

Endothelial Cells Promote Docetaxel Resistance of Prostate Cancer via FGF2/ERG/Akt/mTOR Signaling Pathway

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Research

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Abstract

Background

Docetaxel is a first-line chemotherapy for the treatment of patients with castration-resistant prostate cancer (CRPC). Despite the good initial response of docetaxel, drug resistance will inevitably occur. Mechanisms underlying docetaxel resistance are not well elaborated. Endothelial cells (ECs) have been implicated in the progression and metastasis of prostate cancer (PCa). However, little attention has been paid to the role of ECs in the development of docetaxel resistance in PCa.

Methods

Here, we sought to investigate the function and mechanism of ECs involving in the docetaxel resistance of PCa. The 22Rv1 and C4-2B PCa cell lines were cultured with or without human umbilical vein endothelial cells (HUVEC). The proliferation of each PCa cell line was assessed by CCK8 and EdU assays. Cell viability of each PCa cell line treated with docetaxel was evaluated by CCK8. Apoptosis was measured by flow cytometry. Quantitative reverse transcription (RT)-PCR assay was used to determine the expression of ETS related gene (ERG) in each PCa cell line and FGF2 in HUVEC. The proteins including ERG, Caspase3, PARP, Akt, p-Akt, mTOR and p-mTOR were quantified by western blotting. ERG overexpressing C4-2B cells(C4-2B-ERG) were constructed by transfection with pLenti6.3-ERG lentivirus. C4-2B-ERG cells were knocked down by transfecting with ERG siRNAs. Differentially expressed cytokines between the serum-free media from 22Rv1 and 22Rv1/HUVEC co-culture system were detected by human cytokine array and determined by ELISA assay. Tumors were induced in mice by injecting 22Rv1 cells with or without HUVEC and treated with docetaxel. Tumor growth and apoptosis were examined by immunohistochemistry and TUNEL respectively.

Results

ECs promoted proliferation and inhibited apoptosis in PCa cells (in vitro) and mouse xenograft tumors induced by these cells (in vivo) under docetaxel treatment. ECs secreted FGF2 to induce ERG expression and activate the Akt/mTOR signaling pathway in PCa cells contributing to docetaxel resistance. Blocking FGF2 could reverse the enhancing effects of HUVEC on docetaxel resistance in PCa cells. Inhibition of the Akt/mTOR signaling pathway could alleviate chemoresistance mediated by ERG.

Conclusion

ECs promote docetaxel resistance via FGF2/ERG/Akt/mTOR signaling pathway in PCa cells. Targeting FGF/ FGFR signaling may represent a promising therapeutic strategy to overcome docetaxel resistance.

1. Introduction

Prostate cancer is one of the most common malignant tumors in the male genitourinary system and its mortality rate ranks second in male malignancies(1). For advanced prostate cancer, androgen deprivation therapy (ADT) is the standard of care. However, after 12 to 18 months of ADT, most patients become insensitive to it and gradually develop into castration-resistant prostate cancer (CRPC)(2). In 2004, the landmark study TAX327 demonstrated a significant survival benefit for docetaxel over mitoxantrone, which was the first study to lay the foundation for chemotherapy in CRPC patients. Docetaxel treatment achieved PSA decline, prolonged overall survival (OS), and improved quality of life(3). Recently, STAMPEDE and CHAARTED trials have confirmed that docetaxel combined with ADT as the front-line therapy for metastatic hormone-sensitive prostate cancer (mHSPC) accomplished a dramatic survival advantage over ADT alone(4, 5). These encouraging results further strengthen the position and application of docetaxel in advanced prostate cancer. However, chemoresistance remains a significant obstacle to docetaxel treatment, which significantly decreases its clinical efficacy (6). Mechanisms of docetaxel resistance are not completely understood. Known mechanisms of resistance to docetaxel include increased expression of multidrug resistance (MDR) genes, tubulin alterations, deregulation of growth factors, and intracellular signaling pathways activation, tumor microenvironment, etc(7-11). Identification of specific mechanisms that modulate resistance to docetaxel will facilitate the development of novel therapies and improve responses to currently available therapies.

Swarnali Acharyya *et al*/first described that endothelial cells were involved in the development of chemoresistance of breast cancer via secretion of TNF- α (12). Increasing evidence has demonstrated that endothelial cells contribute to chemoresistance of cancer cells by secreting soluble factors in a paracrine fashion in lymphoma(13-15), glioblastoma(16, 17), colorectal cancer(18, 19), and other cancer types(20-23). Results from these studies showed that secreted factors including exosomes from endothelial cells can activate "cancer-advancing" signaling pathways in cancer cells such as AKT, Wnt, NOTCH, and epithelial-mesenchymal transition pathways in favor of survival under chemotherapy. Our previous studies manifested that endothelial cells promoted the metastasis of prostate cancer by enhancing autophagy and increasing IL-6 secretion(24, 25), which indicated that endothelial cells are associated with prostate cancer progression. However, whether endothelial cells play a role in the development of docetaxel resistance in prostate cancer remains largely elusive.

Therefore, we set out to assess the effect of endothelial cells on the evolution of chemoresistance in prostate cancer. Herein, we demonstrated that endothelial cells significantly promoted prostate cancer cell proliferation and chemoresistance via constructing a co-culture system in vitro. Mechanistically, we showed that endothelial cells-derived FGF2 mediated ERG expression and Akt/mTOR activation in prostate cancer cells. Furthermore, we utilized a subcutaneous xenograft tumor model to validate the results of in vitro experiments in vivo. Overall, these findings demonstrated a synergistic role of endothelial cells in contributing to prostate cancer proliferation and chemoresistance via inducing ERG expression and activating the Akt/mTOR signaling pathway.

2. Materials And Methods

2.1 Cell lines and treatment

Human umbilical vein endothelial cells (HUVEC), 22Rv1, and C4-2B were purchased from the American Type Culture Collection (ATCC). HUVEC was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS; Gibco). 22Rv1 and C4-2B were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin. Cells were maintained at 37 °C and 5% of CO₂ in humidified air. Human FGF-basic(FGF2) (PeproTech, NJ, USA), Human IL-6(PeproTech, NJ, USA), Human IL-8(PeproTech, NJ, USA), Human RANTES (PeproTech, NJ, USA), PD173074 (Selleckchem, TX, USA) and Perifosine (Selleckchem, TX, USA) were used for this study.

2.2 Coculture experiments

Coculture experiments were performed by seeding prostate cancer cells in the lower chamber and HUVEC in the upper chamber of a 6-well or a 24-well transwell apparatus with a 0.4 um pore size (Corning, NY, USA).

2.3 Proliferation assay

22Rv1 and C4-2B cell proliferation was determined using a Cell Counting Kit (Dojindo, Kumamoto, Japan) and EdU DNA Cell Proliferation Kit (RiboBio, Guangzhou, China). For the CCK-8 assay, 2×10^3 cells were seeded in 24-well plates in triplicate. At various time points, 30 μ l CCK-8 solution with 300 μ l PBS was added to each well and incubated at 37°C for 2h. The absorbance at 450 nm was measured with a microplate reader (Bio-Rad Laboratories). EdU experiments were carried out for analyzing the proliferation of 22Rv1 and C4-2B cells after cocultured with HUVEC for 48h according to the manufacturer's instructions.

2.4 Cell viability assay

For cell viability assay, 1×10^4 22Rv1 or C4-2B cells were cultured with or without HUVEC in 24-well plates overnight at 37°C. After 24h, the cells were then treated with docetaxel at the indicated concentrations for 48h. A cell counting kit was used to analyze cell viability, the number of surviving cells was detected at the absorbance of 450 nm by a microplate reader (Bio-Rad Laboratories). Each experiment was performed three times.

