-labeled), pass the sections through PBST, add 50ul of secondary antibody dropwise, and incubate at room temperature for 30 minutes.

9. Wash three times with PBS, 3 minutes each time. Prepare display solution (DAB or AEC). Observe the color change and do not dye it darkly (it can be counter-dyed if it is not enough).

10. After counterstaining the nuclei with hematoxylin for 15 seconds, rinse with tap water.

11. Rehydration and transparency: Place the sections in 80% ethanol for 3 minutes, 90% ethanol for 5 minutes, absolute ethanol for 10 minutes, xylene I for 10 minutes, xylene II for 10 minutes, and xylene III for 10 minutes. Let it dry in a ventilated place for 30 minutes, then seal with neutral resin.

HE staining:

Bake in 1.60% oven for 2 hours.

2. Dewaxing and hydration: Dewax the sections in xylene while hot, xylene I for 10 min, xylene II for 10 min, xylene III for 10 min, absolute ethanol for 10 min, 90% ethanol for 5 min, and 80% ethanol for 3 min.

3. Wash three times with PBS, 3 minutes each time

4. After staining the nuclei with hematoxylin for 1 minute, rinse with tap water.

5. After dyeing with eosin for 1 minute, rinse with tap water.

6. Rehydration and transparency: Place the slices in 80% ethanol for 3 minutes, 90% ethanol for 5 minutes, absolute ethanol for 10 minutes, xylene I for 10 minutes, xylene II for 10 minutes, and xylene III for 10 minutes. Let it dry in a ventilated place for 30 minutes, then seal with neutral resin.

Antibody dilution ratio:

LANA: 1:100 NES: 1:50 CD29: 1:100 CD133: 1:50

Notes:

① For some tissue sections that are easy to detach, you can dry them in a fume hood for 20 minutes after passing through absolute ethanol during hydration, which can effectively prevent detachment.

② When staining nuclear antigens, such as LANA, you can first treat it with 0.1% TritonX-100 for 20-30 minutes to promote the antibody to enter the nucleus and bind to the antigen (cell punching).

③After AEC color development, the slides are generally mounted directly with water-based mounting medium without going through dehydration and transparency.

④After eosin stains the cytoplasm, do not soak it in water, otherwise the staining will easily fade. It should be left dry.

⑤After staining the cell surface maker or cytoplasmic proteins, it is generally necessary to counterstain the nucleus with hematoxylin.

Matrigel

Melt Matrigel on ice, dilute it 1:1 with serum-free medium, coat a 96-well plate with 50 μL per well, and leave it at room temperature for 30 minutes until use; digest and collect cells, and adjust the cell density to a volume of 100 μL containing 2x104 cells. The suspension was seeded in a pre-coated 96-well plate, and tube formation was observed under a microscope for 6-8 hours, and photos were taken for recording.

3D spheroid culture

1.1 3D spheroid culture

(1) Prepare a sterile 0.5% agarose solution, add 50 μL agarose solution to the 96-well plate while it is hot, and leave it at room temperature for 30 minutes before use;

(2) After digesting the cells, count the cells, adjust the cell density to 1.5-2.0x105 cells/mL, add 100 μL cell suspension to each 96-well plate pre-coated with agarose, and mesenchymal stem cells in In this non-adhesive environment, cells spontaneously assemble into spheroids and are cultured for 4 days. During this period, the medium is changed once or 100 μL of culture medium is added;

(3) After four days, collect the spheroid into centrifuge tubes for subsequent 3D migration and invasion experiments or tissue embedding.

1.2 3D spheroid blood vessel sprouting experiment

(1) Prepare a sterile 0.5% agarose solution, add 50 μL agarose solution to the 96-well plate while it is hot, and leave it at room temperature for 30 minutes before use;

(2) After digesting the cells, count the cells, adjust the cell density to 1.5-2.0x104 cells/mL, add 100 μL cell suspension to each 96-well plate pre-coated with agarose, and mesenchymal stem cells in In this non-adhesive environment, cells spontaneously assemble into spheroids and are cultured for 2 days;

(3) Collect spheroids, mix them with 1:2 diluted Matrigel, and seed them in a 48-well plate. After the Matrigel solidifies, add 200 μL of culture medium and culture for 3 days. Use a microscope to record the budding of spheroids.

