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🌐 WNK2 may promote ovarian cancer progression by upregulating POU5F1B

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yanzhou Wang

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Disclaimer

Transcriptome sequencing analysis was performed by Beijing Novogene Institute (Beijing, China), so the protocol for this experiment is not provided here.

Abstract

To solidify WNK2's oncogenic role and highlight its therapeutic potential in OC, we performed transcriptome sequencing to identify WNK2-regulated mRNAs and noncoding RNAs. Candidate targets were validated via qRT-PCR and Western blot. Functional assays (CCK-8, colony formation, Transwell and xenograft models) assessed the role of POU5F1B and its ability to rescue WNK2 knockdown effects.

Materials

Cell culture, Tissue and nude mice

Human OC cell lines: CAOV3, American Type Culture Collection (ATCC, Manassas, VA, USA, HTB-75). A2780, Shanghai Zeye Biotechnology, ZYH60089. Tissue chip (Shanghai Outdo Biotech Co., Ltd. HOvaC160Su01). Forty 6-week-old immunodeficient female nude mice (Beijing Huafukang Experimental Animal Co., Ltd).

Dulbecco's Modified Eagle's Medium, fetal bovine serum, penicillin-streptomycin antibiotic solution, Phosphate-Buffered Saline, trypsin, Serum-Free Cell Freezing Medium (Gibco, Gaithersburg, MD, USA, 11965092, A5669701, 15140122, 10010023, 25300054, 12648010, all sterile).

96, 24, 12, 6 well plates, 1.5 mL, 15 mL, 50 mL centrifuge tube (Corning, USA, 3599, 3524, 3531, 3516, 430791, 9415, 430909 all sterile). 1 mL, 200 μ L and 10 μ L Pipette tip (Beyotime, FTIP01C, FTIP200, FTIP011 all sterile).

Cell transfection and lentivirus transduction

POU5F1B-overexpression plasmids (Tsingke Biotechnology Co., Ltd.), WNK2-overexpression plasmid (Genechem Co., Ltd), siRNAs targeting WNK2 and POU5F1B (RiboBio), Lipofectamine™ 3000 Transfection Reagent (Invitrogen, L3000015), puromycin (LEAGENE, CA0070). Kanamycin, Ampicillin (Beyotime Y259322-50mg, ST007). Opti-MEM medium (Gibco, Gaithersburg, MD, USA, 31985070).

Quantitative real-time PCR

Trizol (Invitrogen, 15596-026), Chloroform (Sigma, 67-66-3), Isopropanol (Sigma, 563935), Ethanol (Sigma, 64-17-5), Nuclease-free Water (Beyotime, R0021), HiScript III 1st Strand cDNA Synthesis Kit (+gDNA wiper) (Vazyme, R312-02*), SYBR (Invitrogen, L3000015).

Western blotting

RIPA Lysis Buffer, Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail A,B, BCA Assay Kit, Loading Buffer, Filter paper, Polyvinylidene Difluoride (PVDF) Membrane, Precast Gel, Tris-Buffered Saline with Tween 20, Blocking Buffer, Kanamycin (Beyotime P0013E, P1070-25mg, P1081, P1087, P0010S, P0289-2ml, FFP51, FFP32, 8012011, ST671-500ml, P0233), Running Buffer, Transfer buffer, Super ECL Substrate (Dakewe, P0014C, 8015071, 8061011). Prestained Protein Ladder/Marker (Thermo Fisher, 26616), Methanol (Sigma 34860).

anti-WNK2 (cat: ab192397, 1:500, Abcam), anti-POU5F1B (cat: ab230429, 1:1,000, Abcam), anti-actin (cat#3700, 1:1,000, CST), anti-mouse (cat#7076, 1:5,000, CST), and anti-rabbit (cat#7074, 1:5,000, CST) antibody.

Immunohistochemistry

Formalin, Antigen Retrieval Buffer, Phosphate Buffered Saline, immunohistochemistry in two-step kit, 3,3'-diaminobenzidine, Hematoxylin (Boster, AR1068, AR0023, AR0192-10, SV0001, SA2025, AR0005), Xylene-based



Mounting Medium (Solarbio, G8590).