2.5 Apoptosis analysis

Cells were dissociated by 0.25% trypsin-EDTA and harvested by centrifugation, rinsed once with PBS, and suspended in binding buffer. Annexin V and PI staining were performed as per the manufacturer's instructions (BD Biosciences). After incubation, cells were measured by Beckman Coulter Accuri Cytometers C6 flow cytometer (BD), and analyzed using the Accuri CFlow software. The experiments were repeated three times.

2.6 Quantitative real-time PCR (qRT-PCR)

Total RNA of HUVEC, 22Rv1, and C4-2B was extracted using TRIzol (Life Technologies, Carlsbad, CA). Reverse transcript PCR for mRNA was carried out using Prime Script RT Master Mix (Takara, Otsu, Shiga, Japan) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa, Japan) according to the manufacturer's instructions. Primers used were: ERG sense, 5'CGCAGAGTTATCGTGCCAGCAGAT3'; antisense, 5'CCATATTCTTTCACCGCCCACTCC3'; GAPDH sense, 5'AATGTCACCGTTGTCCAGTTG3'; antisense, 5'GTGGCTGGGGCTCTACTTC3'; FGF2 sense, 5'AGAAGAGCGACCCTCACATCA3'; antisense, 5'CGGTTAGCACACACTCCTTTG3'; Expression levels were normalized to the expression of GAPDH RNA. All experiments were performed in triplicate.

2.7 Western blot

Total proteins were extracted from tissues and primary cells in lysis buffer on ice. The lysates were cleared by centrifugation at 12,000 g at 4°C for 15 min, and the protein concentrations were measured by BCA protein assay kit. Protein extracts were heated at 95°C with 5 × SDS-PAGE Loading Buffer (Dingguo, WB-0091) for 10 min and separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After blocking with 5% skim milk, primary antibodies against ERG (Cell Signaling Technology, #97249, 1:1000 dilution), AR (Cell Signaling Technology, #3202, 1:1000 dilution), Caspase3 (Cell Signaling Technology, #9662, 1:1000 dilution), PARP (Cell Signaling Technology, #9532, 1:1000 dilution), Akt (Cell Signaling Technology, #9272, 1:1000 dilution), phospho-Akt (Ser473) (Cell Signaling Technology, #4060, 1:1000 dilution), mTOR (Cell Signaling Technology, #2972, 1:1000 dilution), phospho-mTOR (Cell Signaling Technology, #2971, 1:1000 dilution), GAPDH (Sangon #D110016, 1:3000 dilution) were used at 4°C overnight. After being washed three times with TBS-T, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour and visualized by enhanced chemiluminescence assay (ECL, Thermo).

2.8 Lentivirus construction and infection

The coding sequence of ERG mRNA (NM_001136154.1) was synthesized and cloned into pLenti6.3 lentivirus overexpression vectors. All lentiviruses were constructed by Shanghai Obio Technology Company. After packaging, pLenti6.3-ERG lentivirus and negative control lentivirus were used to infect C4-2B cells, for the construction of ERG overexpressing C4-2B (C4-2B-ERG) and negative control C4-2B cells.

2.9 RNA interference

The siRNA targeting ERG mRNA or negative control siRNAs were designed and constructed by GenePharma Company. ERG siRNA sequences are as follows: siERG1: sense 5'CCACGGUUAUGCAUGCUATT 3', antisense 5' UAGCAUGCAUUAACCGUGGAG3'. siERG2: sense 5' GCUAUGGAGUACAGACCAUTT 3', anti-sense: 5' AUGGUCUGUACUCCAUAAGCTT3'. C4-2B-ERG cells were transfected with ERG siRNAs and negative control siRNAs according to the manufacturer's protocol.

2.10 Cytokine Antibody Arrays and ELISA assay

Human Cytokine Antibody Arrays G-Series 1000 (Raybiotech) was used according to the manufacturer's instructions. Briefly, serum-free media from prostate cancer cells culture and HUVEC/prostate cancer cells coculture were collected and incubated with the blocked glass chips for 2h at room temperature. After development, the signals (532 nm excitation) were scanned and extracted using the InnoScan 300 Microarray Scanner (Innopsys, Inc. France). The results were analyzed using the RayBiotech Q Analyzer program.

HUVEC cells were cultured in DMEM with 10% fetal bovine serum until 80% of confluency. These cells were then washed with PBS and cultured with or without prostate cancer cells in fresh serum-free media. Cell culture media were collected after 24h. FGF2 level was determined by using a commercial human FGF2 ELISA kit (RayBiotech, GA, USA) according to the manufacturer's instructions.

2.11 Immunohistochemical Staining

Paraffin sections were dewaxed in xylene and rehydrated in graded ethanol, followed by incubation with non-specific protein blocking solution 1% bovine serum albumin in PBS for 45 min at room temperature, and incubated with primary antibody against ki67 (Cell Signaling Technology, #9449, 1:400) overnight at 4 °C. The sections were then incubated with HRP-conjugated secondary antibody for 60 min at room temperature followed by treatment with diaminobenzidine (Sigma-Aldrich) working solution and counterstaining with hematoxylin.

2.12 TUNEL assay

The sections were dewaxed with xylene twice for 15 min and then treated with a graded series of alcohol (100%, 95%, 85%, 70%, and 50% ethanol in double-distilled H₂O) and rehydrated in PBS (pH 7.5). Apoptotic cells were detected according to the protocol of the TUNEL kit (Roche, Mannheim, Germany). DAPI was then used for counterstaining of the nuclei and images were obtained by laser scanning microscopy.

2.13 Animal experiment

Five to six-week-old male BALB/c nude mice were purchased from Animal Center of the Chinese Academy of Sciences (Shanghai, China) and all animal studies were carried out in compliance with guidelines of the Chinese Council on Animal Care. Protocols were approved by the Medical Science Ethics Committee of Shanghai General Hospital. 5×10^6 22Rv1 cells mixed with or without 2.5×10^6 HUVEC were suspended in 200 μ l PBS diluted Matrigel (100 μ l PBS+100 μ l Matrigel), which were subcutaneously inoculated into the right flanks of mice. When tumors reached a mean size of 150 mm³, the mice were treated with docetaxel 20mg/kg intraperitoneally once a week for four weeks. Every six days, the length (L) and width (W) of tumors were measured using calipers, and their volumes were calculated using the equation ($L \times W^2/2$).

2.14 Statistical analysis

The data were expressed as mean \pm SD and the two groups were compared with the *t*-test. Statistical analysis was performed by using the SPSS software package (version 19.0). A P value < 0.05 indicated a significant difference.

3. Results

3.1 Endothelial cells promote the proliferation of prostate cancer cells with or without docetaxel treatment.

We established a coculture system (media as control) to investigate the effect of endothelial cells on the growth of prostate cancer cells (Figure 1A). 22Rv1 and C4-2B cells were cultured with or without HUVEC. CCK8 assays revealed that 22Rv1 and C4-2B cells cocultured with HUVEC increased cell proliferation capability compared with the corresponding monoculture group, respectively (Figure 1B and C). Moreover, as shown in Figure 1D and 1E, 22Rv1 or C4-2B cells cocultured with HUVEC incorporated more EdU than the monoculture group (58% vs 32% for 22Rv1 and 52% vs 34% for C4-2B, respectively). To investigate whether HUVEC has a synergistic impact on docetaxel resistance in prostate cancer cells, we measured cell viability of 22Rv1 and C4-2B cultured with or without HUVEC after 48h of docetaxel treatment at the indicated concentration. Interestingly, 22Rv1 and C4-2B cocultured with HUVEC demonstrated greater degrees of docetaxel resistance than the monoculture group (Figure 1F and 1G). Collectively, these results indicated that endothelial cells may enhance the proliferation of prostate cancer cells before and after docetaxel treatment.

