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

Angiogenesis Assay (II)

I. Objective of the experiment

To evaluate the impact of VEGFA changes in drug-resistant cell lines on the microenvironment by performing a tube formation assay using Human Umbilical Vein Endothelial Cells (HUVEC) cultured with conditioned media from untreated T24 wild-type cells, low/medium/high drug-resistant T24-RC48 cells, and corresponding drug-treated T24 wild-type and T24-RC48 cells.

II. Experimental content

2.1 Experimental design

Conditioned media from untreated T24 wild-type cells, low/medium/high drug-resistant T24-RC48 cells, and corresponding drug-treated T24 wild-type and T24-RC48 cells were used to culture HUVECs for the tube formation assay. By observing and recording the growth status and interactions of the various cell lines in the co-culture system for both untreated and drug-treated groups, we analyzed whether the drug inhibited angiogenesis in the drug-resistant T24-RC48 cells in the treated group, and the differential impact of changes in VEGFs expression in the drug-resistant T24-RC48 cells compared to the ordinary T24 cells on the surrounding microenvironment between the two groups. This investigation aimed to determine whether VEGF changes in drug-resistant cells significantly affect angiogenesis-related processes and the composition and function of the entire cellular microenvironment, thereby providing insights into the mechanisms of microenvironment regulation by drug-resistant cells.

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.

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)

Human Umbilical Vein Endothelial Cells (HUVECs), Passage 3

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)

Matrigel (Corning Matrigel Matrix, Growth Factor Reduced, GFR)

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 One day in advance, transfer Matrigel from -20°C to a 4°C refrigerator for overnight slow thawing. All subsequent operations are performed in the ultra-clean workbench using pre-cooled pipette tips and centrifuge tubes.

5.2 Twenty-four hours in advance, take two flasks each of T24 wild-type cells and low/medium/high drug-resistant T24-RC48 cells at 70% confluence. For each cell type, replace the medium in one flask with serum-free medium, and in the other flask with serum-free medium containing 300μg/ml Ivonescimab. After 24 hours of culture, collect the supernatants to obtain a total of 8 conditioned media samples.

5.3 Twelve hours in advance, starve HUVEC cells for 12 hours using DMEM medium with 1% FBS.

5.4 Mix 4.8ml Matrigel with 2.4ml DMEM medium at a 2:1 ratio. Using pre-cooled pipette tips, slowly add 300μl of the mixture to each of the 24 wells along the wall, avoiding bubbles. Transfer the 24-well plate to a 37°C incubator and incubate for 30 minutes to allow the Matrigel to polymerize and solidify completely.

5.5 Take HUVECs in the logarithmic growth phase, discard the old medium, and wash once with PBS. Add trypsin to digest the cells. Once the cells become rounded, add complete medium to stop digestion. Gently pipette to form a single-cell suspension and distribute equally into 8 new centrifuge tubes. Centrifuge at 1000 rpm for 5 minutes and discard the supernatant.

5.6 Resuspend the cell pellets from the 8 tubes in the 8 different conditioned media respectively, pipetting to mix thoroughly. Count the cells and adjust the density to 3×10^5 cells/ml.

5.7 After 30 minutes, the Matrigel should be fully polymerized and solidified. According to the planned layout (see Table 1 below), add 0.5 ml of cell suspension to each well of the 24-well plate sequentially. Each conditioned media group has 3 replicate wells.

Table 1 Layout of the 24-well Plate

WT Treated	WT Treated	WT Treated	WT Untreated	WT Untreated	WT Untreated
LowR Treated	LowR Treated	LowR Treated	LowR Untreated	LowR Untreated	LowR Untreated
MedR Treated	MedR Treated	MedR Treated	MedR Untreated	MedR Untreated	MedR Untreated

HighR Treated	HighR Treated	HighR Treated	HighR Untreated	HighR Untreated	HighR Untreated

*WT: Wild Type T24; LowR: Low-resistant T24-RC48; MedR: Medium-resistant T24-RC48; HighR: High-resistant T24-RC48

5.8 Observe tube formation under an inverted phase-contrast microscope at 2h, 4h, and 12h after plating. Analyze the tube formation images using ImageJ software.