Cell Counting Kit-8 (CCK-8), Crystal Violet Staining Solution, (Beyotime, C0039, Y268091-100g), Transwell Chamber / Insert (Corning, CLS3421), Isoflurane (Sigma, 26675-46-7), Digital Caliper (Sigma, Z136115).

Procedure

- 1 Inoculate 30 mL of LB medium in a 50 mL centrifuge tube with 1 μ L of bacterial stock and 30 μ L of kanamycin or ampicillin. Incubate the culture overnight in a shaker incubator.
- 2 Harvest the bacterial cells by centrifugation at 12,000 rpm for 12 minutes. Carefully decant the supernatant and retain the bacterial pellet.
- 3 Column Equilibration: Add 500 μ L of BL solution to the CP3 adsorption column. Centrifuge at 12,000 rpm for a brief spin (~30 seconds). Discard the flow-through and place the column back into the collection tube.
- 4 Resuspend the bacterial pellet completely in 1 mL of P1 Solution by gentle pipetting.
- 5 Add 1 mL of P2 Solution to the tube and mix gently by inversion until the solution becomes clear and viscous, indicating complete cell lysis.
- 6 Add 1.4 mL of P3 Solution to neutralize the lysate and mix immediately. A white precipitate will form. Centrifuge the mixture at 12,000 rpm for 10 minutes.
- 7 Carefully transfer the supernatant from the previous step to the equilibrated CP3 column. Centrifuge at 12,000 rpm for 1 minute. Discard the flow-through.
- 8 Add 600 μ L of PW Wash Solution (ensure ethanol has been added to the PW solution as specified) to the column. Centrifuge at 12,000 rpm for 1 minute. Discard the flow-through.
- 9 Repeat the wash step (Step 8) once.
- 10 Place the CP3 column back into the collection tube and centrifuge at 12,000 rpm for 2 minutes to remove any residual wash buffer completely.
- 11 Finally, transfer the CP3 column to a clean microcentrifuge tube. Add 50 μ L of Ultrapure Water directly onto the center of the column membrane. Let it stand for 2 minutes, then centrifuge at 12,000 rpm for a brief spin to elute the purified plasmid DNA.
- 12 Measure the plasmid concentration using a microvolume spectrophotometer.

Transfection

- 13 Seed cells in a 6-well plate and culture for 24 hours until they reach 60-70% confluence.
- 14 Prepare two microcentrifuge tubes: Tube A: Dilute 5 μ L of si-RNA (or 3000 ng of plasmid DNA) in 500 μ L of Opti-MEM Reduced-Serum Medium. Tube B: Dilute 5 μ L of Lipo3000 transfection reagent in 500 μ L of Opti-MEM. Mix gently and incubate for 5 minutes at room temperature.
- 15 Combine the contents of Tube A and Tube B. Mix gently and incubate the mixture for 20 minutes at room temperature to allow transfection complex formation.
- 16 Medium Change: Replace the cell culture medium with serum-free medium. Then, add the siRNA (or plasmid) complex mixture dropwise to each well of the 6-well plate.
- 17 After 6 hours of transfection, replace the serum-free medium with fresh complete medium containing serum.

CCK-8 Proliferation Assay

- 18 Twenty-four hours after transfection, wash the cells twice with PBS and digest them with trypsin.
- 19 Count the cells using a hemocytometer. Count the cells in at least five large squares, calculate the average, and multiply by 10^4 to obtain the cell density (cells/mL).
- 20 Seed 5,000 cells per well in a 96-well plate. For each group, prepare three replicate wells. Calculate the total volume of cell suspension needed for all replicates of a group, mix the suspension well by pipetting, and add 200 μ L to each well.
- 21 Place the 96-well plate in the incubator.
- 22 Perform the CCK-8 proliferation assay on Day 0, 2, 4, and 6.
- 23 Prepare the CCK-8 working solution by mixing the CCK-8 stock solution with culture medium at a 1:10 ratio.
- 24 For the assay, add 100 μ L of the CCK-8 working solution to each well.



- 25 Incubate the plate at 37°C for 1 hour.
- 26 Measure the absorbance (OD) at 450 nm using a microplate reader.
- 27 Plot a proliferation curve based on the obtained OD values.