3.2 Endothelial cells inhibit the apoptosis of prostate cancer cells after docetaxel treatment.

We performed flow cytometry to calculate the apoptosis rates of 22Rv1 and C4-2B cultured with or without HUVEC after treatment with 10nM docetaxel for 48h. Surprisingly, the apoptosis rates of 22Rv1 and C4-2B in the coculture group decreased significantly compared with the monoculture group, which suggested that HUVEC could shield prostate cancer cells from docetaxel-induced apoptosis (Figure 2A and 2B). Moreover, western blotting assay showed that the expression of apoptosis proteins including cleaved-PARP and cleaved caspase-3 was down-regulated markedly in the coculture group treated with docetaxel compared with the monoculture group treated with docetaxel (Figure 2C and 2D). Taken together, these data manifested that endothelial cells can inhibit the apoptosis of prostate cancer cells after docetaxel treatment.

3.3 ERG is a key mediator for endothelial cells-induced docetaxel resistance in prostate cancer cells.

Recent studies have revealed that ERG can promote resistance to docetaxel in different prostate cancer cell lines (26, 27). Accordingly, the expression of ERG in 22Rv1 and C4-2B cells cocultured with HUVEC was determined, respectively. Interestingly, qPCR and western blot assay demonstrated that the expression of ERG increased in prostate cancer cells with prolonged coculture time (Figure 3A and 3B). This finding indicated that endothelial cells could induce ERG expression in prostate cancer cells in an AR independent manner. To investigate whether ERG expression has an impact on docetaxel resistance, we constructed ERG overexpressing C4-2B cells (C4-2B-ERG) by lentiviral transfection. Cell viability assays

revealed that ERG overexpression enabled more C4-2B cells to survive upon the same dose of docetaxel while knocking down ERG could restore their sensitivity to docetaxel (Figure 3C). Annexin V/PI assay was used to assess apoptosis following docetaxel treatment in C4-2B with or without ERG overexpression. The percentage of apoptotic cells was much lower in C4-2B-ERG cells than that in C4-2B cells (Figure 3D and 3E). Consistently, cleaved-caspase3 and cleaved-PARP also decreased in C4-2B-ERG cells compared with C4-2B cells by western blot analysis (Figure 3F). Collectively, these data suggested that endothelial cells enhance the docetaxel resistance of prostate cancer cells through increasing ERG expression.

3.4 FGF2 secreted from endothelial cells to PCa/ECs co-culture system induces ERG expression and docetaxel resistance in prostate cancer cells.

To determine which factor contributes to the expression of ERG in prostate cancer cells cocultured with HUVEC, we conducted cytokines array according to the manufacturer's protocol. As shown in Figure 4A and 4B, a series of cytokines were elevated in the culture media of 22Rv1 cocultured with HUVEC compared with 22Rv1 cultured alone. Among these factors, IL-6, IL-8, RANTES(CCL5), and bFGF(FGF2) have been identified as cytokines that are associated with chemotherapy resistance(28-31). Next, we examined the effect of IL-6, IL-8, CCL5, and FGF2 in the regulation of ERG expression by adding them to the culture media of prostate cancer cells, respectively. As demonstrated in Figure 4C, FGF2 upregulated the expression of ERG in 22Rv1 and C4-2B cells by western blotting analysis, while IL-6, IL-8, and CCL5 did not (data not shown). Interestingly, the expression of FGF2 increased in HUVEC cocultured with prostate cancer cells compared with HUVEC cultured alone measured by qPCR and ELISA analysis (Figure 4D and 4E). Moreover, blocking FGF2 by using PD173074 could reverse the enhancing effects of HUVEC on ERG expression and docetaxel resistance in prostate cancer cells (Figure 4F and 4G). Taken together, these results revealed that FGF2 derived from HUVEC may play a key role in inducing the expression of ERG and promoting chemoresistance to docetaxel in prostate cancer cells.

3.5 Endothelial cells induced docetaxel resistance via FGF2/ERG/ Akt/mTOR signaling pathway in prostate cancer cells.

Previous studies have demonstrated that the Akt/mTOR signaling pathway plays an important role in docetaxel-resistant CRPC(32). Therefore, we hypothesized that this pathway might be involved in ERG-mediated chemoresistance to docetaxel in prostate cancer cells. As expected, phosphorylation of Akt and mTOR increased in ERG-overexpressing C4-2B cells compared with normal C4-2B cells by western blot analysis, which indicated that ERG was involved in the activation of Akt/mTOR signaling pathway (Figure 5A). We subsequently examined the phosphorylation status of Akt and mTOR in C4-2B cells cocultured with HUVEC and found that HUVEC induced the phosphorylation of Akt and mTOR in C4-2B cells (Figure 5B). Meanwhile, FGF2 treatment also upregulated the phosphorylation of Akt and mTOR in C4-2B cells (Figure 5C). To further investigate the key role of Akt/mTOR in chemoresistance mediated by ERG, we used Perifosine (an Akt inhibitor) in ERG-overexpressing C4-2B cells. Flow cytometry analysis indicated that the percentage of apoptotic C4-2B-ERG cells increased when they were treated with docetaxel plus Perifosine compared to docetaxel alone (Figure 5D). Furthermore, cell viability assay

showed that Perifosine could decrease docetaxel resistance induced by ERG (Figure 5E). Collectively, these findings suggested that ERG enhances docetaxel resistance via the Akt/mTOR signaling pathway in prostate cancer cells.

3.6 Endothelial cells promote the docetaxel resistance of prostate cancer cells in vivo.

To further validate the above findings in vivo, 22Rv1 cells with or without HUVEC were injected subcutaneously into the right flanks of athymic BALB/c nude mice. As shown in Figure 6A-C, co-injection of 22Rv1 cells with HUVEC dramatically enhanced tumor growth rate and sustained tumor proliferation in mice under docetaxel chemotherapy in comparison with 22Rv1 uni-injection group. Immunohistochemistry analysis revealed that the expression of Ki67, pAkt, pmTOR increased markedly in tumors derived from 22Rv1 co-injected with HUVEC compared to 22Rv1 injected alone following chemotherapy with docetaxel (Figure 6D). Besides, the TUNEL assay demonstrated that the percentage of TUNEL positive cells in tumors from the co-injection group was lower than that from the uni-injection group following docetaxel chemotherapy (Figure 6E). Collectively, these data suggested that endothelial cells not only promote prostate cancer cells proliferation but also shield prostate cancer cells from docetaxel-induced apoptosis in vivo.

3.7 Summary of the mechanism underlying endothelial cells promoting docetaxel resistance in prostate cancer cells.

Endothelial cells cocultured with prostate cancer cells can secrete FGF2 into the culture media. FGF2 boosts the expression of the ERG gene in prostate cancer cells subsequently. ERG protein activates Akt/mTOR signaling pathway contributing to docetaxel resistance in prostate cancer cells ultimately.

