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DERL3 participates in the progression of renal clear cell carcinoma by promoting epithelial-mesenchymal transition through regulating TGFB1 pathway

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Abstract

Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) and Western blot were used to detect the expression of DERL3 in ccRCC cell lines. Immunohistochemistry (IHC) was used to stain ccRCC specimens and adjacent normal tissue specimens to verify the differential expression of DERL3 in ccRCC tissues and adjacent normal tissues. The effects of DERL3 on the growth, invasion and migration of ccRCC were verified in vitro by MTT, plate cloning and cell migration assays. The expression levels of DERL3 and epithelial-mesenchymal transition-related marker proteins were analyzed by Western blot.

Materials

The renal clear cell carcinoma samples and adjacent cancer tissue samples involved in this study were obtained from the Provincial Hospital Affiliated to Shandong First Medical University from August 2024 to October 2024. These samples were all fresh pathological tissues from patients undergoing radical nephrectomy or partial nephrectomy.
The human renal tubule epithelial cell line HK-2 and several ccRCC cell lines (ACHN, Caki–1, 786–O, and A498) were acquired from the Shanghai Cell Bank of the Chinese Academy of Sciences.
Main experimental instruments and equipment
Experimental instruments and equipment names (models and equipment sources)
(1) Carbon dioxide incubator (BPN-80CH): Yiheng Instrument
(2) Clean bench (SW-CJ-1FD): Sujing Antai Air Technology Co., Ltd.
(3) Inverted biological microscope (XD-202): Nanjing Jiangnan Yongxin Company
(4) Inverted fluorescence microscope (CKX53): OLYMPUS Company
(5) Centrifuge (DM04129): SCILOGEX Company
(6) Dual-function refrigerator (BCD-187IQ): Hisense Company
(7) Desktop high-speed refrigerated centrifuge (H1650R): Changsha High-tech Industrial Development Zone Xiangyi Centrifuge Instrument Co., Ltd.
(8) Metal bath (KR116): Tiangen Biochemical Technology (Beijing) Co., Ltd.
(9) Vortex oscillator (VX100): Labnet Company
(10) Small centrifuge (OSE- MC8): Tiangen Biochemical Technology (Beijing) Co., Ltd.
(11) Fluorescence quantitative PCR instrument (LightCycler96): Roche
(12) Full wavelength spectrophotometer (OSE-260): Tiangen Biochemical Technology (Beijing) Co., Ltd.
(13) Vertical swing shaker (SK-L180-E): SCILOGEX (USA)
(14) Left-right swing shaker (SK-R1807-S): SCILOGEX (USA)
(15) Chemiluminescence instrument (Tanon-4600): Beijing Yuanpinghao Biotechnology Co., Ltd.
(16) Electrophoresis instrument (JY300E): Beijing Junyi Oriental Electrophoresis Equipment Co., Ltd.
(17) Electrophoresis tank (JY-ZY5): Beijing Junyi Oriental Electrophoresis Equipment Co., Ltd.
(18) Incubator (DNP-9272): Shanghai Jinghong Experimental Equipment Co., Ltd.
(19) Microplate reader (CMAX PLUS): Meigu Molecular Instruments (Shanghai) Co., Ltd.
(20) Embedding machine (KD-BM): Jinhua Kedi Instrument Equipment Co., Ltd., Zhejiang Province
(21) Pathology slicer (KD-2258): Jinhua Kedi Instrument Equipment Co., Ltd., Zhejiang Province
(22) Slide spreader (KD-P): Jinhua Kedi Instrument Equipment Co., Ltd., Zhejiang Province
(23) Freezing table (KD-BL): Jinhua Kedi Instrument Equipment Co., Ltd., Zhejiang Province
(24) Oven (PHG-9070A): Shanghai Jinghong Experimental Equipment Co., Ltd.
(25) Slides and coverslips (10212432C): Jiangsu Shitai Experimental Equipment Co., Ltd.
(26) Upright optical microscope (OLYMPUS CK31): Olympus Corporation, Japan
(27) Imaging system (TVO.63XC-MO): Mingmei Mshot Co., Ltd.
(28) Digital full-scan instrument (Pannoramic SCAN): 3D Histech Co., Ltd.
Main reagents and consumables related to the experiment
Reagents and consumables related to the experiment (source)
(1) DMEM-Ham's F12 (CM10092): Zhongke Maichen (Beijing) Technology Co., Ltd.
(2) Pancreatin (T1300): Solebao Technology Co., Ltd. (Beijing)