Colony Formation Assay

- 28 Seed 1,000 tumor cells per well in a 12-well plate. Before seeding, ensure the cell suspension is thoroughly mixed by pipetting to guarantee a single-cell suspension without clumps.
- 29 Place the 12-well plate in the incubator and culture for two weeks to allow colony formation.
- 30 After two weeks, wash the colonies twice with PBS.
- 31 Fix the colonies with 4% paraformaldehyde for 10 minutes.
- 32 Stain the fixed colonies with crystal violet for 5 minutes.
- 33 Rinse the wells with PBS until the excess crystal violet is washed away and the background is clear.
- 34 Photograph the stained colonies and count them.

Transwell Invasion Assay

- 35 Place the Transwell chamber (with a Matrigel-coated membrane) into a 24-well plate. Add complete medium (containing serum or chemoattractants) to the lower chamber.

- 36 48 hours post-transfection, digest the cells with trypsin. Neutralize the trypsin with complete medium, then centrifuge the cell suspension at 5,000 rpm for 5 minutes. Resuspend the cell pellet in serum-free medium and perform a cell count.
- 37 Aspirate a cell suspension containing 20,000 cells and carefully add it to the upper compartment of the Transwell chamber.
- 38 Incubate the plate in a cell culture incubator for 48 hours. After incubation, gently wipe off the non-invading cells from the upper side of the membrane using a cotton swab. Then, place the chamber in 4% paraformaldehyde (PFA) to fix the invaded cells for 10 minutes, followed by staining with crystal violet for 5 minutes.
- 39 Rinse the chamber with PBS to remove any excess crystal violet stain. Observe the membrane under a microscope, photograph the invaded cells on the lower surface, and count them.

RNA Extraction

- 40 Wash the cells twice with PBS. Add 500 μ L of Trizol to each well and incubate at room temperature for 5 minutes for lysis. Then, transfer the Trizol lysate to a microcentrifuge tube.
- 41 Add 100 μ L of chloroform to each tube and vortex vigorously. Let it stand for 3 minutes, then centrifuge at 12,000 rpm for 15 minutes.
- 42 Carefully transfer the upper, clear aqueous phase to a new microcentrifuge tube. Add an equal volume (approximately 300 μ L) of isopropanol to the new tube. Mix well and place at -80°C for 30 minutes to precipitate the RNA.
- 43 Thaw the tubes at room temperature, then centrifuge at 12,000 rpm for 10 minutes. A white RNA pellet should be visible at the bottom of the tube. Carefully discard the supernatant.
- 44 Add 1 mL of 75% ethanol (prepared with nuclease-free water) to gently wash the pellet. Centrifuge at 12,000 rpm for 5 minutes.
- 45 Repeat the previous washing step.
- 46 Discard the supernatant. Centrifuge the tube at 12,000 rpm for 3 minutes to remove any residual ethanol.



- 47 Air-dry the pellet at room temperature for 3-5 minutes. Then, dissolve the RNA in an appropriate volume of nuclease-free water (DEPC-treated, sterilized water).
- 48 Measure the RNA concentration using a microvolume spectrophotometer. The A260/A280 ratio of the extracted RNA should be between 1.8 and 2.0.

Reverse Transcription

- 49 First-Step Mixture Preparation: Pipette 1 µg of RNA and 4 µL of gDNA wiper Mix into a tube.
- 50 Add nuclease-free water to bring the total volume to 16 µL. Mix gently and heat at 42°C for 2 minutes.
- 51 Composition of the First-Step Mixture: Reagent Amount, RNA: 1 µg, DNA wiper Mix: 4 µL, Nuclease-free water: Up to 16 µL, Second-Step Reaction Setup: Add 4 µL of 5× HiScript II qRT SuperMix to the previous 16 µL mixture. Composition of the Second-Step Mixture: Reagent: Volume First-Step Mixture: 16 µL, 5× HiScript II qRT SuperMix: 4 µL.
- 52 Incubate the 20 µL reaction mixture at 37°C for 15 minutes, followed by 85°C for 5 seconds to obtain cDNA. Dilute the synthesized cDNA 5-fold and store at 4°C.