4. Discussion

The tumor microenvironment is recognized to profoundly influence cellular response to chemotherapy. Endothelial cells are key components of the tumor microenvironment. Endothelial cells can secrete a variety of cytokines, which play important roles in tumor progression and metastasis(33). Recent studies have demonstrated that endothelial cells can adjust their response to chemotherapy. Meng F *et al* (34) reported that endothelial cells from mouse liver cancer enhance their survival and migration in response to chemotherapeutic stress via an NF- κ B-Akt-dependent manner. AKIYAMA K *et al* (35) described that endothelial cells from melanoma acquire drug resistance to paclitaxel by multidrug resistance 1 (MDR1) upregulation via VEGF signaling. However, to the best of our knowledge, few studies have focused on the role of endothelial cells in modulating chemoresistance of cancer cells to docetaxel(17, 20). Therefore, appreciating the important experimental evidence of endothelial cells' involvement in the chemoresistance of prostate cancer can have important therapeutic implications. In this study, we have identified endothelial cells as a mediator contributing to docetaxel resistance of prostate cancer cells for the first time. Endothelial cells secrete FGF2 in the tumor microenvironment to enhance the expression of ERG in prostate cancer cells. ERG protein stimulates the phosphorylation of Akt and mTOR subsequently. The activation of the Akt/mTOR signaling pathway promotes the docetaxel resistance of prostate cancer

cells ultimately (Fig 7). Our results confirmed that endothelial cells contribute to the chemoresistance of prostate cancer cells in a paracrine manner. This new finding will shed valuable insights into the role of endothelial cells in the development of chemoresistance of prostate cancer cells and highlight the importance of endothelial cells as a therapeutic target in prostate cancer treatment.

The interaction of endothelial cells with tumor cells has been shown to mediate AR-independent invasion of prostate cancer cells(24). By analogy, we hypothesized that the communication between endothelial cells and prostate cancer cells may also play a role in modulating chemoresistance of prostate cancer. Consistently, we found that endothelial cells can enhance the chemoresistance of prostate cancer cells. Mechanistically, endothelial cells induce ERG expression in prostate cancer cells. ERG has been demonstrated to affect several parameters of microtubule dynamics and inhibit effective drug-target engagement of docetaxel with tubulin (26). Interestingly, we have discovered a novel way that induces ERG expression in prostate cancer. Through a combination of cytokines array, quantitative PCR, and ELISA assay, we confirmed that FGF2 was the key factor that facilitated the development of chemoresistance of prostate cancer cells cocultured with endothelial cells. Gan, Wientjes (36) demonstrated that the expression of basic fibroblast growth factor correlates with resistance to paclitaxel in human tumors including prostate cancer. However, they did not further explore the underlying mechanism. Here, we identified a new signaling pathway that connects FGF2 and docetaxel resistance of prostate cancer. We showed that endothelial cells-derived FGF2 can induce ERG expression in prostate cancer cells in an AR-independent manner. This finding provides new evidence to the role of FGF2 in promoting chemoresistance of prostate cancer. However, when FGF2 antagonists were used to block the interactions between endothelial cells and prostate cancer cells, the expression of ERG could not be completely reversed. This may be due to there are other factors that exist in the tumor microenvironment to modulate the expression of ERG. Nevertheless, there are few reports focused on the regulation of ERG expression by cytokines. Kao C *et al* (37) suggested that ERG expression could be modulated by EGF. Other AR-independent ways regulating ERG expression are needed to be further investigated in the future. Similarly, the enhancing effects of HUVEC on docetaxel resistance could not be completely reversed as well when FGF2 antagonists were added. This indicates that although FGF2 plays a significant role in mediating chemoresistance, there are other factors needed to be further investigated as well. Intriguingly, the expression of FGF2 in endothelial cells was elevated when cocultured with prostate cancer cells compared with endothelial cells cultured alone. This finding implied that prostate cancer cells can give instructions to their microenvironment components to adjust responses to external circumstances in favor of survival. Thereciprocal crosstalk between tumor and microenvironment therefore substantially affects tumor cell biology. Although the ways and mechanisms by which prostate cancer cells act on endothelial cells are not the subject of this study. This reflection points out a new direction that needs further study in the future.

FGF2 has been manifested to activate Akt/mTOR signaling pathway in earlier studies(38, 39). In this study, we confirmed that ERG was able to induce Akt phosphorylation, which was consistent with the previous report(40). The activation of the Akt/mTOR pathway contributed to the docetaxel resistance of prostate cancer, which was corroborated in previous studies(41, 42). Herein, docetaxel resistance of ERG-

overexpressing prostate cancer cells was significantly reversed when Perifosine, the inhibitor of the Akt/mTOR signaling pathway was added, which was in agreement with the previous report(32). Our recent findings and previous reports will pave the way for further study of Akt inhibitors combined with docetaxel in the treatment of CRPC.

Although there are some findings in this study, there are several limitations as well. First, we could not investigate the FGF2-ERG-Akt/mTOR signaling axis in clinical samples due to the availability of CRPC specimens. Second, ERG expression status could not be verified in vivo because the mouse xenograft tumors we obtained were generally negative in immunohistochemical staining for ERG. Third, we did not acquire the direct molecular evidence to show how FGF2 activates ERG expression in detail. Fourth, we did not validate the efficacy of FGF2 antagonists to overcome docetaxel resistance in vivo due to limited funding. Notwithstanding these inadequacies, we still demonstrate that endothelial cells-derived FGF2 plays an important part in promoting docetaxel resistance of prostate cancer cells through in vitro and in vivo experiments.

Conclusions

In summary, our findings highlight that endothelial cells play a significant role in enhancing the proliferation and inhibiting the docetaxel-induced apoptosis of prostate cancer cells in vitro and in vivo. Specifically, we demonstrated that FGF2 secreted from endothelial cells can upregulate ERG expression in prostate cancer, which then activates the Akt/mTOR signaling pathway and promote docetaxel resistance of prostate cancer subsequently. Besides, we propose that endothelial cells, as a key component of the tumor microenvironment, contribute to docetaxel resistance of prostate cancer cells in a paracrine fashion, which may provide new insights for the development of novel anticancer therapies. Furthermore, evidence from in vitro studies suggests that inhibitors of FGF/ FGFR signaling combined with docetaxel warrant further investigation as a potential therapeutic option for the treatment of patients with advanced prostate cancer.

List Of Abbreviations

PCa - prostate cancer

CRPC - castration-resistant prostate cancer

HUVEC - human umbilical vein endothelial cells

ECs - endothelial cells

FGF2 - basic fibroblast growth factor

ERG - ETS related gene

ADT - androgen deprivation therapy

OS - overall survival

mHSPC - metastatic hormone-sensitive prostate cancer

MDR - multidrug resistance

ATCC - American Type Culture Collection

DMEM - Dulbecco's Modified Eagle Medium

FBS - fetal bovine serum

Declarations

Ethics approval and consent to participate

Animal research has been approved and carried out in strict accordance with the institutional ethical guidelines of the Committee on the Use of Live Animals of Shanghai General Hospital.

Consent for publication

Not applicable

Availability of data and materials

Please contact author for data requests.

Competing interests

The authors declare that they have no competing interests.

