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Protein Z Promotes Proliferation in Vascular Endothelial Cell Mediated by Chemokine C-X-C-Motif Receptor 4

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Preeclampsia (PE) is a multisystem progressive disease unique to pregnancy, seriously affecting maternal and infant health. Previous studies have shown that PE is associated with changes in vascular endothelial cells, but the specific mechanism is unclear. In this study, we assessed the effects of chemokine C-X-C-motif receptor 4 (CXCR4) on endothelial cells and explored the potential mechanisms. Human placental microvascular endothelial cells (HPVEC) were exposed to protein Z (PZ), qRT-PCR and Western blotting were used to detect the expression of proliferation related genes and proteins, such as PI3K/Akt/ERK. Meanwhile, qRT-PCR and Western blotting were used to detect the expression of anticoagulation markers PGI2 and t-PA. Then, HPVEC were transfected with CXCR4 siRNA or NC siRNA. The expression of proliferation related genes and proteins were also detected. Finally, PZ and CXCR4 were co-cultivated with different fluorescent labels, the binding sites of the two proteins were observed under confocal laser scanning microscopy (CLSM). PZ promoted the proliferation and expression of anticoagulant markers PGI2 and t-PA in HPVEC. CXCR4 silencing could inhibit the proliferation of HPVEC which stimulated by PZ. CLSM showed that the binding site of PZ and CXCR4 was located on the cell membrane. In conclusion, our results suggested that PZ promote the expression of PGI2/t-PA and affect the PI3K/Akt/ERK signaling pathway by binding with CXCR4 which improved our understanding of the molecular mechanisms involved in HPVEC.

Keywords: chemokine C-X-C-motif receptor 4; preeclampsia; proliferation; protein Z
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Introduction

Preeclampsia (PE) not only seriously affects maternal health and endangers fetal life, but also increases the risk of cardiovascular and cerebrovascular diseases in the future (Brouwers et al. 2018). The occurrence of PE is related to chorionic microvascular lesions (Mastrolia et al. 2014). Abnormal microvascular coagulation is relevant to endothelial cells and coagulation factors. Studies have shown that endothelial cells have an antithrombotic effect during proliferation, so regulating endothelial cell proliferation can regulate coagulation mechanisms and prevent thrombosis (Sfriso et al. 2017; Markowicz-Piasecka et al. 2019).

Protein Z (PZ) is an anticoagulant factor that plays an important role in the coagulation process and is associated with the occurrence of thrombosis (Karakoyun et al. 2017; Ghozlan et al. 2019). Clinical studies have shown that low PZ levels are closely related to the occurrence of deep vein thrombosis and ischemic stroke (Zhang et al. 2017a). Once the PZ level decreases, it may lead to the formation of thrombosis (Zhang et al. 2017b). The preliminary experimental results of our group confirmed that PZ has the effect of promoting the proliferation of vascular endothelial cells, but the specific mechanism is still unclear.

Stromal cell-derived factor-1 (SDF-1) has been reported to bind to the cell receptor Chemokine C-X-C-motif receptor 4 (CXCR4), causing cell migration and chemotactic recruitment (Cheng et al. 2017). Activated CXCR4 can activate G protein mediated signaling pathways, such as RAS, JAK/STAT, PI3K kinase pathways, to achieve cell proliferation (Yu and Cui 2016; Cui et al. 2019). Whether PZ, which also has the function of promoting endothelial cell proliferation, targets CXCR4 to regulate this process, and the results are worth looking forward to. In the current study, we investigated the molecular mechanism of endothelial cell proliferation. We found that PZ activated PI3K/Akt/ERK signaling by binding with CXCR4, so as to facilitate endothelial cell proliferation and

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reduce the risk of thrombosis.