Western blotting

- 53 Protein Extraction: After removing the cells from the incubator, wash them twice with PBS. Add the prepared protein lysis buffer to the culture dish, then scrape the cells off the surface and transfer the lysate to a microcentrifuge tube. Lyse the cells on ice for 30 minutes.
- 54 Pre-cool a centrifuge to 4°C. Centrifuge the tubes at 12,000 rpm for 15 minutes. Carefully collect the supernatant (containing the soluble protein) and discard the pellet (cell debris).
- 55 Standard Curve Preparation: Prepare a series of dilutions from the standard protein solution. Pipette 2 µL of each diluted standard and 18 µL of nuclease-free water into separate wells of a 96-well plate. Then, add 200 µL of the freshly prepared BCA working reagent (prepared by mixing Reagent A and Reagent B at a 50:1 ratio) to each well. Incubate the plate at 37°C for 30 minutes.
- 56 Measure the absorbance (OD) at 562 nm using a microplate reader. Plot the standard curve using the known standard protein concentrations and their corresponding absorbance values.

- 57 **Sample Preparation and Loading:** The protein samples were normalized to the same mass and volume. The required volume of each protein sample was calculated based on the total protein amount. Subsequently, a volume of 5× Loading Buffer equal to one-fifth of the final total volume was added to each sample. The mixtures were then denatured by heating at 95°C in a water bath for 10 minutes. The prestained protein marker and prepared protein samples were loaded into the wells of the gel from left to right.
- 58 **SDS-PAGE Electrophoresis:** Electrophoresis was initiated at a constant voltage of 90 V while the samples migrated through the stacking gel. After approximately 20 minutes, when the samples had entered the separating gel, the voltage was switched to 130 V and maintained for 60 minutes to achieve optimal separation.
- 59 **Membrane Transfer:** Following electrophoresis, the proteins were transferred onto a membrane using the semi-dry transfer method. Prior to assembly, the PVDF membrane was activated by briefly immersing it in methanol. The transfer stack was assembled in the following order (from top to bottom): filter paper, the gel, the activated PVDF membrane, and another piece of filter paper. Care was taken to remove all air bubbles between each layer. The transfer was then conducted for 25 minutes.
- 60 **Blocking:** Upon completion of the transfer, the PVDF membrane was incubated in a blocking solution of 5% skim milk for two hours at room temperature to prevent non-specific antibody binding.
- 61 **Primary Antibody Incubation:** After blocking, the membrane was washed with TBST to remove excess milk. It was then incubated with primary antibodies diluted in the appropriate buffer as follows: WNK2 (1:500), POU5F1B (1:1000), AKT (1:1000), and GAPDH (1:2000). The incubation was carried out overnight at 4°C.
- 62 **Washing:** The membrane was washed three times with TBST, for 10 minutes each wash, to remove unbound primary antibody.
- 63 **Secondary Antibody Incubation:** The membrane was then incubated with an appropriate HRP-conjugated secondary antibody at room temperature for one hour.
- 64 **Washing:** Following secondary antibody incubation, the membrane was washed three times with TBST, for 5 minutes per wash, to remove any unbound secondary antibody.
- 65 **Detection:** The immunoreactive protein bands were visualized using an Enhanced Chemiluminescence (ECL) substrate and detected by imaging with a chemiluminescence imaging system.

Immunohistochemical (IHC) Staining for Tissue Microarrays (TMAs)

- 66 **Baking:** Bake the paraffin-embedded tissue microarray (TMA) slides in a 60°C oven for 6 hours.

- 67 Deparaffinization and Rehydration: Immerse the TMA slides in xylene for 15 minutes, then transfer them to fresh xylene for another 15 minutes. Subsequently, immerse the slides sequentially in 100% ethanol, 95% ethanol, 85% ethanol, and 75% ethanol, for 3 minutes each. Rinse with TBST for 10 minutes.
- 68 Blocking of Endogenous Peroxidases: Apply 3% H₂O₂ onto the tissue sections and incubate for 10 minutes to block endogenous peroxidase activity. Rinse with TBST for 10 minutes.
- 69 Antigen Retrieval: Place the TMA slides in a glass coplin jar filled with antigen retrieval buffer. Perform heat-induced epitope retrieval (HIER) by boiling the jar in a pressure cooker for 20 minutes. Then, allow the jar to cool down to room temperature naturally. Rinse the slides with TBST for 10 minutes.
- 70 Primary Antibody Incubation: Apply the diluted WNK2 primary antibody (1:50 dilution) onto the tissue sections and incubate overnight at 4°C.
- 71 Washing: Wash the TMA slides three times with TBST, for 10 minutes each wash, to remove unbound primary antibody.
- 72 Secondary Antibody Incubation: Incubate the slides with a rabbit secondary antibody at room temperature for 1 hour. Then, wash with TBST for 10 minutes.
- 73 Detection and Counterstaining: Develop the signal using DAB substrate for 30 seconds. Counterstain the nuclei with hematoxylin for 10 seconds. Rinse the slides with TBST for 10 minutes.
- 74 Dehydration and Clearing: Dehydrate the TMA slides by sequentially immersing them in 75% ethanol, 85% ethanol, 95% ethanol, and 100% ethanol, for 3 minutes each. This is followed by a final clearing step in xylene for 15 minutes.
- 75 Mounting: Mount the slides with neutral balsam mounting medium, taking care to avoid air bubbles.

