Experiment time: 2025-07-20, 18:00 - 2025-07-25, 21:30

Angiogenesis Assay (I)

I. Objective of the experiment

In order to evaluate the influence of VEGFA changes in drug-resistant cell lines on the microenvironment, a tube formation assay was performed by co-culturing T24 and T24-RC48 cell lines with Human Umbilical Vein Endothelial Cells (HUVEC).

II. Experimental content

2.1 Experimental design

To investigate the mechanisms of drug-resistant T24 cells in microenvironment regulation, we co-cultured the wild-type T24 cell line and the drug-resistant T24-RC48 cell line with human vascular endothelial cells (HUVEC) for tube formation experiments. By monitoring the growth patterns and interactions of all cell lines in the co-culture system, we analyzed how the altered VEGFA expression levels in the drug-resistant T24-RC48 cell line compared to the normal T24 cell line affected the surrounding microenvironment. This approach aimed to determine whether changes in VEGFA expression in drug-resistant cells significantly impact angiogenesis-related processes and the composition/function of the entire cellular microenvironment.

2.2 Sample types

T24 wild-type cell lines

Highly drug-resistant T24-RC48 cell line.

2.3 Experimental principle

Under the stimulation of angiogenic factors, endothelial cells can self-assemble into tubular networks. When tumor cells (T24 and drug-resistant strain T24-RC48) are co-cultured with human vascular endothelial cells (HUVEC), VEGFAs (vascular endothelial growth factor A) secreted by the tumor cells exert paracrine effects on adjacent HUVEC cells. As a key angiogenic signal, VEGFA activates endothelial cell proliferation, migration, and lumen-forming capabilities, driving their connection to form three-dimensional tubular structures. By comparing the differences in tube formation induced by drug-resistant strains versus wild-type strains, this experiment directly reflects the regulatory efficacy of VEGFA expression changes in the angiogenic microenvironment, revealing how drug resistance reshapes the tumor microenvironment through altered secretion of pro-angiogenic factors.

International Directed Evolution Competition Lab Notebook

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III. Materials and reagents

3.1 Materials

The 1000μl and 100μl pipette tips and 15ml centrifuge tubes were purchased from Axygen

24-well plates: Corning

Micropipettes: Eppendorf

T25 culture flasks were purchased from Corning

Human bladder transitional cell carcinoma cell line (T24)

Low/medium/high drug-resistant T24-RC48 cell lines

3.2 Reagents

PBS and trypsin were purchased from Gibco, USA

McCoy's 5A medium, DMEM medium (Shanghai Yuanpei Biotechnology Co., Ltd., China)

Fetal bovine serum (FBS, Gibco)

IV. Experimental instruments

Benchtop room temperature low speed centrifuge (Eppendorf)

Ultra-clean workbench (Suzhou purification equipment factory)

37℃,5% CO_2 cell culture chamber (Thermo Fisher Scientific)

Cell counter

V. Experimental steps

5.1 McCoy's5A medium was mixed with DMEM medium at a ratio of 1:1 and 10% FBS was added to make a complete medium.

5.2 Trypsin was used to digest T24, T24-RC48 and HUVEC cells in the logarithmic growth phase respectively.

5.3 Resuspend the cells in complete medium and count the cells using a hemocytometer and adjust the cell concentration to 2×10^5 cells/ml.

5.4 According to Table 1 24 orifice plate layout, T24 cells, T24-RC48 cells and HUVEC cells were mixed in different proportions and added into the 24 orifice plate respectively, with a total

volume of 500μl per hole. The specific proportions were:

1) T24 and HUVEC were mixed at 1:1: 250 μl T24 cell suspension and 250 μl HUVEC cell suspension were added to each well.

2) T24 and HUVEC were mixed at 1:4: 100μl T24 cell suspension and 400μl HUVEC cell suspension were added to each well.

3) T24 and HUVEC were mixed in a ratio of 4:1: 400μl T24 cell suspension and 100μl HUVEC cell suspension were added to each well.

4) T24-RC48 and HUVEC were mixed in a ratio of 1:1, 1:4 and 4:1 as above.