Materials and Methods

Cell culturing and PZ treatment

Human Placental Microvascular Endothelial Cells (HPVEC) were obtained from zhong qiao xin zhou biotech (Shanghai, China) and cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C and 5% CO_2 atmosphere. PZ was obtained from zeye biotech (Shanghai, China). After the treatment for 1 day, cells were collected for assays. The experimental procedure was approved by the Institutional Ethics Review Board of the Yijishan hospital of Wannan Medical College (No. WNMC-FY-20230910).

SiRNA and transfection

Based on the CXCR4 nucleotide sequence in GenBank, the siRNA target sequence is designed as follows: 5′-GATCGGCAGTCCATGTCATCTACATGTATTC AAGAGAGATGACATGGACTGCTTTTTTA-3′; 5′-AGC TTAAAAAAGCAGTCCATGTCATCTACATCTCTTGAA TGTAGATGACATGGACTGCC-3′. CXCR4 siRNA was synthesized by Sango Biotech (Shanghai, China). SiRNA was transfected by lentiviral vector according to the instruction of manufacturer.

Cell proliferation assay

To evaluate the effects of PZ for cell proliferation, HPVEC were treated with PZ firstly. Then HPVEC were transfected by lentiviral vector silencing CXCR4 before treated with PZ. The concentration of PZ used for the experiments was 3 μg/ml. Cell Counting Kit 8 (CCK-8, Sigma-Aldrich) was used to count viable cells and detect the cell proliferation of HPVEC. The assay was performed following the manufacturer's instructions.

RNA extraction and qRT-PCR

HPVEC were harvested at properly time points, and total RNA was extracted by Trizol reagent (Invitrogen). The 20 μL reaction system includes RNA, oligo dT, 5 × Buffer, Dithiothreitol (DTT), dNTPs and RNase-Free water. The product cDNA was used for qRT-PCR, with fluorescent molecules as probes (Takara), according to manufacturer's instructions. The assay was carried out on ABI 7500 thermocycler (Applied Biosystems). Primers are as described: PGI2 (Prostaglandin-I-2) forward: 5′-ACCCCCAC TGAAAAAGATGA-3′; PGI2 reverse: 5′-CCTTCTAA GTGGTTGGAACA-3′; t-PA forward: 5′-CCTTCACTGTC TGCCTAACTCCTTCGTGTGTTCC-3′; t-PA reverse: 5′-TGT CTCCAGCACACAGCATGTTGTCGGTGAC-3′; CXCR4 forward: 5′-ACTACACCGAGGAAATGGGCT-3′; CXCR4 reverse: 5′-CCCACAATGCCAGTTAAGAAGA-3′; GAPDH forward: 5′-AGGTCGGTGTGAACGGATTTG-3′; GAPDH reverse: 5′-GGGGTCGTTGATGGCAACA-3′; PI3K forward: 5′-TATTTGGACTTTGCGACAAGACT-3′; PI3K reverse: 5′-TCGAACGTACTGGTCTGGATAG-3′; AKT forward: 5′-AGCGACGTGGCTATTGTGAAG-3′; AKT reverse: 5′-GCCATCATTCTTGAGGAGGAAGT-3′; ERK1/2 forward: 5′-AGGCTGTTCCCAAATGCTG-3′; ERK1/2 reverse: 5′-CGGGTCGTAATACTGCTCC-3′; Bcl-x forward: 5′-GTGAGTGGACGGTCAGTGGTG-3′; Bcl-x reverse: 5′-TTGGACAATGGACTGGTTGTTGA-3′.

Western blotting

Total proteins of the cell were extracted by a BBproExtra Kit (Bestbio, China). The quantification of the protein was measured by a BCL kit (Pierce, Rockford, IL). 10 μg of protein was loaded onto stacking gel and then transferred to the nitrocellulose membrane after separating gel electrophoresis. Subsequently, the membranes were incubated with primary and secondary antibodies. Finally, the proteins were detected by enhanced chemiluminescence (ECL) method. The primary antibodies include anti-PGI2 (1:800, Abcam), anti-t-PA (1:800, Abcam), anti-CXCR4 (1:800, Abcam), anti-GAPDH (1:800, Abcam), anti-PI3K (1:800, Abcam), anti-AKT (1:800, Abcam), anti-ERK1/2 (1:800, Abcam), anti-Bcl-x (1:800, Abcam). All of them were incubated overnight at 4°C. The Horseradish Peroxidase (HRP)-conjugated secondary antibodies (1:4,000, Sigma-Aldrich) was incubated for 2 hours at 37°C.