VI. Experimental results

Observation at 2 hours post-plating showed that HUVECs began to migrate and initially form tubular network structures in all groups, with no significant differences observed between groups (Figure 1 a-h). Observation at 4 hours post-plating revealed further extension and maturation of the tubular structures. Under drug-free conditions, conditioned media from medium and high drug-resistant T24-RC48 cells promoted more significant tube formation compared to the parental T24 cells (Figure 1 i-p). Observation at 12 hours post-plating showed that the tubular networks tended to mature and stabilize. The pro-angiogenic effect was most pronounced in the high drug-resistant T24-RC48 conditioned media group (Figure 1 q-x).

T-test analysis of the number of junctions in groups with the same conditioned media at each time point revealed that only the high-resistant group showed a significant reduction in the number of junctions when treated with Ivonescimab compared to untreated controls ($P < 0.05$) at the 2h, 4h, and 12h time points (Figure 1 y). One-way ANOVA comparing the number of junctions in untreated drug-resistant groups versus the wild-type group at each time point showed that at 4 hours, both the medium and high-resistant groups had a significantly increased number of junctions compared to the wild-type group ($P < 0.05$); at 12 hours, the high-resistant group showed a highly significant increase in the number of junctions compared to the wild-type group ($P < 0.01$) (Figure 1 z).