(3) Penicillin-streptomycin (P1400): Solebao Technology Co., Ltd. (Beijing)
(4) Fetal bovine serum (FCS500): EXcellBio
(5) PBS buffer (G4202): Wuhan Saiweier Biotechnology Co., Ltd.
(6) T25 culture flask (07-8025): Biolglx
(7) 10 cm culture dish (07-3100): Biolglx
(8) 50 ml centrifuge tube (23-2263): Crystalgen
(9) 15 ml centrifuge tube (23-2266): Crystalgen
(10) 2 ml cryotube (430659): Beijing Lanjieke Biotechnology Co., Ltd.
(11) FastQuant cDNA first-strand synthesis kit (genome-free) (KR116): Tiangen Biochemical Technology (Beijing) Co., Ltd.
(12) SuperReal Fluorescence Quantification Premix Reagent (SYBR Green) (FP205): Tiangen Biochemical Technology (Beijing) Co., Ltd.
(13) TRNzol Universal Total RNA Extraction Reagent (DP424): Tiangen Biochemical Technology (Beijing) Co., Ltd.
(14) DNase/RNase-free deionized water (RT121): Tiangen Biochemical Technology (Beijing) Co., Ltd.
(15) Chloroform: Sinopharm Chemical Reagent Co., Ltd.
(16) Isopropanol: Sinopharm Chemical Reagent Co., Ltd.
(17) Anhydrous ethanol: Sinopharm Chemical Reagent Co., Ltd.
(18) Glycine (G8200): Solebao Technology Co., Ltd. (Beijing)
(19) Sodium chloride (10019318): Sinopharm Chemical Reagent Co., Ltd.
(20) Tris (MB3739): Meilun Biotechnology Co., Ltd. (Dalian)
(21) Sodium dodecyl sulfate (MB2479): Meilun Biotechnology Co., Ltd. (Dalian)
(22) Skimmed milk powder (CN7861): Coolbo Technology Co., Ltd. (Beijing)
(23) Western primary antibody diluent (G2025): Wuhan Saiweier Biotechnology Co., Ltd.
(24) Western secondary antibody diluent (G2009): Wuhan Saiweier Biotechnology Co., Ltd.
(25) Immobilon Western HRP Substrate Luminol Reagent (WBKLS0500): Millipore
(26) SDS-PAGE protein loading buffer (1X) (P0015A): Biotech Biotech Co., Ltd. (Shanghai)
(27) SDS-PAGE protein loading buffer (5X) (P0015): Biotech Biotech Co., Ltd. (Shanghai)
(28) Western and IP cell lysis buffer (P0013J): Biotech Biotech Co., Ltd. (Shanghai)
(29) PMSF: Biotech Biotech Co., Ltd. (Shanghai)
(30) 6-well plate (07-6006): Biolglx
(31) Xylene: Sinopharm Chemical Reagent Co., Ltd.
(32) EDTA antigen retrieval solution: Wuhan Saiweier Biotechnology Co., Ltd.
(33) PBS Buffer: Wuhan Saiweier Biotechnology Co., Ltd.
(34) Sodium citrate buffer: Solebao Technology Co., Ltd. (Beijing)
(35) 30% H2O2 methanol: Sinopharm Chemical Reagent Co., Ltd.
(36) BSA: Solebao Technology Co., Ltd. (Beijing)
(37) Primary antibody: Anti-DERL3 antibody (ab78233): Abcam
(38) Secondary antibody: HRP-labeled goat anti-rabbit IgG (GB23303): Servicebio
(39) MCCOY'S 5A (CM10051): Zhongke Maichen (Beijing) Technology Co., Ltd.
(40) MEM (CM10010): Zhongke Maichen (Beijing) Technology Co., Ltd.
(41) MTT kit (G4101): Wuhan Saiweier Biotechnology Co., Ltd.
(42) 96-well plate (07-6096): Biolglx
(43) 24-well plate (07-6024): Biolglx