Confocal laser scanning microscopy

PZ and CXCR4 were labeled with different fluorescent, and co-cultured for 12 and 24 hours, then were made to slides. The binding sites of two proteins were observed under laser confocal laser scanning microscopy.

Statistical analysis

All data were statistically processed using SPSS 24.0 software, and quantitative data were expressed as $\bar{x} \pm s$. ANOVA was used for comparison between multiple groups, with $p < 0.05$ indicating statistically significant.

Results

PZ stimulated the proliferation of HPVEC and up-regulated expression of PGI2 and t-PA

Due to the fact that proliferation is essential for angiogenesis and anticoagulation, in the next step we investigated the effect of PZ for the activity of cells, including proliferation, expression of anticoagulation markers PGI2 and t-PA. As shown in Fig. 1A, the results of CCK8 assay indicated that PZ treatment led to increased proliferation of HPVEC. In Fig. 1B and C, the results of qRT-PCR and western blotting shown that the expression of gene and protein of PGI2 and t-PA were up-regulated.

PZ activated PI3K/Akt/ERK pathway in HPVEC

It is well known that PI3K/Akt/ERK signaling pathway is critical for the regulation of endothelial cell proliferation. Thus, we examined whether PZ affects this pathway in HPVEC. As shown in Fig. 2A, the results of qRT-PCR

revealed the expression of PI3K/Akt/ERK and BCL-x genes were up-regulated by the PZ treatment. In Fig. 2B, the results of western blotting are consistent with qRT-PCR results, showing that the protein level of PI3K/Akt/ERK and BCL-x were promoted by PZ.

PZ stimulated the proliferation and activated signaling pathway by targeting CXCR4

In this section of study, knockdown of CXCR4 was performed to investigate whether PZ induces proliferation of HPVEC and activates PI3K/Akt/ERK signaling by targeting CXCR4. After CXCR4 knockdown, the mRNA level of CXCR4 was down-regulated and the results shown in Fig. 3A, then, HPVEC were treated with PZ. Moreover, as shown in Fig. 3B, PZ treatment promoted HPVEC proliferation, however, this process was suppressed by CXCR4 knockdown. Additionally, the mRNA level of PI3K/Akt/ERK and BCL-x were found to be suppressed by CXCR4 knockdown, and suppression of PI3K/Akt/ERK and BCL-x protein level were confirmed by western blotting, the results shown in Fig. 3C,D.

The binding site of PZ and CXCR4 were located on cell membrane

To investigate the interaction of PZ and CXCR4, a co-cultivation of PZ and CXCR4 which labeled different fluorescent was performed. In this study, we can observe the specific binding location of two proteins. As shown in Fig. 4, the binding site of PZ and CXCR4 were located on cell membrane, and as time went on, they still remained on the surface of the membrane.

Discussion

PE is related to placental circulatory disorders and hypoperfusion, and its complications include abnormal coagulation, which seriously endangers the health of pregnant women (Melchiorre et al. 2022). PZ and vascular endothelial cells play a certain role in anticoagulation and control of thrombosis formation, especially the anticoagulant function of vascular endothelial cells during proliferation (Al-Amer 2022). This function is achieved through vasodilation, inhibition of platelet aggregation, synthesis of antithrombin, and other substances (Neubauer and Zieger