1.3 3D spheroid migration experiment

The collected 3D cell spheroids were seeded into adhesive and non-adhesive (0.5% agarose-coated) 24-well culture plates, cultured in serum-free medium, and after 48

hours, the cells were observed and recorded from the 3D cell spheroids. The amount of cells that migrated out.

1.3 3D spheroid invasion experiment

Pre-coat the 12μm Transwell chamber with Matrigel diluted 1:10 and leave it at room temperature for 30 minutes until use. The collected 3D cell spheres were inoculated and resuspended in serum-free medium, and 10 cell spheres were inoculated into each small spoon. Medium containing 20% FBS was added to the lower layer, and after 48 hours, the cells in the upper layer were wiped off with cotton balls. If you want to count the number of cells invaded in the lower layer of each cell ball, fix the spoon with 4% paraformaldehyde at room temperature for 15 minutes, wash it three times with PBS, and then stain it with 0.1% crystal violet stain for 5 minutes at room temperature, and then rinse with running water. Observe under the microscope and take photos to record. If you need to run flow cytometry to identify the type of cells that have invaded, soak a small spoon in 0.25% trypsin, digest for 2-3 minutes, resuspend in complete culture medium, centrifuge for staining, and perform the experimental steps of flow cytometry analysis.

1.4 Immunofluorescence and HE staining of 3D spheroid tissue sections

(1) After fixing the collected spheroids with 4% paraformaldehyde, they are pre-embedded in agarose for routine tissue paraffin embedding processing, and then paraffin sections are obtained;

(2) Bake the paraffin sections of KS tissue in a 65°C oven for 2-4 hours;

(3) Put the baked slices into xylene I (10 min), xylene II (10 min), xylene III (10 min), absolute ethanol (10 min), and 90% ethanol (5 min) in sequence while they are still hot. min), 80% ethanol (3 min) for dewaxing and hydration;

(4) After washing the sections with 1xPBS, place them in a pressure cooker containing citrate antigen retrieval solution for antigen retrieval;

(5) After washing three times with PBS, treat with 0.1% Tritox-100 for 20 minutes. Wash with PBS; block with 5% BSA at room temperature for 30 minutes;

(6) Drop the diluted primary antibody on the tissue and incubate at 4°C overnight or at room temperature for 2-4 hours;
(7) After washing the sections three times with 1xPBS, add the corresponding diluted fluorescent secondary antibody dropwise and incubate at room temperature for 2 hours;
(8) Finally, counterstain the nuclei with Hoechst 33342 (1:10000) for 3 minutes, wash three times with 1xPBS, dry the water, seal the slides with fluorescent mounting medium, and store in a refrigerator at 4 degrees under closed light;
(9) Use a fluorescence microscope to obtain the staining results.

1.5 HE staining of 3D spheroid tissue sections
(1) Bake tissue paraffin sections in a 65°C oven for 2-4 hours;
(2) Put the baked slices into xylene I (10 min), xylene II (10 min), xylene III (10 min), absolute ethanol (10 min), and 90% ethanol (5 min) in sequence while they are still hot. min), 80% ethanol (3 min) for dewaxing and hydration;
(3) After washing the sections with 1xPBS, stain them with hematoxylin for 1 min, rinse with running water, stain with water-soluble eosin for 2 min, and rinse with running water;
(4) Dehydration and transparency: 80% ethanol (3 min), 90% ethanol (5 min), absolute ethanol (10 min), xylene I (10 min), xylene II (10 min); air dry Afterwards, the slides were sealed with neutral resin, and the staining effect was observed under a microscope.