Nude mice model establishment

- 76 The twenty female nude mice were randomly divided into four groups (n=5 per group): shNC, shWNK2, shWNK2 + Lv-NC, and shWNK2 + Lv-POU5F1B.
- 77 A 100 µL cell suspension containing approximately 1×10^7 cells was subcutaneously injected into the right flank of each mouse.



- 78 Tumor formation was observed on day 12 post-injection. Tumor size was measured daily thereafter.
- 79 After seven days, when the tumors had grown to approximately 1.5 cm in diameter, the mice were euthanized by cervical dislocation.
- 80 The tumors were subsequently excised and photographed. Finally, the tumor tissues were fixed, paraffin-embedded, and sectioned by specialized staff from the Department of Breast Surgery at Southwest Hospital.

Acknowledgements

Funding

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Competing interests

The authors declare no competing interests.

Equipment

Ultrapure Water System (MILLIPORE), Constant Temperature Shaker TS-1000 (Qilin Bell Instrument Manufacturing Co., Ltd.), Upright Microscope BX53 (Olympus Co., Ltd. Beijing), Oven Thermo Fisher Scientific (Massachusetts, USA), Rice Cooker (Demash, Germany), Cell Culture Incubator Thermo Fisher Scientific (Massachusetts, USA), Fluorescence Microscope, Olympus BX53 (Tokyo, Japan), Microvolume Spectrophotometer, Thermo Fisher Scientific (Massachusetts, USA), Rapid Blotting System, Bio-Rad Fluorescence Quantitative PCR System, Bio-Rad (California, USA), VILBER Fluorescence/Chemiluminescence Imaging System. (Biocomc Beijing), Constant Temperature Water Bath, (Shanghai Yike Instrument), Microplate Reader (Thermo Fisher Scientific Massachusetts, USA).

Procedure

Cell transfection and lentivirus transduction

- (1). Inoculate 30 mL of LB medium in a 50 mL centrifuge tube with 1 μ L of bacterial stock and 30 μ L of kanamycin or ampicillin. Incubate the culture overnight in a shaker incubator.
- (2). Harvest the bacterial cells by centrifugation at 12,000 rpm for 12 minutes. Carefully decant the supernatant and retain the bacterial pellet.
- (3). Column Equilibration: Add 500 μ L of BL solution to the CP3 adsorption column. Centrifuge at 12,000 rpm for a brief spin (~30 seconds). Discard the flow-through and place the column back into the collection tube.
- (4). Resuspend the bacterial pellet completely in 1 mL of P1 Solution by gentle pipetting.
- (5). Add 1 mL of P2 Solution to the tube and mix gently by inversion until the solution becomes clear and viscous, indicating complete cell lysis.
- (6). Add 1.4 mL of P3 Solution to neutralize the lysate and mix immediately. A white precipitate will form. Centrifuge the mixture at 12,000 rpm for 10 minutes.