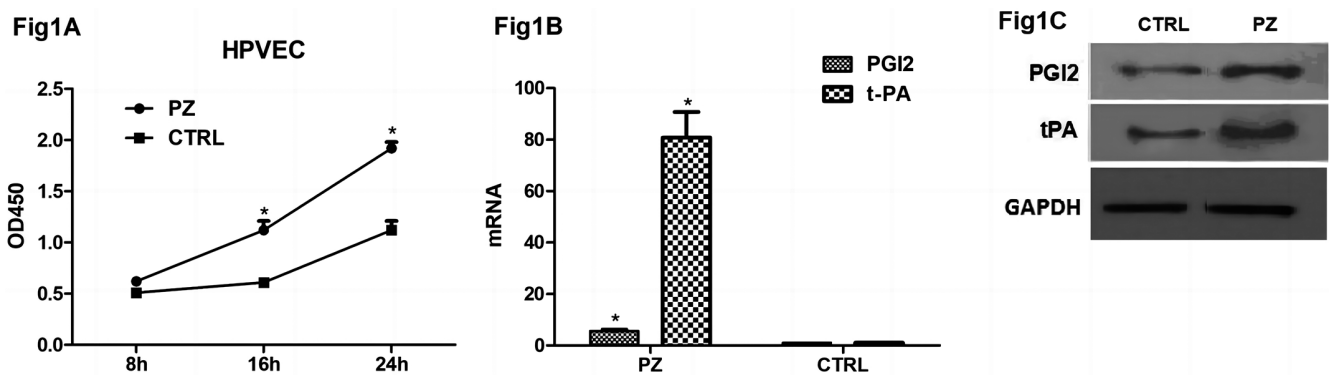

Fig.1. PZ stimulated the proliferation of HPVEC and up-regulated expression of PGI2 and t-PA.
A: The CCK8 assay indicated that PZ treatment led to increased proliferation of HPVEC. B and C: The results of qRT-PCR and western blotting shown that the expression of gene and protein of PGI2 and t-PA were up-regulated. Data are presented as mean ± standard deviation of three independent experiments. *$p < 0.05$ vs. control group.

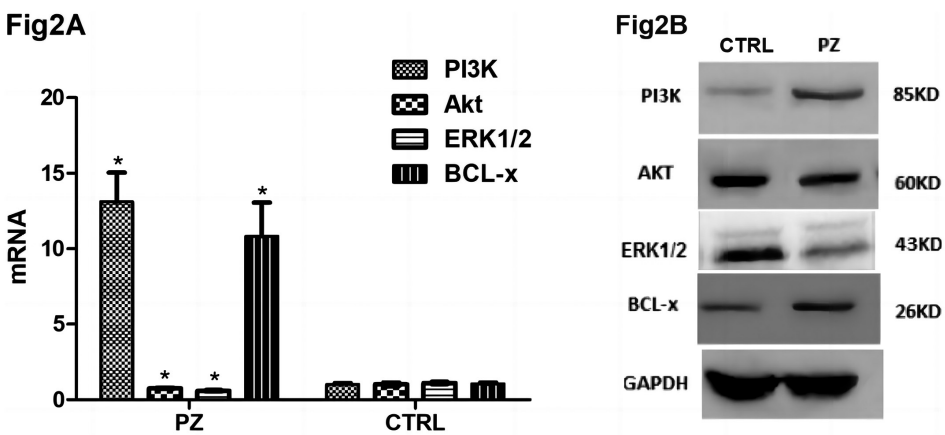

Fig.2. PZ activated PI3K/Akt/ERK pathway in HPVEC.
A: qRT-PCR revealed the expression of PI3K/Akt/ERK genes were up-regulated by the PZ treatment. B: Western blotting showed that the protein level of PI3K/Akt/ERK were promoted by PZ. Data are presented as mean ± standard deviation of three independent experiments. *$p < 0.05$ vs. control group.