1.6 3D spheroid kidney capsule transplantation experiment
Collect 100 3D cell spheroids (spheroids) and wash them three times with PBS. Then inoculate it onto gelatin sponge and culture it for 24 hours until the cells adhere closely to the sponge. First, nude mice were anesthetized with chloral hydrate, a small incision was made on the back of the chest, and the mouse's kidney was squeezed out. Then a small incision was made with an intravenous infusion needle, and the gelatin sponge with attached cells was transplanted under the renal capsule of the mouse. The intravenous infusion needle is roasted with an alcohol

lamp, and the renal membrane is burned with the needle to seal it. Then use surgical sutures to sew the fascial layer and skin wound. After 28 days, the mouse kidney capsules were removed and sent for paraffin-embedded sections.

3D Spheroid formation

Day1: Coat round bottom 96-well plates (Nest, #701101) with agarose

- **Prepare sterile 0.5% (w/v) agarose solution**

0.5% Low melting gel (Solarbio #A8350) in ddH2O, treated with high pressure sterilization, and maintained in 60℃drying oven .

- **Coated with agarose in 96 well plates**

Using the multi-channel pipette, add 50μL agarose solution to 96-well plate while hot (*Once the material solidifies, the agarose gel can dissolve by heating in microwave oven.*), and set aside at room temperature for 30 min until the gel is set (*use them within 2 days after preparing*).

Day2: Spheroids Generation

- **Preparing the cells**

Oral MSCs (PDLSCs) should be used under P5 (*The higher the cell passage, the more difficult for cells to form into spheroids*). PDLSCs or KSHV infected PDLSCs are digested as single-cell suspensions, count cell using a hemocytometer, then dilute the cell suspension to obtain about 2 x 10^4 cells/ml.

- **Spheroids formation**

Using the multi-channel pipette, add 100μL cell suspension per well to pre-coated 96 well plates. Oral MSCs (PDLSCs) spontaneously assemble into spheroids of cells in this non-adherent environment, spheroids will begin to appear within 18-36 h. Just few hours after seeding, most of the wells should contain small spheroids that will progressively converge into one large spheroid (*If you want to accelerate the spheroids time, the 96 well plate could centrifuge at 500 × g for 5–10 min*). Spheroids were grown at 37 °C up to 4 d in a humidified atmosphere with 5% CO_2.

Day6: Spheroids collection

To collect spheroids, the media containing the spheroids were transferred to a 15 ml conical tube, then washed twice with PBS, and centrifuged at 1000 rpm for 5 min. For histology analysis, the spheroids were collected and fixed with 4% PFA following by agarose pre-embedding. The paraffin section of spheroids could be used for IHC or IFA in conventional methods.

(1) Prepare xylene, absolute ethanol, 90% ethanol, 80% ethanol, citrate antigen retrieval solution, and 1xPBS for later use;

(2) Bake the paraffin sections of KS tissue in a 65°C oven for 2-4 hours;

(3) Put the baked slices into xylene I (10 min), xylene II (10 min), xylene III (10 min), absolute ethanol (10 min), and 90% ethanol (5 min) in sequence while they are still hot. min), 80% ethanol (3 min) for dewaxing and hydration;

(4) After washing the sections with 1xPBS, place them in a pressure cooker containing citrate antigen retrieval solution for antigen retrieval;

(5) After washing three times with PBS, treat with 0.1% Tritox-100 for 20 minutes. Wash with PBS; block with 5% BSA at room temperature for 30 minutes;

(6) Drop the diluted Rat anti-LANA on the tissue, and incubate at 4°C overnight or at room temperature for 2-4 hours (LANA--1:100 dilution); wash the sections three times with 1xPBS, and then use Goat anti-Rat IgG (H+L), 555 secondary antibody was incubated at room temperature for 2 h;