- (7). Carefully transfer the supernatant from the previous step to the equilibrated CP3 column. Centrifuge at 12,000 rpm for 1 minute. Discard the flow-through.
- (8). Add 600 μ L of PW Wash Solution (ensure ethanol has been added to the PW solution as specified) to the column. Centrifuge at 12,000 rpm for 1 minute. Discard the flow-through.
- (9). Repeat the wash step (Step 8) once.
- (10). Place the CP3 column back into the collection tube and centrifuge at 12,000 rpm for 2 minutes to remove any residual wash buffer completely.
- (11). Finally, transfer the CP3 column to a clean microcentrifuge tube. Add 50 μ L of Ultrapure Water directly onto the center of the column membrane. Let it stand for 2 minutes, then centrifuge at 12,000 rpm for a brief spin to elute the purified plasmid DNA.
- (12). Measure the plasmid concentration using a microvolume spectrophotometer.

Transfection

- (1). Seed cells in a 6-well plate and culture for 24 hours until they reach 60-70% confluence.
- (2). Prepare two microcentrifuge tubes:

Tube A: Dilute 5 μ L of si-RNA (or 3000 ng of plasmid DNA) in 500 μ L of Opti-MEM Reduced-Serum Medium.

Tube B: Dilute 5 μ L of Lipo3000 transfection reagent in 500 μ L of Opti-MEM. Mix gently and incubate for 5 minutes at room temperature.
- (3). Combine the contents of Tube A and Tube B. Mix gently and incubate the mixture for 20 minutes at room temperature to allow transfection complex formation.
- (4). Medium Change: Replace the cell culture medium with serum-free medium. Then, add the siRNA (or plasmid) complex mixture dropwise to each well of the 6-well plate.
- (5). After 6 hours of transfection, replace the serum-free medium with fresh complete medium containing serum.

CCK-8 Proliferation Assay

- (1). Twenty-four hours after transfection, wash the cells twice with PBS and digest them with trypsin.
- (2). Count the cells using a hemocytometer. Count the cells in at least five large squares, calculate the average, and multiply by 10^4 to obtain the cell density (cells/mL).

- (3). Seed 5,000 cells per well in a 96-well plate. For each group, prepare three replicate wells. Calculate the total volume of cell suspension needed for all replicates of a group, mix the suspension well by pipetting, and add 200 μ L to each well.
- (4). Place the 96-well plate in the incubator.
- (5). Perform the CCK-8 proliferation assay on Day 0, 2, 4, and 6.
- (6). Prepare the CCK-8 working solution by mixing the CCK-8 stock solution with culture medium at a 1:10 ratio.
- (7). For the assay, add 100 μ L of the CCK-8 working solution to each well.
- (8). Incubate the plate at 37°C for 1 hour.
- (9). Measure the absorbance (OD) at 450 nm using a microplate reader.
- (10). Plot a proliferation curve based on the obtained OD values.

Colony Formation Assay

- (1). Seed 1,000 tumor cells per well in a 12-well plate. Before seeding, ensure the cell suspension is thoroughly mixed by pipetting to guarantee a single-cell suspension without clumps.
- (2). Place the 12-well plate in the incubator and culture for two weeks to allow colony formation.
- (3). After two weeks, wash the colonies twice with PBS.
- (4). Fix the colonies with 4% paraformaldehyde for 10 minutes.
- (5). Stain the fixed colonies with crystal violet for 5 minutes.
- (6). Rinse the wells with PBS until the excess crystal violet is washed away and the background is clear.
- (7). Photograph the stained colonies and count them.

Transwell Invasion Assay

- (1). Place the Transwell chamber (with a Matrigel-coated membrane) into a 24-well plate. Add complete medium (containing serum or chemoattractants) to the lower chamber.
- (2). 48 hours post-transfection, digest the cells with trypsin. Neutralize the trypsin with complete medium, then centrifuge the cell suspension at 5,000 rpm for 5 minutes. Resuspend the cell pellet in serum-free medium and

perform a cell count.

(3). Aspirate a cell suspension containing 20,000 cells and carefully add it to the upper compartment of the Transwell chamber.

(4). Incubate the plate in a cell culture incubator for 48 hours. After incubation, gently wipe off the non-invading cells from the upper side of the membrane using a cotton swab. Then, place the chamber in 4% paraformaldehyde (PFA) to fix the invaded cells for 10 minutes, followed by staining with crystal violet for 5 minutes.

(5). Rinse the chamber with PBS to remove any excess crystal violet stain. Observe the membrane under a microscope, photograph the invaded cells on the lower surface, and count them.

