Experiment time: 2025-06-25, 18:00 - 2025-06-28, 21:00

Transwell Invasion Assay (II)

I. Objective of the experiment

In this experiment, transwell invasion was used to compare the invasive capabilities of the T24 wild-type cell line with its low-, medium-, and high-drug-resistant T24-RC48 sublines, and to explore the relationship between drug resistance and cell invasion behavior.

II. Experimental content

2.1 Experimental design

Sample type: T24 wild-type cell line and its low-, medium-, and high-drug-resistant T24-RC48 sublines.

Cell density: 50,000 cells per well.

Repeat times: 3 duplicate holes per group.

Matrix gel: Transwell chambers were pre-coated with Matrigel matrix gel (diluted 1:8)

2.2 Measurement principle

The Transwell invasion assay enhances the migration experiment by incorporating a Matrigel barrier. In this setup, the upper chamber of the Transwell microplate is pre-coated with Matrigel to simulate the extracellular matrix environment. Cells must secrete proteases to degrade the gel before migrating through the permeable membrane into the lower chamber. The invasion capability of cells is assessed by quantifying the number that successfully penetrate the gel barrier.

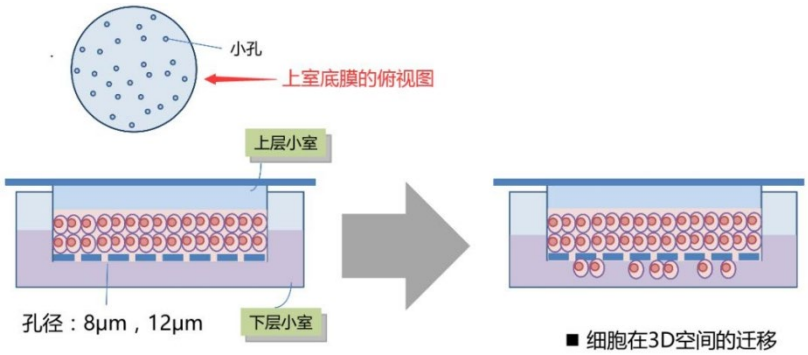

Figure 1 Transwell chamber principle

III. Materials and reagents

3.1 Materials

T25 cell culture bottle

Pipette tips and 15ml centrifuge tubes were purchased from Axygen

Serological pipettes were purchased from a domestic company

Micropipettes: Eppendorf

Transwell chamber (8μm aperture)

Low-, medium- and high-drug-resistant T24-RC48 cell lines

3.2 Reagents

PBS, trypsin and fetal bovine serum were purchased from Gibco, USA

Matrigel matrix gel was purchased from Corning, part number 354234

McCoy's 5A medium purchased from Shanghai Yuanpei Biotechnology Co., Ltd., China

4% polyformaldehyde

Crystal violet

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

Inverted phase contrast microscope

V. Experimental steps

5.1 The T24 wild-type and the low-, medium-, and high-resistant T24-RC48 cell lines were seeded into T25 cell culture flasks, respectively.

5.2 After 24 hours, the complete medium of each flask was replaced with McCoy's 5A medium containing 1% FBS for starvation treatment for 24 hours.

5.3 Under condition 4℃ (on ice), Matrigel was diluted with serum-free McCoy's 5A cell culture medium at 1:8.

5.4 Take 60μl and add it uniformly to the upper surface of the Transwell chamber. Incubate in the 37℃ incubator for 3h to allow the Matrigel to polymerize and form a gel layer.

5.5 Following polymerization, the excess liquid was carefully aspirated from the upper chamber,

and 100μl of low serum medium was added to each chamber. The plate was then returned to the incubator for 30 minutes to carry out substrate membrane hydration.

Tips:

(1) The gun head is gently pushed along the wall of the chamber to push out Matrigel without bubbles, and avoid poking the filter membrane of the chamber.

(2) The volume of the added Matrigel glue should not be too large, and the polycarbonate film can be soaked.

(3) Matrigel is easy to solidify at too high or too low temperature, so the gun head and other equipment required for operation should be pre-cooled at 4℃.