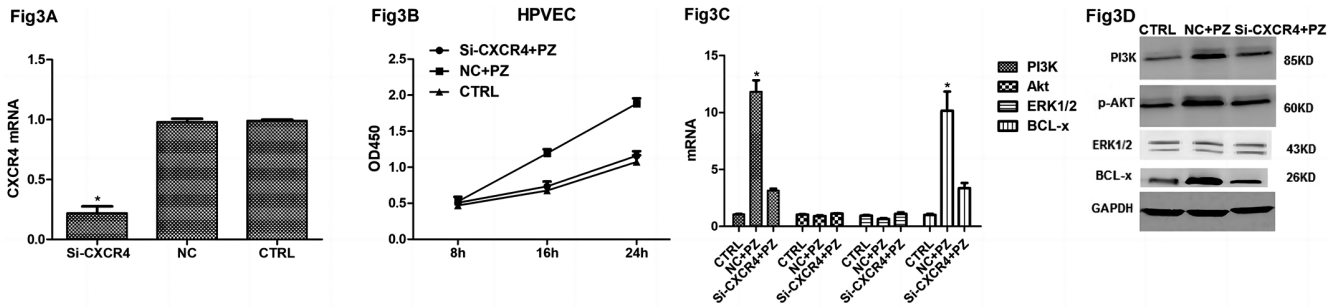

Fig.3. PZ stimulated the proliferation and activated signaling pathway by targeting CXCR4.
A: The expression of CXCR4 mRNA was down-regulated after CXCR4 knockdown. B: PZ treatment promoted HPVEC proliferation, however, this process was suppressed by CXCR4 knockdown. C and D: The mRNA and protein level of PI3K/Akt/ERK were found to be suppressed by CXCR4 knockdown. Data are presented as mean ± standard deviation of three independent experiments. *$p < 0.05$ vs. control and NC group.

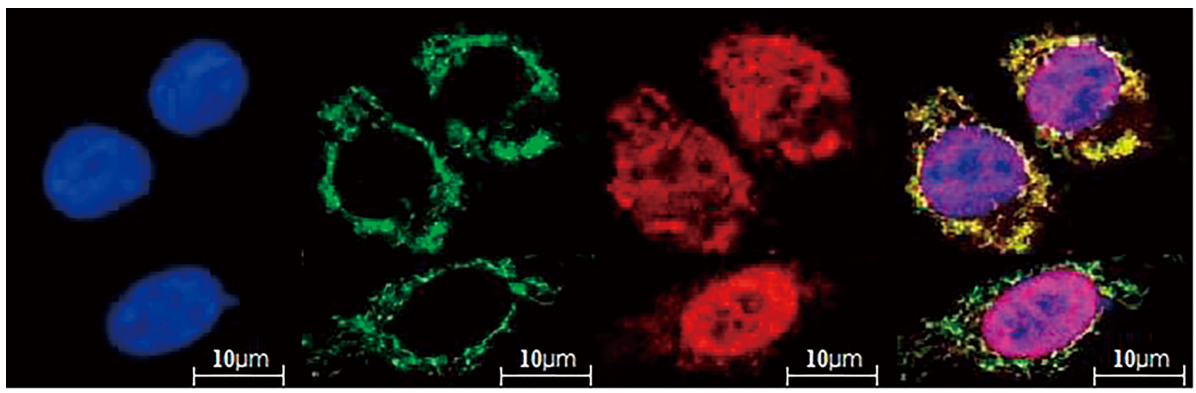

Fig.4. The specific binding location of two proteins.
The binding site of PZ and CXCR4 were located on cell membrane.

2022). Studies have shown that some anticoagulant proteins in the body can act on vascular endothelial cells, promote their proliferation and release a large amount of vasodilators, reduce microvascular resistance, alleviate microcirculation disorders, and prevent the thrombosis formation effectively (Uchiba et al. 2004; Minhas et al. 2010; Xue et al. 2011). However, more molecular details of this process remained to be revealed.