(44) 4% paraformaldehyde (P0099): Biotech Biotechnology Co., Ltd. (Shanghai)
(45) 0.1% crystal violet stain (G1064): Solebo Technology Co., Ltd. (Beijing)
(46) transwell chamber (3422): Corning

Paraffin section immunohistochemistry

1 1. Drying: Mark the experimental sections and place them in a 60°C oven for 2 hours or 37°C overnight.
2. Dewaxing paraffin sections to water: Put the sections into xylene I for 15min-xylene II for 15min-anhydrous ethanol I for 5min-anhydrous ethanol II for 5min-95% alcohol for 5min-90% alcohol for 5min-80% alcohol for 5min-70% alcohol for 5min-distilled water.
3. Antigen repair: Place the tissue sections in a repair box of sodium citrate repair solution in a microwave oven for antigen repair. During this process, excessive evaporation of the buffer should be prevented and the sections should not be dried. After natural cooling, place the slides in PBS (PH7.4) and shake and wash on a decolorizing shaker 3 times, each time for 5min.
4. Block endogenous peroxidase: Place the sections in 3% hydrogen peroxide solution, incubate at room temperature in the dark for 20min, place the slides in PBS (PH7.4) and shake and wash on a decolorizing shaker 3 times, each time for 5min.
5. 5% BSA blocking: Add blocking solution to the slices to cover the entire tissue; at the same time, prevent the blocking solution from flowing away and drying the slices, and block in a 37°C incubator for 30 minutes.
6. Primary antibody incubation: Gently shake off the blocking solution, add PBS to the slices according to a certain ratio of primary antibodies, and lay the slices flat in a humidified box for overnight incubation at 4°C. (Add a small amount of water to the humidified box to prevent the antibody from evaporating).
Antibody Manufacturer Item No.
Anti-DERL3 antibody Abcam ab78233
7. Secondary antibody incubation: Place the slides in PBS (PH7.4) and shake and wash on a decolorizing shaker 3 times, 5 minutes each time. After the slices are slightly dried, add the secondary antibody of the corresponding species to the primary antibody in the circle, cover the tissue, and incubate at room temperature away from light for 50 minutes.
Antibody Manufacturer Item No.
HRP-labeled goat anti-rabbit IgG Servicebio GB23303
8. DAB color development: Place the slide in PBS (PH7.4) and shake on a decolorizing shaker to wash 3 times, 5 minutes each time. After the slices are slightly dried, add freshly prepared DAB color development solution (TIANGEN, PA140212) in the circle, control the color development time under the microscope, positive is brown-yellow, and rinse the slices with tap water to stop color development.
9. Counterstaining of cell nuclei: Harris hematoxylin (BOSTER, AR1108) counterstaining for about 5 minutes (the time can be controlled by the degree of staining), washing with tap water, 1% hydrochloric acid alcohol differentiation for a few seconds, washing with tap water, ammonia water back to blue, and washing with running water.

10. Dehydration and sealing: Put the slices into 75% alcohol for 6 minutes, 85% alcohol for 5 minutes, 95% alcohol for 5 minutes, anhydrous ethanol I for 5 minutes, anhydrous ethanol II for 5 minutes, and xylene I for 10 minutes to dehydrate and make them transparent. Take the slices out of xylene and let them dry slightly, then seal them with neutral gum.
11. Microscopic examination and photography: Observe the slices under a fluorescence microscope and collect images.