(7) After washing the sections three times with PBS, drop diluted Mouse anti-PDGFRA or Nestin on the tissue, and incubate at 4°C overnight or at room temperature for 2-4 hours (PDGFRA--1:100 dilution, Nestin--1: 100 dilution); after washing the sections three times with PBS, incubate them with Goat anti-Mouse IgG (H+L), 488 secondary antibody at room temperature for 2 hours;

(8) After washing the sections three times with PBS, drop diluted Rabbit anti-CD31 or PDPN on the tissue, and incubate at 4°C overnight or at room temperature for 2-4 hours (CD31--1:200 dilution, PDPN--1:200 dilute); wash the sections three times with PBS, incubate them with Donkey anti-Rabbit IgG (H+L), 647 secondary antibody for 2 hours at room temperature; finally counterstain the nuclei with Hoechst33342 (1:10000) for 3 minutes, wash them three times with 1xPBS, and dry After drying the water, seal the slides with fluorescent mounting medium and store in a refrigerator at 4 degrees with the light closed;

(9) Use a fully automatic slide scanner (AxioScan.Z1) or a fluorescence microscope to obtain the staining results.

The cells were seeded on cell slides, and after their growth was stable, they were fixed with 4% paraformaldehyde at room temperature for 15 min, and washed three times with PBS. Treat with 0.1% Triox-100 at room temperature for 15 min, and wash three times with PBS. Filter 1% BSA with a 0.45 μm filter, then block the slide with filtered 1% BSA at room temperature for 15 minutes, apply the diluted primary antibody, and incubate at 4°C overnight or at room temperature for 2-4 hours. Wash three times with PBS, apply the corresponding fluorescent secondary antibody, and incubate at room temperature for about 2 hours. Wash three times with PBS, and finally counterstain the nuclei with Hoechst 33342 (1:10000) for 1 min. Wash three times with PBS. After drying the water, mount the slides with fluorescent mounting medium and store them in a refrigerator at 4 degrees in the dark. Obtain pictures using a fluorescence microscope.

Soft Agar Assay

For analysis of colony formation, cell suspensions containing 0.3% agar were seeded in each well of 24-well plates containing an underlay of 0.6% agar in complete medium. Cultures were supplemented with complete medium per week and colonies were scored 21 days after seeding the cells.

Colony formation in soft agar

First, a base layer containing 0.5% agarose medium and 5% FCS was poured into six-well plates. Then, 10,000 cells were mixed with 0.4% agarose in Earl's minimal essential medium (EMEM) containing 5% FCS to form a single-cell suspension. After being seeded, the plates were incubated for 2 weeks. To establish clones, single colonies were picked and transferred into 96-well plates for expansion.

Analysis of in vitro clonogenic capacity by soft agar assay. The sorted SP and NSP cells were seeded at a density of 100 cells/well in 24-well plates with soft agar. The upper agar layer consisted of 0.35% agarose in DMEM supplemented with 10% FBS, and the base layer was made up of 0.6% agarose. A total of 500 μl fresh medium was added to the cells twice a week. After 3 weeks of culture at 37°C, the colonies were stained with 0.5 ml 0.005% crystal violet for 1 h. Colonies were examined under phase contrast microscopy (IX50; Olympus Corporation, Tokyo, Japan). Colonies with a diameter >75 μm or containing >50 cells were counted. The colony formation rate was calculated as follows: Colony formation rate (%)=number of colonies/number of seeded cells x100. Experiments were conducted in triplicate.

Anchorage-independent cell transformation assay

HaCaT cells were stably transfected with NFAT3 or mutant NFAT3-S259A for an EGF-induced cell transformation assay. Cells (8 × 103) were cultured in DMEM with 0.3% agar (Sigma-Aldrich), 20 ng/ml EGF (BD Biosciences,San Jose, CA, USA)

and 10% FBS. The cells were maintained at 37 °C in a 5% CO2 incubator for 10 days and then colonies were counted and scored using Image-Pro Plus software as described by Colburn et al.