In the present study, we found that cell proliferation can be stimulated by PZ. This is consistent with previous research which published that the anticoagulant protein exerted positive effect for proliferation of endothelial cell (Uchiba et al. 2004). Besides, our results indicated that PZ promoted the expression of anticoagulant markers, such as PGI2 and t-PA. Importantly, results from qRT-PCR and western blotting revealed that PZ stimulated cell proliferation through PI3K/Akt/ERK signaling pathway. This new insight into the mechanism of PZ provides a novel therapeutic agent for PE.

Next, we focused on the research of the regulation mechanisms of endothelial cells by PZ. Because the pathogenesis of PE is related to abnormal placental blood vessels, HPVEC is more suitable for research. HPVEC and PZ were co-cultivated for 24 hours. For the first time, PZ was found to be bind with CXCR4 receptor on cell membrane. Thus, we speculated that there may be an interaction between PZ and CXCR4. For deeper insight into the molecular change of HPVEC, lentiviral vector silencing CXCR4 was constructed and intervened with HPVEC. We identified that CXCR4 knockdown inhibited cell proliferation by PZ treatment. Evidence from qRT-PCR and western blotting indicated that the mRNA and protein levels of PI3K/Akt/ERK were suppressed by CXCR4 knockdown. Probably the signaling pathway that CXCR4 mediated regulation of PI3K/Akt/ERK expression is an essential and critical pathway in proliferation process. To our best of knowledge, the interaction between PZ and CXCR4 is a new mechanism to explain how PZ promotes cell proliferation. Additionally, CXCR4 can be considered as an important switch for this procession. Thus, the development of new therapeutic agents such as PZ by stimulating CXCR4 may be a potential way for the treatment of PE. Additionally, PZ is a Vitamin K-dependent cofactor, so Vitamin K supplements during pregnancy might have some effect on maintaining PZ and preventing PE.

Although there have been many studies on the occurrence and treatment of PE (Agalakova et al. 2021; Medegan and Buhimschi 2024; Zhou et al. 2024), the present study indicated the molecular mechanism of PZ induced cell proliferation. We found that PZ prevents thrombosis by affecting both endothelial cell and anticoagulants. The interaction between PZ and CXCR4 activates PI3K/Akt/ERK

signaling pathway, and induces cell proliferation and expression of anticoagulant markers. Our findings provide new interpretation to explain how PZ improves cell proliferation, but the limitation is that there is no experiment *in vivo*. In the next step, we will conduct the experiment *in vivo* to further confirm these results.

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Conflict of Interest

The authors declare no conflict of interest.

References

Agalakova, N.I., Kolodkin, N.I., Adair, C.D., Trashkov, A.P. & Bagrov, A.Y. (2021) Preeclampsia: Cardiotonic Steroids, Fibrosis, Fli1 and Hint to Carcinogenesis. *Int. J. Mol. Sci.*, **22**, 1941.

Al-Amer, O.M. (2022) The role of thrombin in haemostasis. *Blood Coagul. Fibrinolysis*, **33**, 145-148.

Brouwers, L., van der Meiden-van Roest, A.J., Savelkoul, C., Vogelvang, T.E., Lely, A.T., Franx, A. & van Rijn, B.B. (2018) Recurrence of pre-eclampsia and the risk of future hypertension and cardiovascular disease: a systematic review and meta-analysis. *BJOG*, **125**, 1642-1654.

Cheng, X., Wang, H., Zhang, X., Zhao, S., Zhou, Z., Mu, X., Zhao, C. & Teng, W. (2017) The Role of SDF-1/CXCR4/CXCR7 in Neuronal Regeneration after Cerebral Ischemia. *Front. Neurosci.*, **11**, 590.

Cui, C., Cheng, X., Yan, L., Ding, H., Guan, X., Zhang, W., Tian, X. & Hao, C. (2019) Downregulation of TfR1 promotes progression of colorectal cancer via the JAK/STAT pathway. *Cancer Manag. Res.*, **11**, 6323-6341.