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Figures

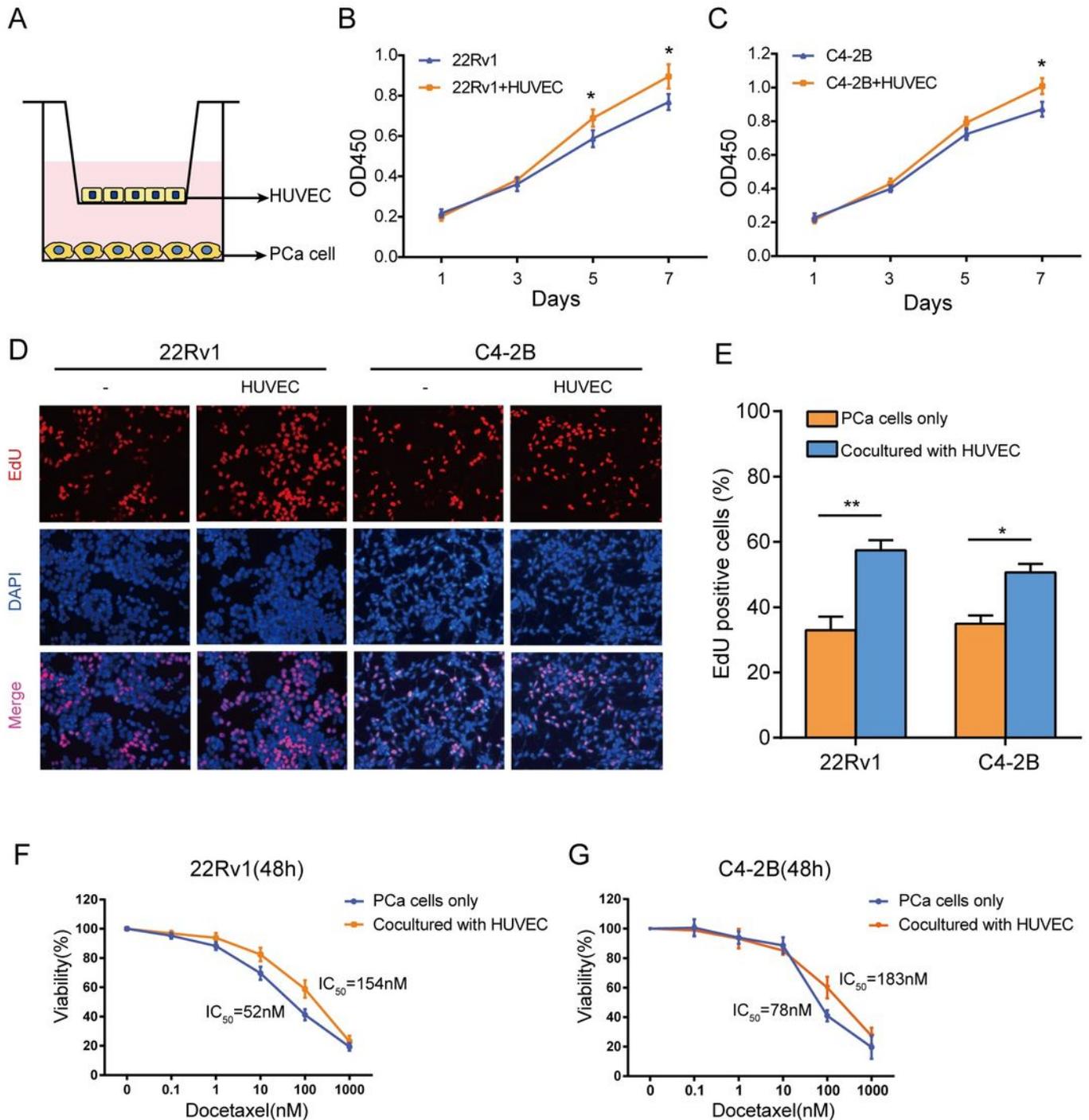


Figure 1

Endothelial cells enhance the proliferation of prostate cancer cells with or without docetaxel treatment. A. This picture showed the construction of the coculture system by using a 0.4 μ m pore Transwell plate. B and C. CCK8 assays revealed that the coculture group with HUVEC increased the growth rate of 22Rv1

and C4-2B compared with the respective monoculture group. D and E. EdU assays demonstrated proliferation differences between 22Rv1 cultured alone and 22Rv1 cocultured with HUVEC, C4-2B cultured alone, and C4-2B cocultured with HUVEC. F and G. Cell viability of 22Rv1 and C4-2B (cultured with or without HUVEC) treated with docetaxel at the indicated concentrations for 48 hours. The data represent the mean \pm SD and differences were tested by the Student's t-test; * $P < 0.05$; ** $P < 0.01$.

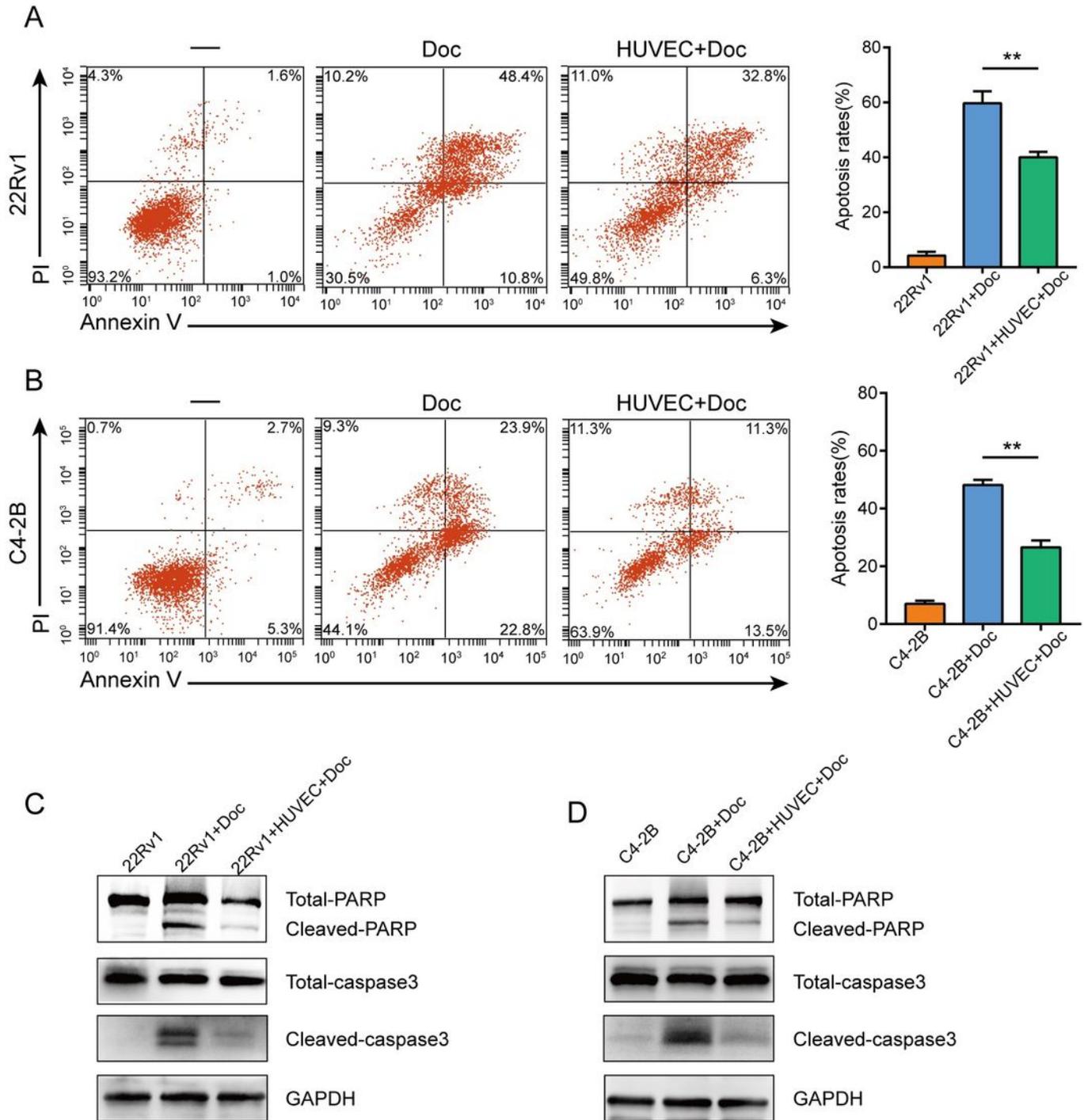


Figure 2

Endothelial cells suppress the apoptosis of prostate cancer cells after docetaxel treatment. A and B. The percentage of apoptosis of 22Rv1 and C4-2B cells cultured with or without HUVEC after 10nM docetaxel treatment for 48h. C and D. Expression of different apoptosis-related proteins in 22Rv1 and C4-2B cultured with or without HUVEC was detected by western blot. Data were presented as mean \pm SD. * P <0.05; ** P <0.01.