Cell culture experiments

2 1 Cell recovery
HK-2 culture conditions: DMEM-Ham's F12+10% FBS+1% double antibody
(1) Take the cryovial out of liquid nitrogen and immediately put it into a 37°C water bath to thaw quickly. After taking it out, spray it with alcohol and put it in a clean bench.
(2) Pipette the thawed cell suspension into a 15ml centrifuge tube containing about 2-3ml culture medium and centrifuge it at 1000 rpm for 5 minutes.
(3) Discard the supernatant and add 1ml culture medium to prepare the cell suspension. Pipette the cell suspension into a 10cm culture dish containing 10ml culture medium, shake it gently back and forth to evenly distribute the cells in the culture dish, mark it and place it in a CO2 incubator for culture. Change the culture medium after the cells adhere to the wall.
2 Cell passaging
(1) Passage when the cell coverage rate in the culture dish reaches 80%-90%.
(2) Remove the original culture medium, wash with PBS buffer 2-3 times, add an appropriate amount of trypsin (just enough to cover the cells), and digest for 1-2 minutes.
(3) After the cells become round, add 2-3 ml of culture medium to stop digestion.
(4) Blow off the cells, transfer to a 15 ml centrifuge tube, and centrifuge at 1000 rpm for 5 minutes.
(5) Discard the supernatant, add 1-2 ml of culture medium, prepare a cell suspension, transfer to a new culture dish, mix well, and continue culturing in a CO2 incubator.
3 Cell freezing
(1) Take out serum-free freezing solution from the refrigerator.
(2) Take cells in the logarithmic growth phase, use trypsin to digest the monolayer cells, and directly transfer the cells to a 15 ml centrifuge tube for the suspended cells.
(3) Centrifuge at 1 000 rpm for 5 min.
(4) Remove trypsin and old culture medium, add an appropriate amount of freezing solution, and gently blow with a pipette to make the cells uniform.
(5) Divide the cells into cryopreservation tubes, 1 ml per tube.
(6) Label the cell name and cryopreservation time on the cryopreservation tubes.
(7) Cryopreservation: Place in a -80°C freezer overnight, remove the cryopreservation tubes, and transfer to a liquid nitrogen container.

Fluorescence quantitative detection experiment

3 1. Total RNA extraction
TRNzol Universal Total RNA Extraction Reagent Instructions (https://www.tiangen.com/asset/imsupload/up0906252001604560423.pdf) Extract RNA. The steps are as follows:
(1) Take a cell sample, add 1 ml of TRNzol reagent, homogenize and let stand at room temperature for 5 min;.
(2) Optional step: Centrifuge at 4°C 12,000 rpm (~13,400×g) for 10 min and take the supernatant. (If the sample contains a large amount of protein, fat, polysaccharide or muscle, plant nodules, etc., it can be removed by centrifugation.)
(3) Add 0.2 ml of chloroform for every 1 ml of Trizol used, cover the tube, shake vigorously for 15 sec, and let it stand at room temperature for 3 min.
(4) Centrifuge at 4°C 12,000 rpm (~13,400×g) for 10-15 min and transfer the upper aqueous phase to a new centrifuge tube. Add an equal volume of isopropanol, mix well, and leave at room temperature for 10 min.
(5) Centrifuge at 4°C, 12,000 rpm for 10 min and remove the supernatant.
(6) Add 1 ml 75% ethanol (RNase-Free ddH2O, prepared immediately before use) to wash the precipitate. For every 1 ml of TRNzol used, use at least 1 ml of 75% ethanol to wash the precipitate.
(7) Centrifuge at 4°C, 12,000 rpm for 5 min and remove the supernatant.
(8) Leave at room temperature for a while to evaporate the residual ethanol.
(9) Add 30-100 µl RNase-Free ddH2O according to experimental needs, pipette and mix repeatedly to fully dissolve the RNA.
2. Reverse transcription
Perform reverse transcription according to the instructions of FastQuant cDNA First Strand Synthesis Kit (Genomic DNA-free) (KR116) (https://www.tiangen.com/asset/imsupload/up0265627001604562652.pdf).
Prepare the genome-free DNA system according to Table 2-1
Table 2-1 Genome-free reaction system
Reagent Volume
5×gDNA Buffer 2 µl
Total RNA 1µg
RNase-Free ddH2O Make up to 10 µl
Incubate at 42°C for 3 min and place on ice.
Prepare the reverse transcription reaction system according to Table 2-2.
Table 2-2 Reverse transcription reaction system
Reagent Volume
10×Fast RT Buffer 2 µl
RT Enzyme Mix 1 µl
FQ-RT Primer Mix 2 µl
RNase-Free ddH2O Make up to 10 µl