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

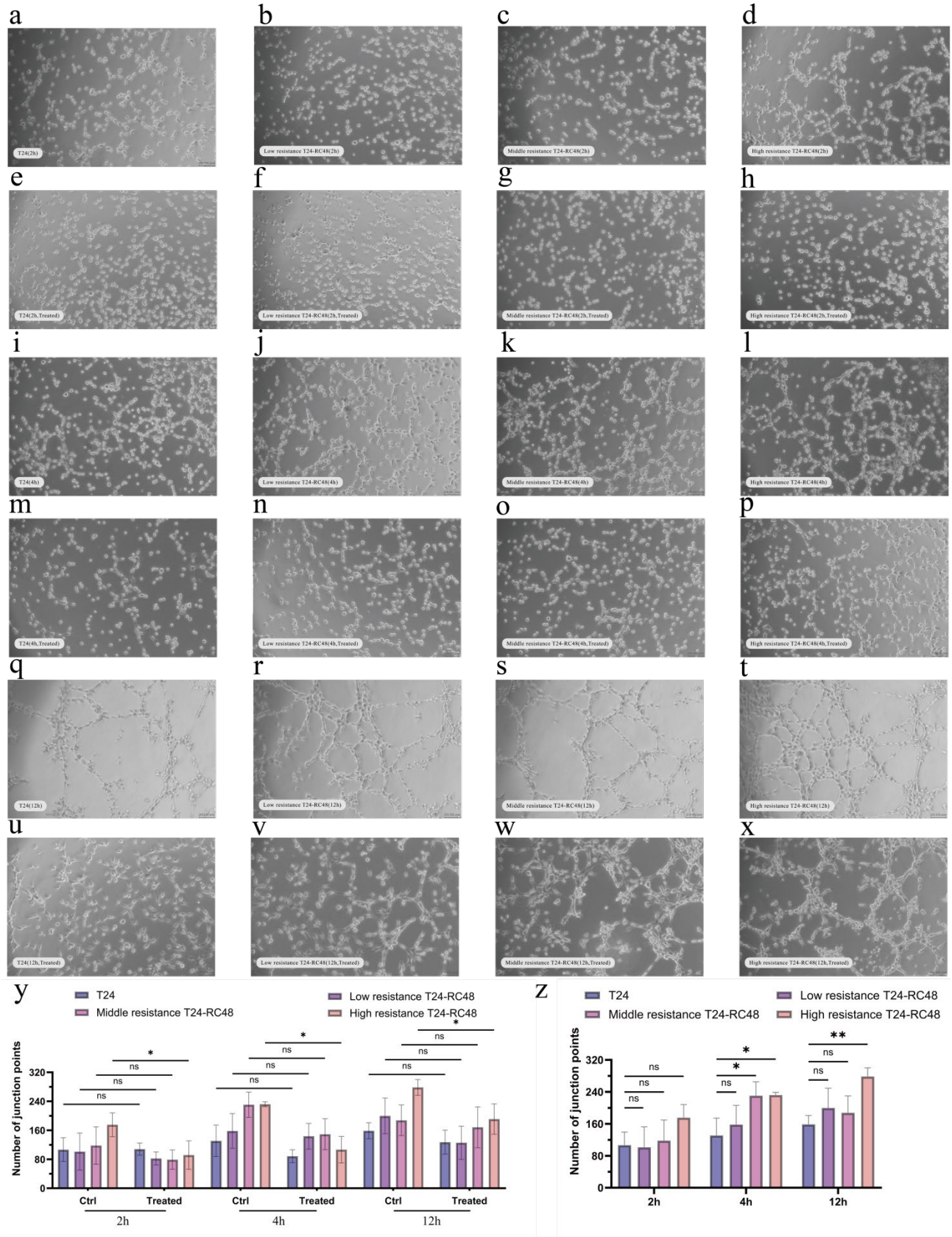

Figure 1. Effect of conditioned media on HUVEC tube formation ability and the inhibitory effect of Ivonescimab.

Figure legend: a-d, Representative images of HUVEC tube formation at 2 hours under untreated conditions.

HUVECs were co-cultured with conditioned media from (a) parental T24, (b) low-resistant T24-RC48, (c) medium-resistant T24-RC48, and (d) high-resistant T24-RC48. e-h, Representative images after 2 h treatment of HUVECs with Ivonescimab (300μg/mL). HUVECs were co-cultured with conditioned media from (e) parental T24, (f) low-resistant T24-RC48, (g) medium-resistant T24-RC48, and (h) high-resistant T24-RC48. i-l, Representative images of HUVEC tube formation at 4 hours under drug-free conditions. HUVECs were co-cultured with conditioned media from parental T24, low-resistant T24-RC48, medium-resistant T24-RC48, and high-resistant T24-RC48. m-p, Representative images after 4 h treatment of HUVECs with Ivonescimab (300μg/mL). HUVECs were co-cultured with conditioned media from (m) parental T24, (n) low-resistant T24-RC48, (o) medium-resistant T24-RC48, and (p) high-resistant T24-RC48. q-t, Representative images of HUVEC tube formation at 12 h under drug-free conditions. HUVECs were co-cultured with conditioned media from parental T24, low-resistant T24-RC48, medium-resistant T24-RC48, and high-resistant T24-RC48. u-x, Representative images of HUVEC tube formation after 12 h treatment with Ivonescimab (300μg/mL). HUVECs were co-cultured with conditioned media from: (u) parental T24, (v) low-resistant T24-RC48, (w) medium-resistant T24-RC48, (x) high-resistant T24-RC48. y, Quantitative analysis of the number of junctions in HUVEC networks with or without Ivonescimab treatment. z, Quantitative analysis of the number of junctions in HUVECs cultured with conditioned media from different cell lines under drug-free conditions over time (2h, 4h, 12h). Data are presented as mean ± standard deviation (n=3). (ns, not significant, *P < 0.05, **P < 0.01)

VII. Results analysis

This experiment systematically evaluated the effects of conditioned media from T24 wild-type and T24-RC48 cells with varying degrees of drug resistance on the tube formation ability of HUVECs through an in vitro angiogenesis assay, and further investigated the inhibitory effect of Ivonescimab on angiogenesis.

Microscopic observation showed that in the early stage of co-culture (2 hours), HUVECs in all groups began to migrate and initially form tubular structures, with no significant differences between groups, suggesting a consistent initial response of endothelial cells. As time progressed to 4 hours and 12 hours, conditioned media from medium and high drug-resistant T24-RC48 cells significantly enhanced the tube formation ability of HUVECs, manifested as more complex and

denser tubular network structures, with the most pronounced effect in the high-resistant group.

Statistical analysis further confirmed the above observations: under drug-free conditions, the number of junctions in the medium and high-resistant groups was significantly higher than in the wild-type group at 4 hours ($P < 0.05$), while the number of junctions in the high-resistant group was highly significantly higher than in the wild-type group at 12 hours ($P < 0.01$). This indicates that drug-resistant cell lines significantly enhance their ability to induce angiogenesis by secreting higher levels of pro-angiogenic factors such as VEGFA.

Furthermore, experimental results from the Ivonescimab-treated group showed that this drug effectively inhibited angiogenesis induced by high-resistant T24-RC48 cells, manifested as a significant reduction in the number of junctions ($P < 0.05$), while the inhibitory effect was not significant in other groups. This indicates that Ivonescimab has a specific inhibitory effect on angiogenesis under conditions of high VEGF expression, further confirming the role of VEGF in the regulation by drug-resistant cells.