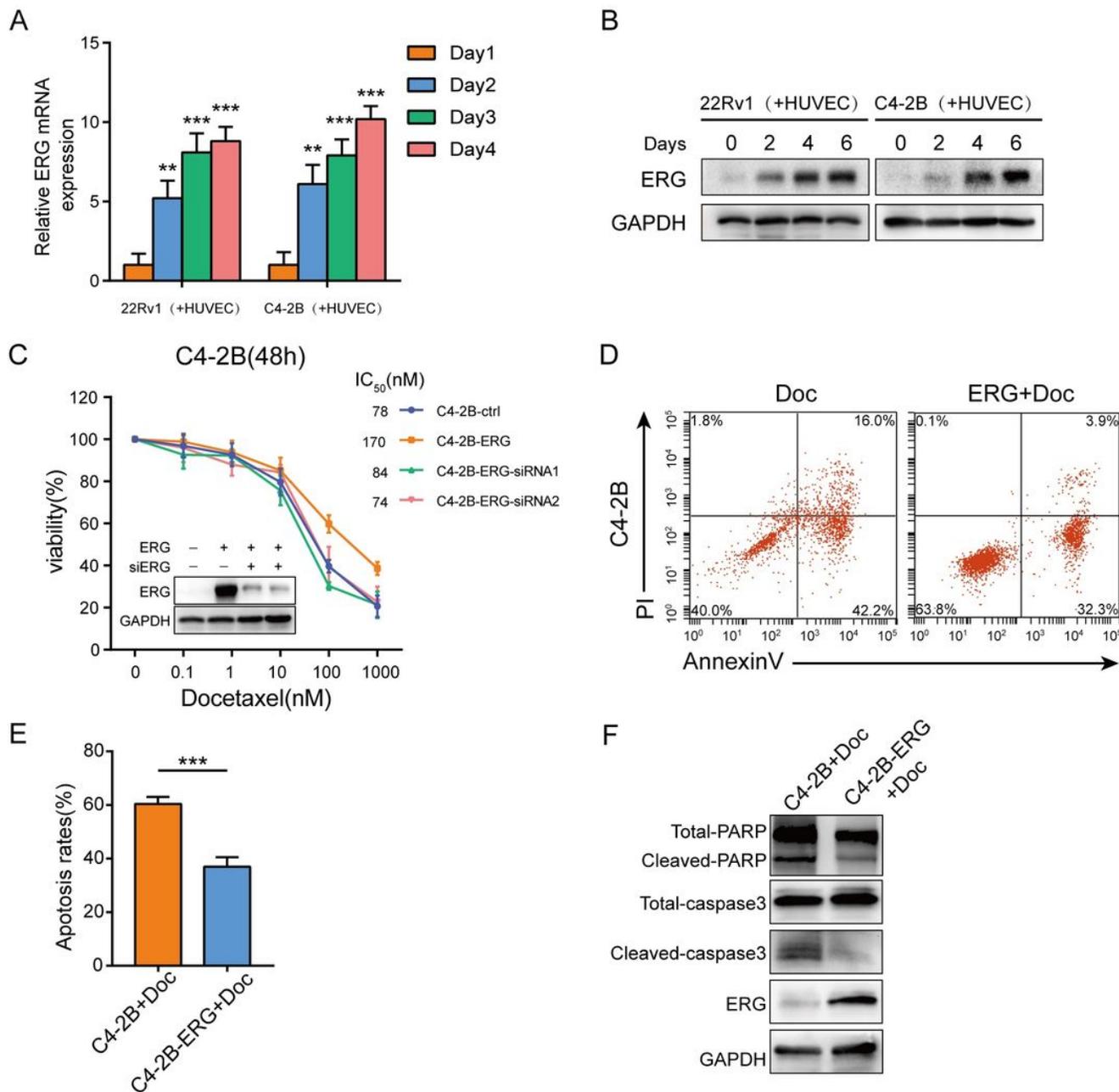


Figure 3

ERG is a key mediator for endothelial cells-induced docetaxel resistance in prostate cancer cells. A and B, 22Rv1, and C4-2B cells were cocultured with HUVEC for the indicated days, qPCR and western blot analysis were performed to assess ERG expression. C, Cell viability of C4-2B cells transfected with ERG

and siRNA targeting ERG incubated with the indicated dose of docetaxel for 48h. Western blot was conducted to measure ERG expression. D and E, The percentage of apoptotic C4-2B and C4-2B-ERG cells treated with 10nM docetaxel for 48h. F, The expression of total/cleaved PARP and caspase-3 in C4-2B and C4-2B-ERG cells treated with docetaxel was determined by western blotting. Data were presented as mean \pm SD * P <0.05; ** P <0.01; *** P <0.001.

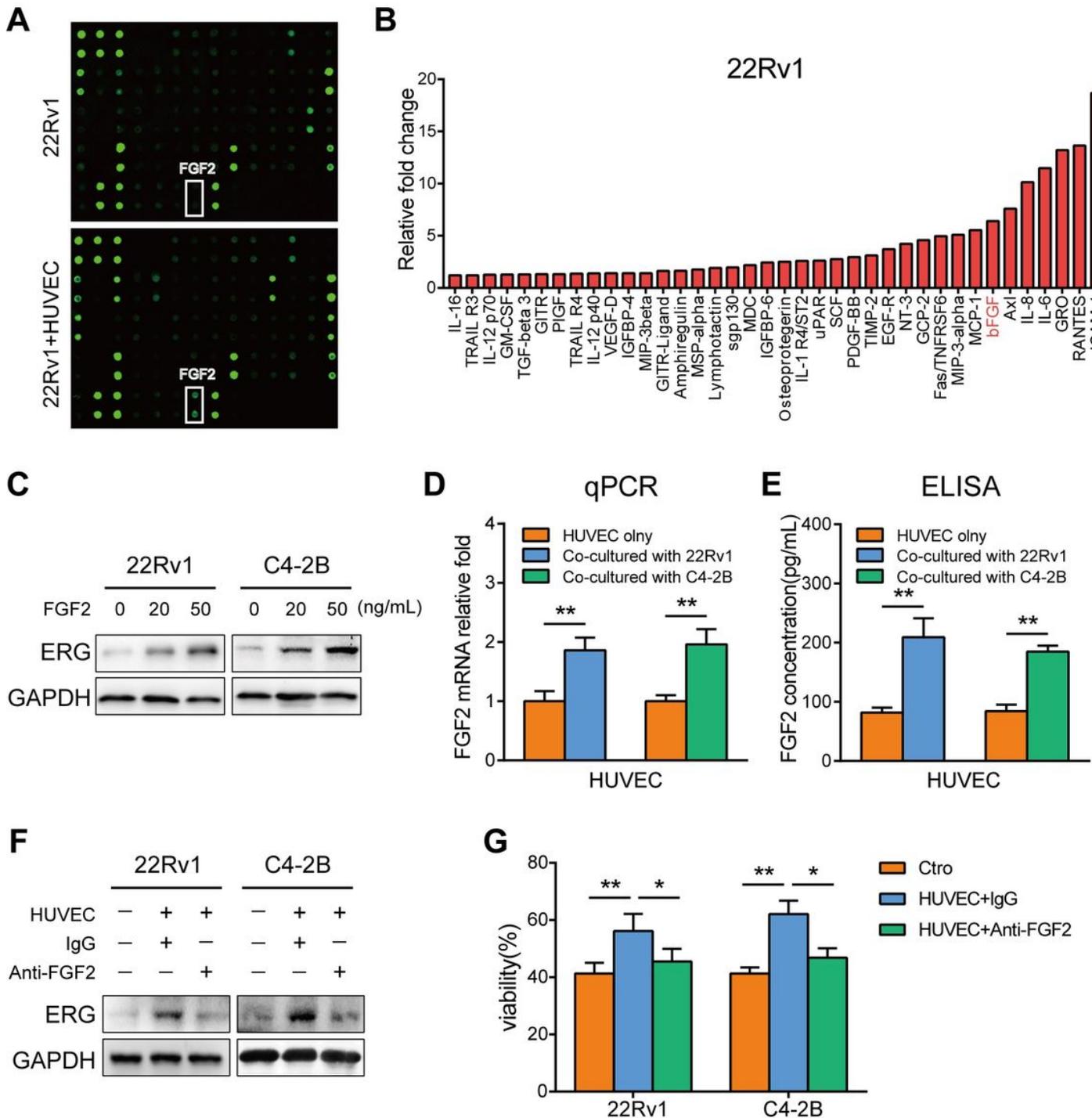


Figure 4

FGF2 secreted from endothelial cells induces ERG expression and docetaxel resistance in prostate cancer cells. A. The cytokines array analysis. The conditioned media collected from 22Rv1, 22Rv1, and HUVEC