RNA Extraction

(1). Wash the cells twice with PBS. Add 500 μ L of Trizol to each well and incubate at room temperature for 5 minutes for lysis. Then, transfer the Trizol lysate to a microcentrifuge tube.

(2). Add 100 μ L of chloroform to each tube and vortex vigorously. Let it stand for 3 minutes, then centrifuge at 12,000 rpm for 15 minutes.

(3). Carefully transfer the upper, clear aqueous phase to a new microcentrifuge tube. Add an equal volume (approximately 300 μ L) of isopropanol to the new tube. Mix well and place at -80°C for 30 minutes to precipitate the RNA.

(4). Thaw the tubes at room temperature, then centrifuge at 12,000 rpm for 10 minutes. A white RNA pellet should be visible at the bottom of the tube. Carefully discard the supernatant.

(5). Add 1 mL of 75% ethanol (prepared with nuclease-free water) to gently wash the pellet. Centrifuge at 12,000 rpm for 5 minutes.

(6). Repeat the previous washing step.

(7). Discard the supernatant. Centrifuge the tube at 12,000 rpm for 3 minutes to remove any residual ethanol.

(8). Air-dry the pellet at room temperature for 3–5 minutes. Then, dissolve the RNA in an appropriate volume of nuclease-free water (DEPC-treated, sterilized water).

(9). Measure the RNA concentration using a microvolume spectrophotometer. The A260/A280 ratio of the extracted RNA should be between 1.8 and 2.0.

Reverse Transcription

- (1) . First-Step Mixture Preparation: Pipette 1 µg of RNA and 4 µL of gDNA wiper Mix into a tube.
- (2) . Add nuclease-free water to bring the total volume to 16 µL. Mix gently and heat at 42°C for 2 minutes.
- (3) . Composition of the First-Step Mixture: Reagent Amount, RNA: 1 µg, DNA wiper Mix: 4 µL, Nuclease-free water: Up to 16 µL, Second-Step Reaction Setup: Add 4 µL of 5× HiScript II qRT SuperMix to the previous 16 µL mixture. Composition of the Second-Step Mixture: Reagent: Volume First-Step Mixture: 16 µL, 5× HiScript II qRT SuperMix: 4 µL.
- (4). Incubate the 20 µL reaction mixture at 37°C for 15 minutes, followed by 85°C for 5 seconds to obtain cDNA. Dilute the synthesized cDNA 5-fold and store at 4°C.

Western blotting

Protein Extraction:

- (1). After removing the cells from the incubator, wash them twice with PBS. Add the prepared protein lysis buffer to the culture dish, then scrape the cells off the surface and transfer the lysate to a microcentrifuge tube. Lyse the cells on ice for 30 minutes.
- (2). Pre-cool a centrifuge to 4°C. Centrifuge the tubes at 12,000 rpm for 15 minutes. Carefully collect the supernatant (containing the soluble protein) and discard the pellet (cell debris).

Standard Curve Preparation:

- (3). Prepare a series of dilutions from the standard protein solution. Pipette 2 µL of each diluted standard and 18 µL of nuclease-free water into separate wells of a 96-well plate. Then, add 200 µL of the freshly prepared BCA working reagent (prepared by mixing Reagent A and Reagent B at a 50:1 ratio) to each well. Incubate the plate at 37°C for 30 minutes.
- (4). Measure the absorbance (OD) at 562 nm using a microplate reader. Plot the standard curve using the known standard protein concentrations and their corresponding absorbance values.

Sample Preparation and Loading:

- (5). The protein samples were normalized to the same mass and volume. The required volume of each protein sample was calculated based on the total protein amount. Subsequently, a volume of 5× Loading Buffer equal to one-fifth of the final total volume was added to each sample. The mixtures were then denatured by heating at 95°C in a water bath for 10 minutes. The prestained protein marker and prepared protein samples were loaded into the wells of the gel from left to right.

SDS-PAGE Electrophoresis:

(6). Electrophoresis was initiated at a constant voltage of 90 V while the samples migrated through the stacking gel. After approximately 20 minutes, when the samples had entered the separating gel, the voltage was switched to 130 V and maintained for 60 minutes to achieve optimal separation.