Add the reverse transcription system to the gDNA removal system and mix thoroughly.
Incubate at 42°C for 15 min.
Incubate at 95°C for 3 min. Store cDNA at -20°C.
3. Fluorescence quantitative detection
Design specific amplification primers based on the target gene sequence (Table 2-3) and send them to Beijing Dingguo Changsheng Biotechnology Co., Ltd. for synthesis.
Use the cDNA obtained by reverse transcription in 2.2 as a template and according to SuperReal Fluorescence Quantification Premix Reagent (SYBR Green) (FP205) (Table 2-4) (https://www.tiangen.com/asset/imsupload/up0890627001604563015.pdf).
Fluorescence quantitative primer sequence
Primer name Primer sequence (5'-3')
DERL3-F ATGGCGTGGCAGGGACTA
DERL3-R GGTGCGGGTTGAAGTAGAGTT
GAPDH-F GTGGATATTGTTGCCATCAATGACC
GAPDH-R GCCCCAGCCTTCTTCATGGTGGT

TGF-β1-F ACCTGCCACAGATCCCCTAT
TGF-β1-R CCGGTAGTGAACCCGTTGAT
E-cadherin-F CAGGCCTCCGTTTCTGGAAT
E-cadherin-R CAAAATCCAAGCCCGTGGTG
Vimentin-F CTCTGGCACGTCTTGACCTT
Vimentin-R TTGCGCTCCTGAAAAACTGC
α-SMA-F CACAATGTGCGACGAAGACG
α-SMA-R ATGATGCCGTGCTCGATAGG
Fibronectin-F AAGAAGGGCTCGTGTGACAG
Fibronectin-R TCTTGTCCTACATTCGGCGG
GAPDH-F GTGGATATTGTTGCCATCAATGACC
GAPDH-R GCCCCAGCCTTCTTCATGGTGGT

TGF-β1-F ACCTGCCACAGATCCCCTAT
TGF-β1-R CCGGTAGTGAACCCGTTGAT
GAPDH-F GTGGATATTGTTGCCATCAATGACC
GAPDH-R GCCCCAGCCTTCTTCATGGTGGT

E-cadherin-F CAGGCCTCCGTTTCTGGAAT
E-cadherin-R CAAAATCCAAGCCCGTGGTG
Vimentin-F CTCTGGCACGTTCTTGACCTT
Vimentin-R TTGCGCTCCTGAAAAACTGC
α-SMA-F CACAATGTGCGACGAAGACG
α-SMA-R ATGATGCCGTGCTCGATAGG
Fibronectin-F AAGAAGGGCTCGTGTGACAG
Fibronectin-R TCTTGTCCTACATTCGGCGG
GAPDH-F GTGGATATTGTTGCCATCAATGACC

GAPDH-R GCCCCAGCCTTCTTCATGGTGGT

Fluorescence quantitative reaction system
Components 20 µl system
2×SuperReal PreMix Plus 10µl
Forward primer (10 µM) 0.6µl
Reverse primer (10 µM) 0.6µl
cDNA template 1µl
RNase-free ddH2O to 20µl
After mixing evenly, use real-time fluorescence quantitative PCR instrument for detection. The amplification conditions are: 95°C, 15 min; 95°C, 10 s; 60°C, 30 s, a total of 40 cycles. Each sample was repeated three times, and the experimental data were analyzed using the 2-ΔΔCT method.