coculture systems were used for the analysis. B, Histogram showing increased cytokines in 22Rv1 and HUVEC coculture supernatant compared to 22Rv1 culture supernatant. C, Western blot analysis of ERG expression following FGF2 treatment in prostate cancer cells. 22Rv1 and C4-2B cells (1×10^5 /well) were treated with 20ng/mL and 50ng/mL of FGF2 for 48h, respectively. D, Quantitative PCR analysis of FGF2 expression in HUVEC cultured with or without prostate cancer cells. E, ELISA demonstrating different FGF2 concentrations in culture supernatants from HUVEC and HUVEC cocultured with prostate cancer cells. F and G, 22Rv1, and C4-2B cells were cultured with or without HUVEC in the presence or absence of anti-FGF2 for 48h. F, Western blot analysis of ERG expression of prostate cancer cells was conducted. G, Cell viability assay of prostate cancer cells treated with 10nM docetaxel for 48h was performed. Data were presented as mean \pm SD * $P < 0.05$; ** $P < 0.01$;

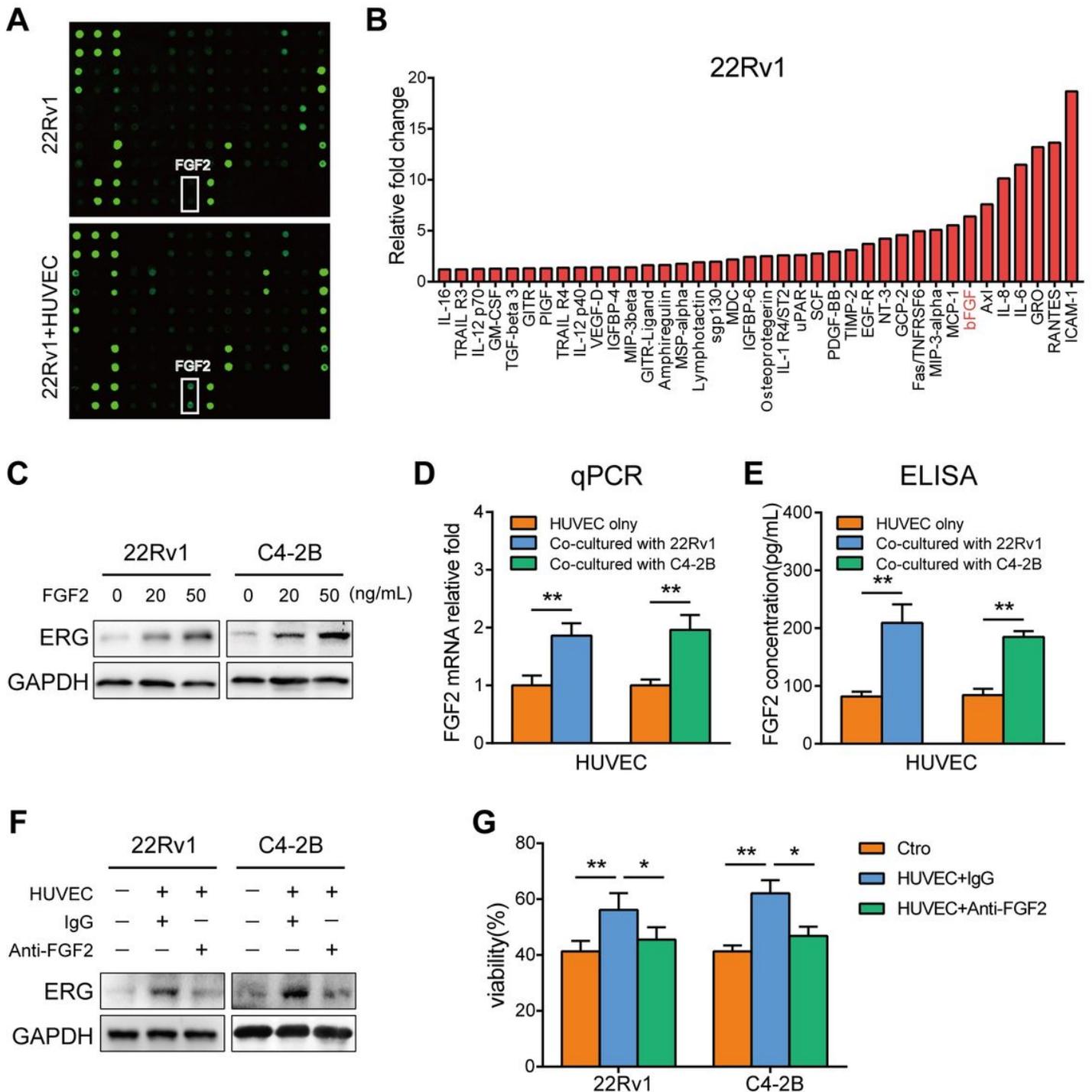


Figure 5

ERG is involved in the regulation of the Akt/mTOR signaling pathway in prostate cancer cells. A. Western blotting analysis of total Akt, phosphorylated Akt (p-Akt), total mTOR, phosphorylated mTOR (p-mTOR) proteins in C4-2B and C4-2B-ERG cells. B, C4-2B cells were cocultured with HUVEC for 48h, and total Akt, phosphorylated Akt (p-Akt), total mTOR, phosphorylated mTOR (p-mTOR) proteins were measured subsequently by western blotting. C, C4-2B cells were treated with 50ng/mL FGF2 for 48h, western blotting analysis was performed subsequently for total Akt, phosphorylated Akt (p-Akt), total mTOR, phosphorylated mTOR (p-mTOR) proteins. D, The apoptosis rates of C4-2B-ERG cells were determined by flow cytometry following docetaxel treatment with or without Akt inhibitor. E, Cell viability of C4-2B-ERG cells treated with docetaxel at the indicated doses with or without Akt inhibitor for 48h. Data were presented as mean \pm SD. **P<0.01; ***P<0.001;