Table 1 Layout of the 24-well Plate (WT: T24; HR: Highly Resistant T24-RC48)

WT: HUVEC= 1: 1	WT: HUVEC= 1: 1	WT: HUVEC= 1: 1	HR: HUVEC= 1: 1	HR: HUVEC= 1: 1	HR: HUVEC= 1: 1
WT: HUVEC= 1:4	WT: HUVEC= 1:4	WT: HUVEC= 1:4	HR: HUVEC= 1: 4	HR: HUVEC= 1: 4	HR: HUVEC= 1: 4
WT: HUVEC= 4:1	WT: HUVEC= 4:1	WT: HUVEC=4:1	HR: HUVEC= 4: 1	HR: HUVEC= 4: 1	HR: HUVEC= 4: 1
blank space	blank space	blank space	blank space	blank space	blank space

Control group setting: A blank control group was set up, that is, the 24 well plate holes without any cells were added with the corresponding volume of culture medium to evaluate the background effect.

5.5 Place the 24-well plate in a 37℃,5% CO_2 incubator for cultivation. After 48 hours, remove the plate from the incubator and observe the cell growth status and tube formation in each well using an inverted microscope. Take photos to document the observations and analyze them using ImageJ software with the angiogenesis analysis plugin.

VI. Experimental results

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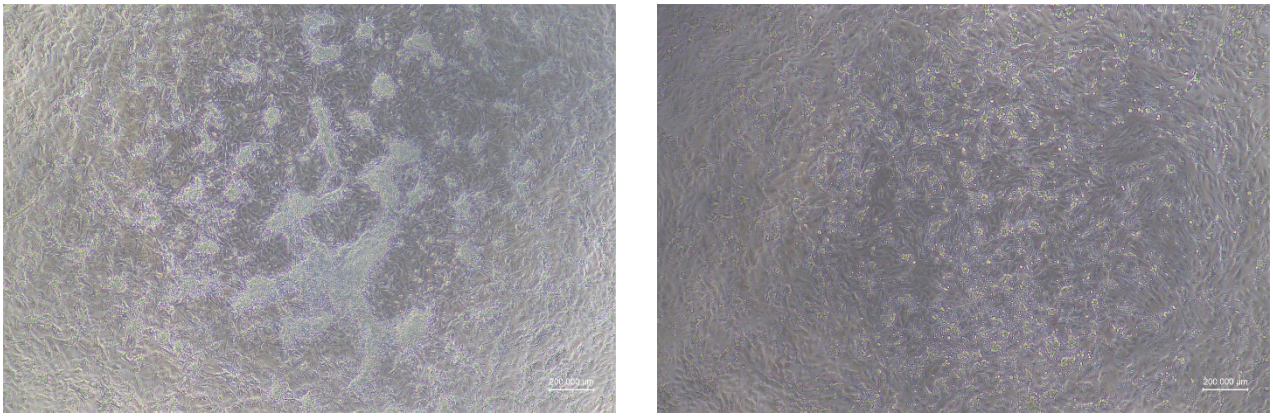

Figure 1 Phase-contrast micrographs of (Left) T24-RC48:HUVEC=1:1 and (Right) T24:HUVEC=1:1 co-cultures after 48 hours

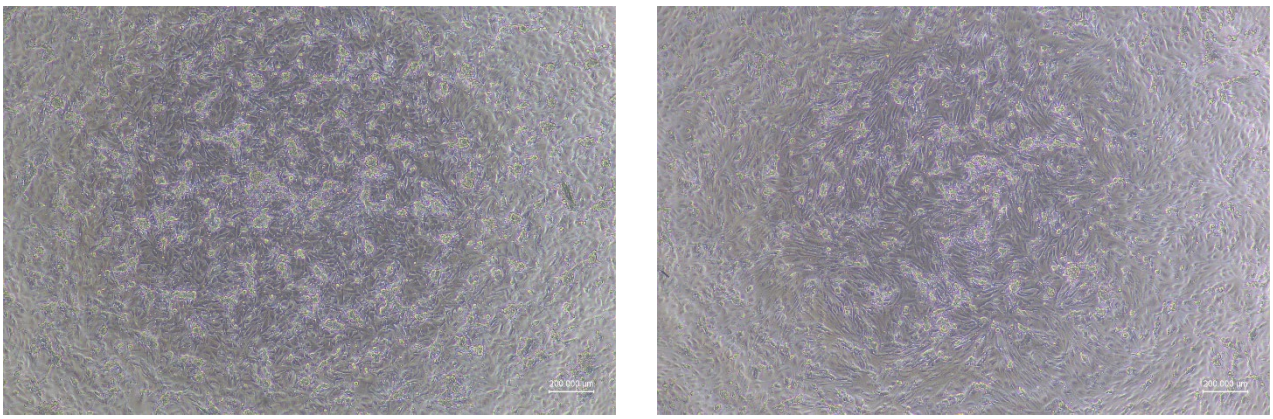

Figure 2 Phase-contrast micrographs of (Left) T24-RC48: HUVEC=1:4 and (Right) T24:HUVEC=1:4 co-cultures after 48 hours