Western Blot

4 1. Protein extraction
1.1 Cell protein extraction
Wash the cells 2-3 times with PBS, and directly add an appropriate amount of 1×SDS-PAGE protein loading buffer. After scraping thoroughly with a cell scraper, treat in a 100°C metal bath for 5-10 minutes.
2. SDS-PAGE electrophoresis
2.1 SDS-PAGE gel preparation
Prepare polyacrylamide gel according to the SDS-PAGE gel preparation kit:
1. Wipe the electrophoresis gel glass plate and install it.
2. Prepare 10% lower separation gel according to the instructions.
3. Mix the above reagents evenly, carefully inject the separation gel into the gap between the glass plates along the glass plate, cover the surface with distilled water, and let it stand at room temperature for 60 minutes.
4. After the separation gel is completely polymerized and solidified (as a clearly visible liquid level appears between the separation gel and the distilled water), gently pour out the upper distilled water and use filter paper to absorb the water on the surface of the gel.
5. Prepare the upper 5% concentrated gel according to the instructions.
6. Mix the above reagents evenly, add them slowly to the glass plate, insert the comb slowly from one side of the glass plate to avoid bubbles, and let it stand for about 50 minutes at room temperature.
7. After the concentrated gel is completely solidified, gently pull out the comb in the gel and gently rinse the sample well with distilled water.
2.2 Electrophoresis
Determine the loading order and loading amount according to the experimental requirements; first set 80V and start electrophoresis. After the sample bromophenol blue

runs to the separation gel, increase the voltage to 120V. Electrophoresis can be terminated when the bromophenol blue runs to the bottom of the glass plate.
2.3 Transfer
Use a standard wet transfer device to cover the PVDF membrane to the target protein area. The transfer current is set to 250mA and the transfer time is 90 minutes.
2.4 Blocking
After the transfer is completed, transfer the membrane to 5% blocking solution and block it on a shaker for 1 hour.
2.5 Primary antibody incubation
Refer to the instructions of the primary antibody and dilute the primary antibody with Western primary antibody diluent according to the appropriate ratio. Incubate overnight at 4°C, wash 3 times with 1×TBST after incubation, 10 minutes each time.
Antibody Name Manufacturer Item No. Dilution Ratio

GAPDH Zhongshan Jinqiao TA-08 1:1000
DERL3 Huaan Bio ER62512 1:1000

GAPDH Zhongshan Golden Bridge TA-08 1:1000
TGF-β1 Proteintech 21898-1-AP 1:1000
E-cadherin Proteintech 60335-1-Ig 1:1000
Vimentin Proteintech 10366-1-AP 1:1000
α-SMA Proteintech 67735-1-Ig 1:1000
Fibronectin Proteintech 66042-1-Ig 1:1000

2.6 Secondary Antibody Incubation:
Refer to the instructions of the secondary antibody and dilute the secondary antibody with Western secondary antibody diluent according to the appropriate ratio. Incubate on a shaker at room temperature for 1 hour, wash 3 times with 1×TBST after incubation, 10 minutes each time.
Antibody Name Manufacturer Item No. Dilution Ratio

Mouse Antibody Zhongshan Jinqiao ZB-2305 1:2000
Rabbit Antibody Zhongshan Jinqiao ZB-2301 1:2000

2.7 Exposure
Put the PVDF membrane into the chemiluminescence instrument, mix the exposure solution A and B 1:1, and evenly drop them on the membrane for exposure.

Cell lentivirus infection experiment

5 Experimental steps for cell lentivirus infection
1. When the cells grow to the logarithmic growth phase, trypsinize the cells. Prepare the cell suspension after centrifugation, count the cells and spread them on a 6-well plate for

overnight culture.
2. On the second day, when the cells grow to 40%-50%, lentivirus infection is performed. The MOI gradient of the pre-experimental setting is 5, 10, 15, 30. Calculate the required virus volume.
3. Replace the culture medium after 24 hours and continue to culture.
4. After 3 days, when the cells reach 100% fusion, observe the infection efficiency of the cells in each well under a fluorescence microscope, and determine the optimal MOI value of 15 for subsequent experiments.
5. Infect the cells with the infection multiplicity determined in the pre-experiment. Calculate the required volume of virus stock solution, incubate the transfection at 37°C for 24 hours, replace with fresh culture medium, and transfect for 48h-72h.