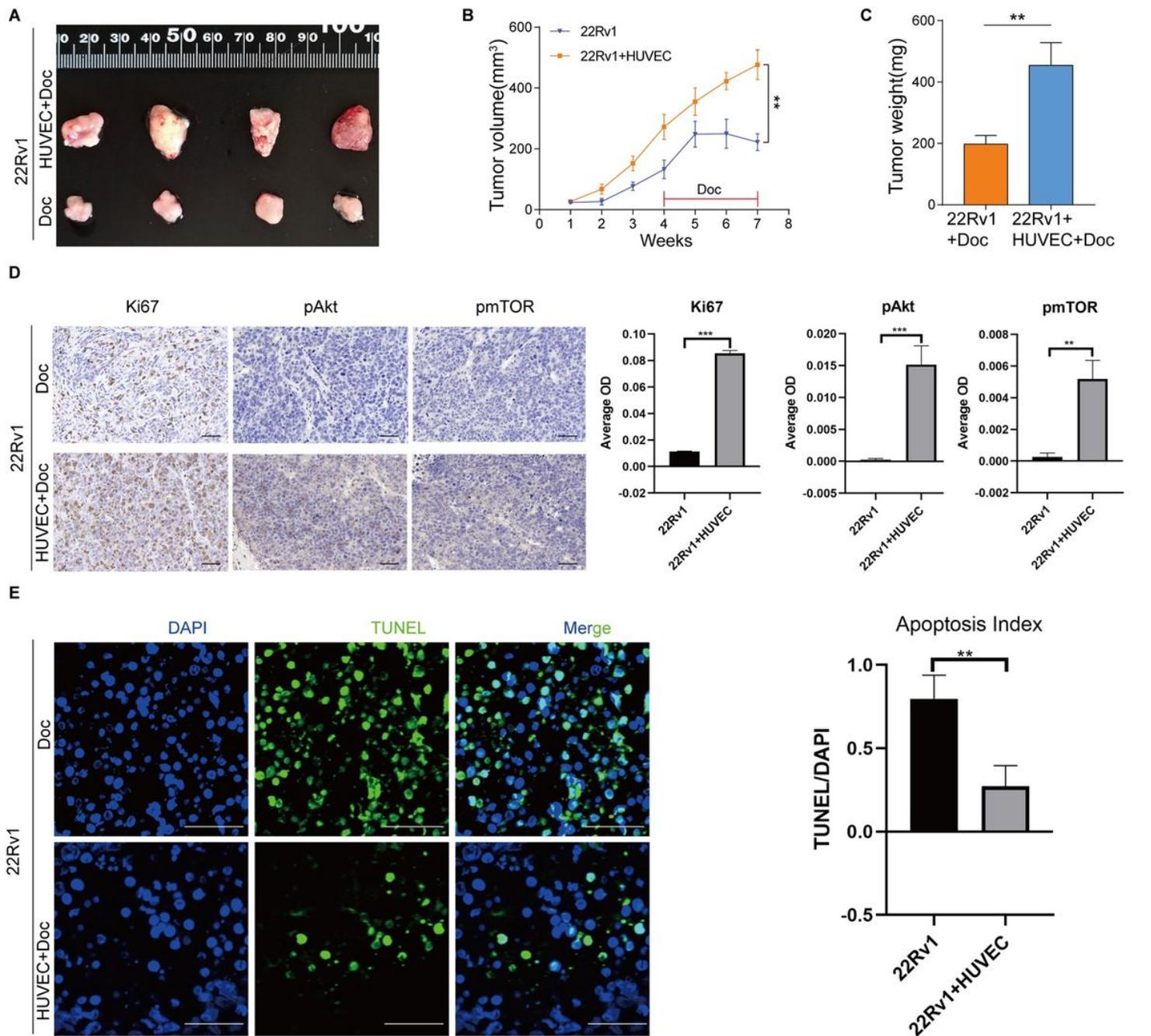


Figure 6

Endothelial cells promote the docetaxel resistance of prostate cancer cells in vivo. A-C. 22Rv1 cells and 22Rv1 cells combined with HUVEC were subcutaneously injected into the right flanks of athymic BALB/c nude mice, respectively. Mice were administered with docetaxel by intraperitoneal injection once a week for four weeks. Tumor volumes were calculated and tumor weight was measured. D, Immunohistochemistry staining of Ki67, pAkt, pmTOR in excised xenograft tumors. Scale bars, 50 μ m. E, Representative fluorescent images of TUNEL-positive apoptotic tumor cells. Scale bars, 50 μ m. Data were presented as mean \pm SD; **P<0.01.

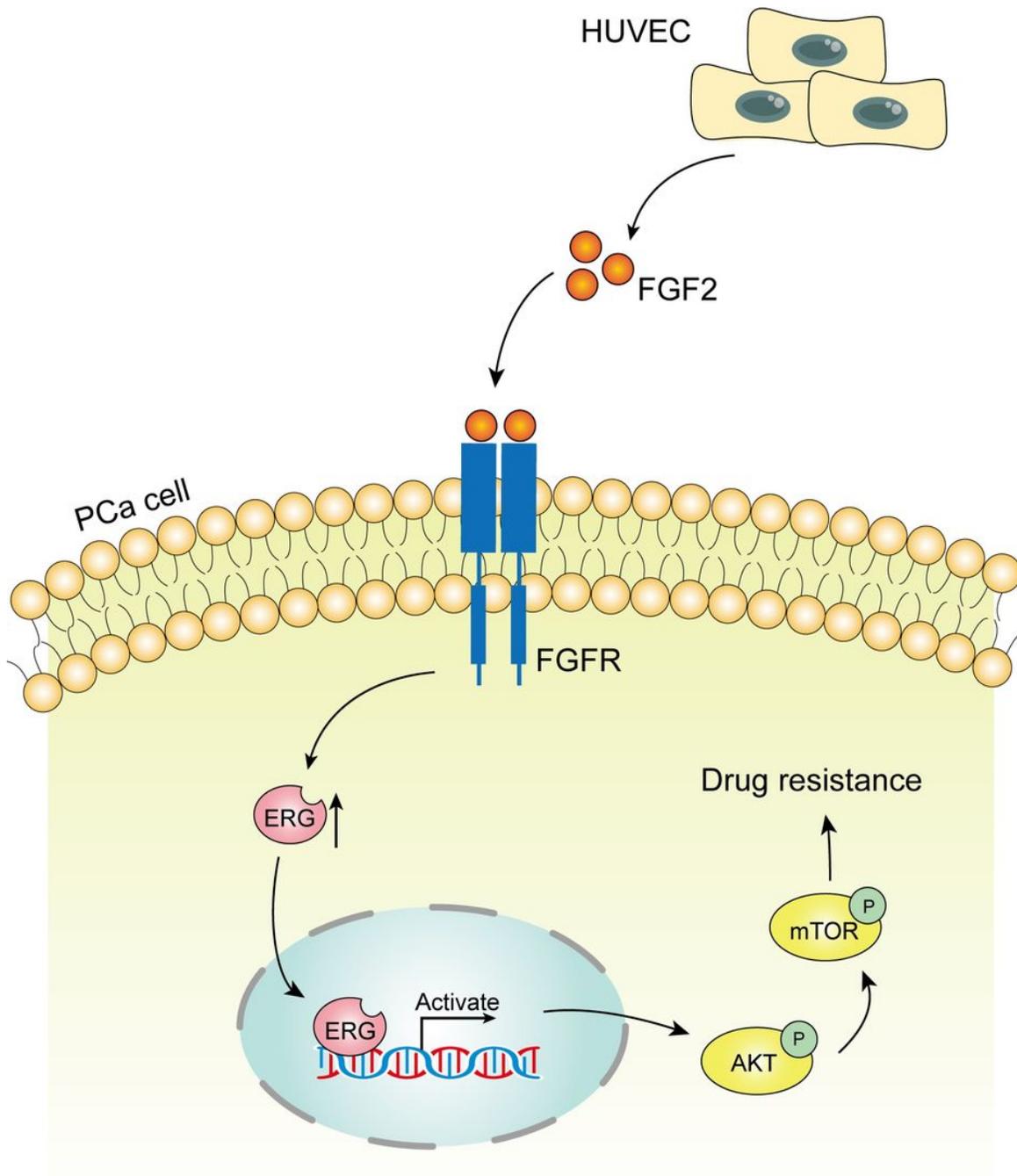


Figure 7

Schematic model of the hypothesized mechanism by which endothelial cells promote docetaxel resistance of prostate cancer cells.