Soft agar assay

Soft agar assay was performed as previously described with minor modifications [5]. Briefly, a total of
2×104 cells suspended in 1 ml of 0.3% top agar (Cat.#A5431, Sigma, St. Louis, MO) were plated onto one well of 0.5% base agar in 6 well-plates and cultured for 2-3 weeks. Colonies with a diameter > 50 μm were counted and photographed with a microscope.

Soft agar assay

Soft agar assay was performed as previously described with minor modifications [5]. Briefly, a total of
2×104 cells suspended in 1 ml of 0.3% top agar (Cat.#A5431, Sigma, St. Louis, MO) were plated onto one well of 0.5% base agar in 6 well-plates and cultured for 2-3 weeks. Colonies with a diameter > 50 μm were counted and photographed with a microscope.

Soft-agar assay

Cells (16104) were suspended in a 0.35% agar solution in DMEM supplemented with 10% FBS, and overlaid onto a 0.5% agar solution in DMEM containing 10% FBS in 35 mm plates prepared 1 day before and incubated in a 37% and 5% CO2 incubator. One day after incubation, 2 ml of DMEM supplemented with 10% FBS was added. Cells grown in soft agar were counted 12 days after plating. Cloning effciency is the number of colonies x100 divided by the numbers of cells plated. Each determination is the average of three experiments with standard deviations.

KSHV-PDLSC vFLIP KO

(1) Design the sgRNA sequence to knock out vFLIP. Referring to the KSHV genome sequence (NCBI: NC_009333.1), intercept the 300 bp fragment of vFLIP including the start codon but excluding the KSHV miRNA gene sequence and the 300 bp fragment of vFLIP including the stop codon but excluding the ORF72 gene sequence. , place the base sequences of these two ends into the online CRISPR design tool (http://cri spr.mit.edu). Select two sgRNA sequences targeting the 5' and 3' ends:

K13-5- sg1 F CACCGACATATACAAGCCGGCACCA

K13-5- sg1 R AAACTGGTGCCGGCTTGTATATGTC

K13-5- sg2 F CACCGGAAACTGGGCACGGATTGACA

K13-5- sg2 R AAACTGTCATCCGTGCCCAGTTTCC

K13-3- sg3 F CACCGATGGTGTATGGCGATAGTGT

K13-3- sg3 R AAACACACTATCGCCATACACCATC

K13-3- sg4 F CACCGGGGAGTGTGATGGGCCGGAA

K13-3- sg4 R AAACTTCCGGCCCATCACACTCCCC

(2) Construct lentiviral vector containing sg sequence. Anneal the sg primer into double strands and connect it to the lentiCRISPR v2 vector containing the BsmBI restriction site. Transform it into a competent state and perform cloning amplification and send it for sequencing to verify that the plasmid is successfully constructed.

(3) Extraction of endotoxin-free plasmid (same as above);

(4) Packaging, infection and screening of lentivirus. ① Carry out transfection when the density of 293T cells in 100 dishes reaches 70-80%. Mix lentiCRISPR v2-sgRNA, psPAX2, and pMD2.G with 10 μg of total DNA in a ratio of 5:3:2 and add 600 μL Opti -MEM medium, mix well and let stand for 5 minutes, add 20 μg of PEI to the mixed solution, let stand for 15 minutes and then add to 100 dish. The culture supernatant was collected 48 hours and 96 hours after transfection. Centrifuge at 8000 rpm at 4 degrees for 10 minutes to remove cell debris, aliquot and store in a -80 degree refrigerator; ② Seed mesenchymal stem cells in a 6-well plate, conduct lentiviral

infection when the cell density reaches about 70%, and inject into the 6 wells Add 4 mL of different combinations of virus-containing supernatants (sg1+ sg3, sg2+ sg3, sg1+ sg4, sg2+ sg4) and polybrene with a final concentration of 8 μg/mL to the plate. Shake the culture plate and centrifuge at 2500 rpm for 60 min. Replace with fresh culture medium after hours. When the transfected cells are passaged, puromycin at a final concentration of 2 μg/mL is added for selection for one week to obtain oral mesenchymal stem cells that stably express sgRNA.