Membrane Transfer:

(7). Following electrophoresis, the proteins were transferred onto a membrane using the semi-dry transfer method. Prior to assembly, the PVDF membrane was activated by briefly immersing it in methanol. The transfer stack was assembled in the following order (from top to bottom): filter paper, the gel, the activated PVDF membrane, and another piece of filter paper. Care was taken to remove all air bubbles between each layer. The transfer was then conducted for 25 minutes.

Blocking:

(8). Upon completion of the transfer, the PVDF membrane was incubated in a blocking solution of 5% skim milk for two hours at room temperature to prevent non-specific antibody binding.

Primary Antibody Incubation:

(9) . After blocking, the membrane was washed with TBST to remove excess milk. It was then incubated with primary antibodies diluted in the appropriate buffer as follows: WNK2 (1:500), POU5F1B (1:1000), AKT (1:1000), and GAPDH (1:2000). The incubation was carried out overnight at 4°C.

Washing:

(10) . The membrane was washed three times with TBST, for 10 minutes each wash, to remove unbound primary antibody.

Secondary Antibody Incubation:

(11) . The membrane was then incubated with an appropriate HRP-conjugated secondary antibody at room temperature for one hour.

Washing:

(12) . Following secondary antibody incubation, the membrane was washed three times with TBST, for 5 minutes per wash, to remove any unbound secondary antibody.

Detection:

(13). The immunoreactive protein bands were visualized using an Enhanced Chemiluminescence (ECL) substrate and detected by imaging with a chemiluminescence imaging system.

Immunohistochemical (IHC) Staining for Tissue Microarrays (TMAs)

- (1). Baking: Bake the paraffin-embedded tissue microarray (TMA) slides in a 60°C oven for 6 hours.
- (2). Deparaffinization and Rehydration: Immerse the TMA slides in xylene for 15 minutes, then transfer them to fresh xylene for another 15 minutes. Subsequently, immerse the slides sequentially in 100% ethanol, 95% ethanol, 85% ethanol, and 75% ethanol, for 3 minutes each. Rinse with TBST for 10 minutes.
- (3). Blocking of Endogenous Peroxidases: Apply 3% H₂O₂ onto the tissue sections and incubate for 10 minutes to block endogenous peroxidase activity. Rinse with TBST for 10 minutes.
- (4). Antigen Retrieval: Place the TMA slides in a glass coplin jar filled with antigen retrieval buffer. Perform heat-induced epitope retrieval (HIER) by boiling the jar in a pressure cooker for 20 minutes. Then, allow the jar to cool down to room temperature naturally. Rinse the slides with TBST for 10 minutes.
- (5). Primary Antibody Incubation: Apply the diluted WNK2 primary antibody (1:50 dilution) onto the tissue sections and incubate overnight at 4°C.
- (6). Washing: Wash the TMA slides three times with TBST, for 10 minutes each wash, to remove unbound primary antibody.
- (7). Secondary Antibody Incubation: Incubate the slides with a rabbit secondary antibody at room temperature for 1 hour. Then, wash with TBST for 10 minutes.
- (8). Detection and Counterstaining: Develop the signal using DAB substrate for 30 seconds. Counterstain the nuclei with hematoxylin for 10 seconds. Rinse the slides with TBST for 10 minutes.
- (9). Dehydration and Clearing: Dehydrate the TMA slides by sequentially immersing them in 75% ethanol, 85% ethanol, 95% ethanol, and 100% ethanol, for 3 minutes each. This is followed by a final clearing step in xylene for 15 minutes.
- (10). Mounting: Mount the slides with neutral balsam mounting medium, taking care to avoid air bubbles.

Nude mice model establishment

- (1) . The twenty female nude mice were randomly divided into four groups (n=5 per group): shNC, shWNK2, shWNK2 + Lv-NC, and shWNK2 + Lv-POU5F1B.
- (2) .A 100 µL cell suspension containing approximately 1×10^7 cells was subcutaneously injected into the right flank of each mouse.
- (3) .Tumor formation was observed on day 12 post-injection. Tumor size was measured daily thereafter.



(4) .After seven days, when the tumors had grown to approximately 1.5 cm in diameter, the mice were euthanized by cervical dislocation.

(5) .The tumors were subsequently excised and photographed. Finally, the tumor tissues were fixed, paraffin-embedded, and sectioned by specialized staff from the Department of Breast Surgery at Southwest Hospital.