(4) Ensure that the liquid surface is level when laying the glue, and the thickness of the glue is uniform. Do not produce bubbles.

5.6 Add 20% serum McCoy's 5A medium 600μl/cell, and then place the Transwell chamber into the 24-well plate with forceps.

5.7 Cells digested with trypsin were prepared into a single cell suspension and the cell concentration was adjusted to 5×10^5 cells/ml.

5.8 Take 100μl cell suspension and add it to the upper chamber of Transwell chamber, with three replicates per group, and continue to culture for 48h.

5.9 Add 800 μl PBS to the blank culture well, gently place the chamber into which the medium has been sucked out, and gently washed (care was taken throughout all washing steps to avoid scraping or dislodging the migrated cells on the lower surface of the membrane).

5.10 Gently wipe off the inner layer of cells in the chamber: use a cotton swab with a slightly loosened tip to ensure thorough cleaning, including the edges. After wiping, rinse the inner wall with PBS.

5.11 Transfer the chamber to a new well containing 800μL of 4% paraformaldehyde (PFA) per well and fix at room temperature for 15 min; then wash twice with PBS, 2 min each time (all washes were performed by gentle immersion without shaking or swishing).

5.12 Transfer the chamber to a blank culture hole with 600ul/ hole of crystal violet dye, and stain

for 10min; after staining, wash off the floating color with clean water twice, each time for 2min.

5.13 Place the stained chamber on a clean glass slide, and pay attention to not completely dry it. Slightly moistening will make the cell morphology better; capture images following a predefined pattern: first along the crosshairs, and then at each of the four corners.

5.14 Use the image analysis software Image J to measure and calculate the number of migrating cells.

VI. Experimental results

In this experiment, after adjusting the cell density (50,000 cells per chamber), the distribution of cells on the lower surface of the Transwell chambers showed significant improvement. Crystalline violet staining revealed dense clusters of migrating cells in the lower chambers of both T24 cell lines and low-, medium-, and high-drug-resistant T24-RC48 cell lines (Figure 2), which was notably higher than the initial experiment's result (14. Cell Invasion (I)).

Crystal violet staining revealed homogeneous dispersion of migrating cells with distinct boundaries and no significant overlap (Figure 2). Quantitative analysis using ImageJ showed that the T24 wild-type group exhibited 721.0±74.39 cells per field, followed by low-drug resistance T24-RC48 cells (808.7±98.99), moderate-drug resistance T24-RC48 cells (1075±141.6), and high-drug resistance T24-RC48 cells (1304±115.0) (Figure 3). Statistical analysis demonstrated no significant difference between wild-type and low-drug resistance groups ($P=0.4558$), while wild-type showed highly significant differences compared to both moderate-drug resistance ($P<0.0001$) and high-drug resistance groups ($P<0.0001$).