(5) Detection of knockout effect. ① PCR identification of knockout effect; KSHV was infected into hPDLSCs expressing and not expressing vFLIP sgRNA. 2 days later, the cells were collected to extract viral genomic DNA, specific primers were designed for PCR amplification, and the PCR amplification products were analyzed by nucleic acid electrophoresis. The specific primer sequences are as follows:

F11 : ACCCTGCGTAAAACAACCG

R11: ACCCAAAGACTGGCTCAT

For the DNA extraction method, refer to the Magen kit instructions. The brief process is: collect cells, discard the culture medium, add 200 μL PBS and 20 μL proteinase K, vortex for 15 s, and add 200 μL Buffer AL to the sample, vortex at high speed for 10 s and then incubate in a 65° C water bath for 15 min, then add 200 μL absolute ethanol and vortex at high speed for 10 s, then perform column purification, and finally use ddH2O to elute and dissolve and measure its concentration. ② qPCR to detect knockout efficiency; hPDLSCs expressing and not expressing vFLIP sgRNA were infected with KSHV, and cells were collected 2 days later and RNA was extracted for quantitative PCR amplification. The knockout efficiency of each combination was determined by calculating the relative expression of vFLIP.

(1) Homologous recombination primer design:

vFLIP CDS area:

ATGGCCACTTACGAGGTTCTCTGTGAGGTGGCGCGGAAACTGGGCACGGATGACAGGGA
AGTGGTATTGTTCCTCCTAAACGTGTTCATACCTCAACCCACACTGGCCCAATTAATTGG
AGCTCTTAGAGCTTTAAAGGAGGAGGGCAGGTTAACGTTTCCCCTGTTAGCGGAATGTCT
GTTTCGTGCAGGTCGCAGAGACCTCTTGCGCGACCTGCTTCACTTAGACCCGCGTTTTTT
AGAGCGCCACCTAGCGGGC
ACAATGAGTTATTTCAGCCCTTATCAGCTCACTGTTCTCCACGTAGACGGGGAGCTGTGT
GCGAGGGATATTAGGTCTTTGATATTTTTAAGCAAGGACACTATAGGGTCTCGCAGCAC
CACAGACATTCTTACACTGGGTGTACTGTATGGAAAACTTAGACCTACTGGGTCCCACTG
ACGTGGATGCCCTAATGTCAATGCTTAGATCTTTGTCAAGAGTAGACCTACAGCGCCAAG
TGCAAACCCTAATGGGCCTTCACCTTTCCGG
CCCATCACACTCCCACACTATCGCCATCACCATAG

Plvx-IRES-ZsGreen1 vector information:

Select two restriction sites, EcoRI and BamHI, through restriction site analysis, and add HA tags to the downstream primers.

Upstream primer: GGATCTATTTCCGGT GAATTC ATGGCCACTTACGAGGTTC

Downstream primer: GGAGGGAGAGGGGCG GGATCC CTA AGCGTAGTCTGGGAC GTCGTATGGGTA TGGTGTATGGCGATAGTG

(2) Clone vFLIP-HA into the PLVX vector. The cDNA of hPDLSCs infected with KSHV was used as a template for PCR amplification, and the vFLIP gene fragment containing the HA tag was obtained. The vFLIP gene fragment containing the HA tag was recombined into the cut Plvx vector by homologous recombinase. The success of plasmid construction was verified by transforming into competent cells, performing clonal amplification and sending for sequencing.

(3) Extraction of endotoxin-free plasmid. Refer to the kit instructions, the brief process is: ① Centrifuge the bacterial solution at 5000g for 10 minutes at room temperature to remove the supernatant; ② Add 500 μL of Solution I containing RNaseA, vortex, and transfer the resulting cell suspension to a new 2 mL centrifuge