Ghozlan, M.F., Mohamed, A.A.E., Eissa, D.S. & Eldawy, H.S. (2019) Low Protein Z Level: A Thrombophilic Risk Biomarker for Acute Coronary Syndrome. *Indian J. Hematol. Blood Transfus.*, **35**, 339-346.

Karakoyun, S., Ozan Gursoy, M., Kalcik, M., Yesin, M., Gunduz, S., Ali Astarcioglu, M., Bayram, Z., Cakal, B., Bayam, E. & Ozkan, M. (2017) The Role of Protein Z and Protein Z-Dependent Protease Inhibitor Polymorphisms in the Development of Prosthetic Heart Valve Thrombosis. *J. Heart Valve Dis.*, **26**, 460-466.

Markowicz-Piasecka, M., Huttunen, K.M., Broncel, M. & Sikora, J. (2019) Sulfenamide and Sulfonamide Derivatives of Metformin - A New Option to Improve Endothelial Function and Plasma Haemostasis. *Sci. Rep.*, **9**, 6573.

Mastrolia, S.A., Mazor, M., Loverro, G., Klaitman, V. & Erez, O. (2014) Placental vascular pathology and increased thrombin generation as mechanisms of disease in obstetrical syndromes. *PeerJ*, **2**, e653.

Medegan Fagla, B. & Buhimschi, I.A. (2024) Protein Misfolding in Pregnancy: Current Insights, Potential Mechanisms, and Implications for the Pathogenesis of Preeclampsia. *Molecules*, **29**, 610.

Melchiorre, K., Giorgione, V. & Thilaganathan, B. (2022) The placenta and preeclampsia: villain or victim? *Am. J. Obstet. Gynecol.*, **226**, S954-S962.

Minhas, N., Xue, M., Fukudome, K. & Jackson, C.J. (2010) Activated protein C utilizes the angiopoietin/Tie2 axis to promote endothelial barrier function. *FASEB J.*, **24**, 873-881.

Neubauer, K. & Zieger, B. (2022) Endothelial cells and coagulation. *Cell Tissue Res.*, **387**, 391-398.

Sfriso, R., Bongoni, A., Banz, Y., Klymiuk, N., Wolf, E. & Rieben, R. (2017) Assessment of the Anticoagulant and Anti-inflammatory Properties of Endothelial Cells Using 3D Cell Culture and Non-anticoagulated Whole Blood. *J. Vis. Exp.*, 56277.

Uchiba, M., Okajima, K., Oike, Y., Ito, Y., Fukudome, K., Isobe, H. & Suda, T. (2004) Activated protein C induces endothelial cell proliferation by mitogen-activated protein kinase activation in vitro and angiogenesis in vivo. *Circ. Res.*, **95**, 34-41.

Xue, M., Chow, S.O., Dervish, S., Chan, Y.K., Julovi, S.M. & Jackson, C.J. (2011) Activated protein C enhances human keratinocyte barrier integrity via sequential activation of epidermal growth factor receptor and Tie2. *J. Biol. Chem.*, **286**, 6742-6750.

Yu, J.S. & Cui, W. (2016) Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. *Development*, **143**, 3050-3060.

Zhang, L., Segal, A.Z., Leifer, D., Silverstein, R.L., Gerber, L.M., Devereux, R.B. & Kizer, J.R. (2017a) Circulating protein Z concentration, PROZ variants, and unexplained cerebral infarction in young and middle-aged adults. *Thromb. Haemost.*, **117**, 149-157.

Zhang, P., Wu, Q.W., Feng, G., Liu, C.S., Zhang, Y.Y. & Pu, C. (2017b) Protein Z and anti-protein Z IgG levels, but not the promotor A13G polymorphism, are associated with preeclampsia. *Clinical and Experimental Obstetrics & Gynecology*, **44**, 929-933.

Zhou, W., Li, X., Li, X., Liu, Y., Song, W. & Yang, Q. (2024) The role of circular RNA in preeclampsia: From pathophysiological mechanism to clinical application. *Life Sci.*, **338**, 122407.