MTT assay

6 1. Cultivate cells to the logarithmic growth phase, digest cells with trypsin, and adjust the cell concentration to 1-10×104/ml cell suspension, inoculate 105 cells/well in a 96-well plate, and place in a CO2 (5%) incubator at 37°C for 24h.
2. Replace with 200μl of the culture medium containing a certain concentration of drugs corresponding to each cell sample, and replace the control group with a medium containing solvent. Set 5 replicates for each sample concentration.
3. Add 20μl of freshly prepared MTT solution with a concentration of 5mg/ml. Incubate in the incubator for 4 hours to reduce MTT to formazan (at this time, filamentous purple crystals can be seen around the cells in the well plate under an inverted microscope).
4. Carefully remove the supernatant, add 100μl of DMSO (dimethyl sulfoxide) to each well, and shake well with a shaker. Use an enzyme marker to measure the 490nm light absorption value, use the solvent-treated cells as the control group, and calculate the drug inhibition rate of the cells according to the formula.

Cell cloning experiment

7 1. When the cells grow to the logarithmic growth phase, trypsinize the cells. Prepare the cell suspension after centrifugation and count the cells.
2. Add 1000 cells/well to a 6cm dish, mix thoroughly, and culture in a 37°C, 5% CO2 incubator.
3. Change the medium every three days and culture for about 10 days.
4. Remove the cells, pour out the culture medium, wash twice with PBS, fix with 4% paraformaldehyde for 30 minutes, and wash twice with PBS.
5. Add 1ml of 0.1% crystal violet stain to each well, stain for 10-30 minutes, and discard the stain.
6. Wash off the stain with tap water, dry, turn the 6cm dish upside down on white paper, and take pictures.
7. Use ImageJ software to analyze and calculate the number of clones.

Transwell experiment

8 1. After cell digestion, resuspend the culture medium to prepare a single cell suspension. After counting the cells, adjust the cells to 2.5×105/ml.
2. Assemble the Transwell migration chamber in a 24-well plate. Add 500μl of 10% FBS culture medium to the lower layer of the Transwell chamber, and add 300μl of cell- and serum-free culture medium to the upper layer.
3. Place in a 37°C incubator and rinse with PBS after 24 hours.
4. Fix with 4% paraformaldehyde solution for 30 minutes and rinse with PBS three times.
5. Add 0.1% crystal violet stain for 10 minutes, rinse with running water, gently wipe off the unmigrated cells in the upper layer with a cotton swab, and dry naturally.
6. Observe and collect images under a fluorescence microscope. For each specimen, randomly select 3 fields of view (100x, 200x) for counting and calculate the average value.

Transwell invasion assay

9 1. First, take out the Matrigel from -20°C or -80°C and place it at 4°C to melt;
2. After freezing and thawing, use a pre-cooled pipette tip to mix the Matrigel matrix into a homogenate;
3. Determine the optimal coating concentration according to the experimental needs and dilute the Matrigel matrix with serum-free culture medium.
4. Coat the diluted Matrigel matrix in the upper chamber of the transwell chamber, 60-100μl per well, and incubate in a 37°C incubator for 2-4 hours.
Note: This step is the invasion experiment in the transwell experiment. In the migration experiment, this step omits the direct cell treatment step.
5. After cell digestion, resuspend the culture medium to prepare a single cell suspension. After cell counting, adjust the cells to 2.5×105/ml.
6. Assemble the Transwell migration chamber in a 24-well plate. Add 500μl of 10% FBS culture medium to the lower layer of the Transwell chamber, and add 300μl of cell- and serum-free culture medium to the upper layer.
7. Place in a 37°C incubator and rinse with PBS after 24 hours.
8. Fix with 4% paraformaldehyde solution for 30 minutes and rinse with PBS three times.
9. Add 0.1% crystal violet stain for 10 minutes, rinse with running water, gently wipe off the unmigrated cells on the upper layer with a cotton swab, and dry naturally.
10. Observe and collect images under a fluorescence microscope. Randomly take 3 fields of view (100x, 200x) for each specimen, count and calculate the average value.