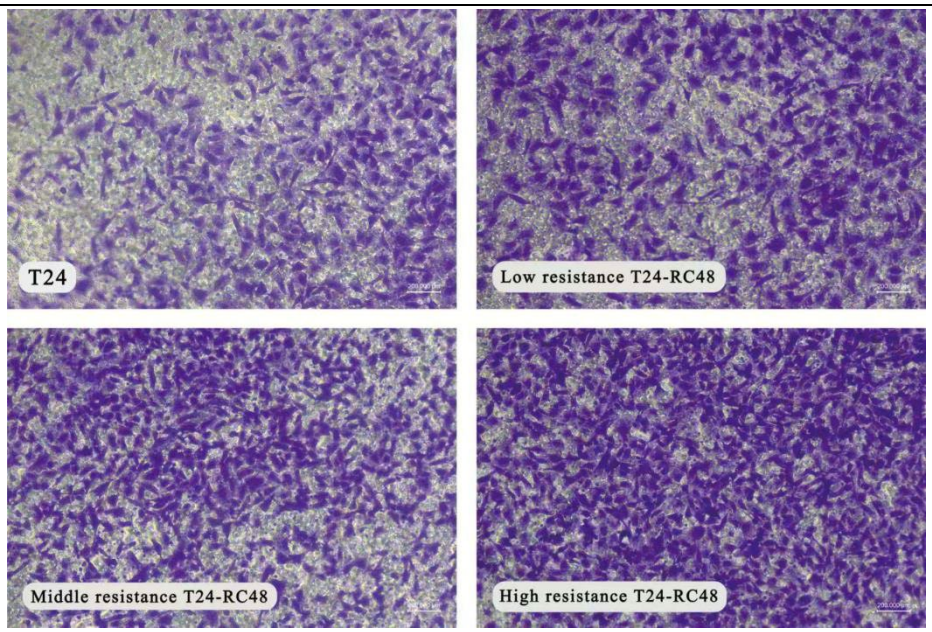

Figure 2 Crystal violet staining of invaded cells in Transwell chambers (observed under an inverted phase-contrast microscope)

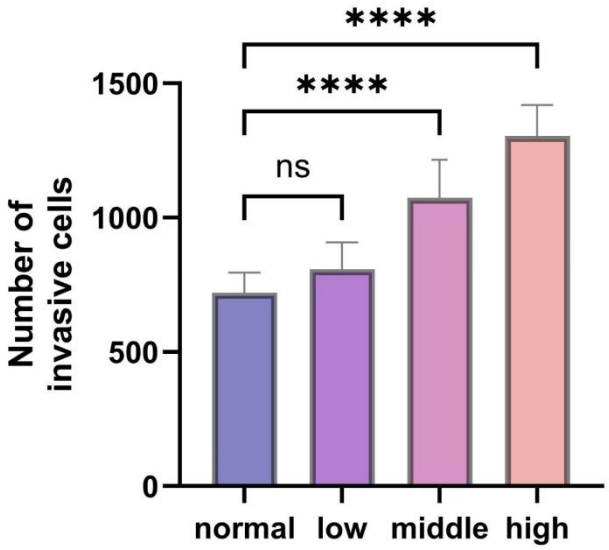

Figure 3 Quantitative analysis of cell invasion in wild-type and drug-resistant T24-RC48 cell lines

VII. Results analysis

Experimental results demonstrated that the invasive capacity of T24-RC48 drug-resistant cell lines showed a significant upward trend with increasing drug resistance, with the high-drug resistance group exhibiting the highest number of invasive cells, indicating a positive correlation between drug resistance and cellular invasiveness. Specific data revealed that wild-type T24 cells migrated 721.0±

74.39 cells per field, while the low-, medium-, and high-drug resistance T24-RC48 groups migrated 808.7±98.99,1075±141.6, and 1304±115.0 cells per field respectively (Figure 3). Statistical analysis showed significant differences between wild-type and medium/high-drug resistance groups (medium/high resistance group $P < 0.0001$), suggesting that enhanced drug resistance may promote cellular invasion through specific molecular mechanisms. Compared to the initial experiment (14. Cell Invasion (I)), this study significantly improved experimental sensitivity and data reliability through the following improvements:

(1) Increase the inoculation amount per well from 10,000 to 50,000 cells to increase the cell base penetrating the matrix gel and avoid false negative results caused by insufficient cell number;

(2) The serum concentration in the lower chamber was increased to 20%, forming a more significant chemotactic gradient, which effectively drove cell migration;

(3) The new Transwell chamber is used to reduce background interference and make the observation of cells after staining more clear.

Further analysis suggests that enhanced drug resistance may be associated with cellular metabolic reprogramming or increased protease secretion capacity, thereby improving the degradation of matrix gel and migration efficiency. The invasive ability of moderately drug-resistant groups was already significantly higher than that of wild-type cells, while the high-drug-resistance group showed further progression in invasive capability, indicating that drug resistance development may activate pro-invasive signaling pathways. Subsequent studies could combine protease activity detection to clarify the molecular correlation between drug resistance and matrix degradation capacity. This experiment provides a new perspective on understanding the mechanism of ADC drug resistance in bladder cancer, demonstrating that drug resistance may accelerate tumor metastasis by enhancing cellular invasiveness. These findings hold potential implications for optimizing clinical intervention strategies.