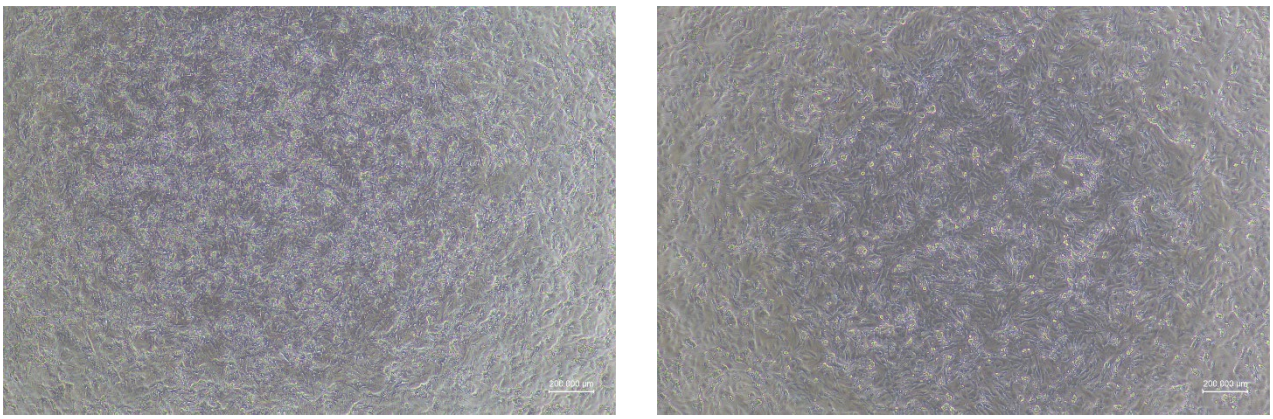

Figure 3 Phase-contrast micrographs of (Left) T24-RC48:HUVEC=4:1 and (Right) T24:HUVEC=4:1 co-cultures after 48 hours

After 48-hour cultivation in all experimental groups (comprising co-culture wells with 1:1, 1:4, and 4:1 ratios of T24/HUVEC cells and T24-RC48/HUVEC cells) along with the blank control group, microscopic observation revealed that all cells within the wells reached full confluence, forming a dense monolayer without observable tubular network structures. The blank control group

showed no cell growth, containing only culture medium in the wells with no background interference or contamination.

The morphological characteristics of HUVEC induced by the wild-type cell group (T24) and the T24-RC48 drug-resistant cell group (T24-RC48) under identical cell ratios (1:1, 1:4, 4:1) showed no significant differences. No significant differences in the effects on tube formation were observed among the different cell seeding ratios (1:1, 1:4, 4:1).

VII. Results analysis

The potential reasons for the failed tube formation in this experiment include: 1) Excessive initial cell density: The total inoculation volume of 1×10^5 cells per well caused excessive proliferation and rapid cell fusion within 48 hours, which inhibited the formation space for the lumen structure. 2) The observation period was limited to 48 hours, potentially missing the critical window for tube formation.

The limitations of this experimental design are as follows: 1) The study exclusively utilized highly drug-resistant T24-RC48 cell lines, lacking comparison with low-and moderate-drug-resistant strains. This limitation prevents evaluation of the gradient relationship between drug resistance levels and VEGFA secretion. 2) Specific staining methods (e.g., VE-cadherin immunofluorescence staining) were not employed, relying solely on light microscopy morphology observation. This approach makes it difficult to identify early-stage/precursor tubular structures.

The following experimental improvements include: 1) Reducing cell seeding density: Limiting total cells per well to 5×10^4 to delay fusion. 2) Adding multi-timed-point analysis: Conducting observations at 6h,12h,24h, and 48h intervals to capture dynamic tube formation. 3) Expanding the experimental group: Incorporating low-and moderate-drug-resistant T24-RC48 cell lines for systematic comparison of drug resistance effects on angiogenesis. 4) Introducing staining techniques to enhance visualization sensitivity of lumen structures.