Tropical Journal of Pharmaceutical Research February 2023; 22 (2): 305-311
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v22i2.12

Original Research Article

Knockdown of PRUNE2 alleviates hypoxia-induced oxidative stress inhibits cell proliferation in trophoblast cells, and reverses LY294002-induced PI3K/AKT pathway inhibition

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Sent for review: 27 October 2022 *Revised accepted: 29 January 2023*

Abstract

Purpose: *To investigate the probable effect of PRUNE2 on viability, motility, and oxidative stress of hypoxia-induced trophoblast cells, and the mechanism of action involved.*
Methods: *Trophoblast cells were treated with 250 μM $CoCl_2$ for 24 h to simulate hypoxia. Then, a cell counting kit (CCK-8), colony formation, and flow cytometry (FCM) were used to determine the role of PRUNE2 in cell viability. Wound closure, as well as Transwell-migration assay, were conducted to evaluate cell motility. Superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), and myeloperoxidase (MPO) levels were determined using the appropriate kits, while the effects of PRUNE2 on PI3K/AKT pathway in trophoblast cells were assessed by immunoblot assay. LY294002 was used to inhibit PI3K/AKT pathway in trophoblast cells.*
Results: *The hypoxia-induced trophoblast cell model was successfully constructed. The data showed that PRUNE2 was overexpressed in hypoxia-induced trophoblast cells, and that ablation of PRUNE2 stimulated proliferation as well as motility of hypoxia-induced trophoblast cells ($p < 0.01$). Depletion of PRUNE2 reduced oxidative stress in the cells. PRUNE2 mediated PI3K/AKT pathway and thus affected the proliferation, motility, and oxidative stress of trophoblast cells ($p < 0.01$). In addition, depletion of PRUNE2 reversed PI3K/AKT signaling and the inhibition of trophoblast proliferation induced by t PI3K/AKT inhibitor LY294002.*
Conclusion: *PRUNE2 depletion alleviates hypoxia-induced oxidative stress, inhibits cell proliferation in trophoblast cells and reverses LY294002-induced PI3K/AKT pathway inhibition. The findings suggest that PRUNE2 could act as a target treatment for PE.*

Keywords: *Preeclampsia, PRUNE2, PI3K/AKT pathway, Trophoblast cells, Oxidative stress*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Preeclampsia (PE) is a pregnancy disorder that occurs after 20 weeks of gestation, and it is characterized by clinical symptoms such as hypertension and proteinuria [1]. It affects 7 - 10 % of pregnancies, and has high maternal and neonatal mortality rates [2]. It is widely believed

that the pathogenesis of PE is related to the migration of trophoblast cells outside the villi and the dysfunction of intrusions into the uterine spiral artery, subsequent disorders of neovascularization, and increased uterine placental vascular resistance [3,4].

Abnormal placental development leads to local hypoxia and ischemia, which is considered to be the main cause of reactive oxygen species overproduction [5]. Under hypoxia, the trophoblast cells are induced by iron death and apoptosis [6]. The exact pathogenesis of PE remains largely unclear. Therefore, there is an urgent need to understand the specific molecular mechanisms of PE to find better treatments and strategies to prevent PE. The BNIP-2 and Cdc42GAP Homology (BCH) domain PRUNE2, also known as BMCC1 and BNIPXL, encodes a 340-kDa protein with a conserved BCH scaffold domain at its C-terminal [7]. Proteins with BCH domains regulate morphogenesis, differentiation, motility, and apoptosis by binding to components of the signaling network (e.g., Rho, Ras, and MAPK signaling). The role of PRUNE2 has been reported in a variety of tumor types. For example, overexpression of PRUNE2 is associated with a favorable prognosis in neuroblastoma and leiomyosarcoma [8]. PRUNE2 also inhibits the progression of colorectal cancer and has been shown to be an inhibitor of human prostate cancer [9]. In addition, PRUNE2 reduces the phosphorylation of AKT and its upstream kinase PDK1 and induced cell apoptosis [10]. Microarray analysis of gene expression profiles in placenta tissues showed that PRUNE2 is up-regulated in the placenta of PE and normal pregnant women, but the function and molecular mechanism of PRUNE2 in PE remain unclear.

A hypoxia-induced trophoblast cell model was constructed to simulate PE. This study was aimed to investigate the possible effects of PRUNE2 on the viability, motility, and oxidative stress of hypoxia-induced trophoblast cells and explore the mechanism.

EXPERIMENTAL

Antibodies and siRNAs

The following antibodies were used: anti-PRUNE2 (1:500 dilutions, ab80262, Abcam), anti-Bcl-2 (1:500 dilutions, ab32124), anti-Bax (1:500 dilutions, ab32503), anti-AKT (1:500 dilutions, ab8805), anti-p-AKT (1:500 dilutions, ab38449), anti-mTOR (1:500 dilutions, ab134903), anti-p-mTOR (1:500 dilutions, ab109268), and anti-β-actin (1:2000 dilutions, ab8226). The siRNAs of PRUNE2 were purchased from Riobio plc (Guangzhou, China). The overexpression plasmids of PRUNE2 were purchased from Addgene. PRUNE2 siRNA: 5'-GGGCCAGAATATCGATGAATT-3'.

Cell culture

The HTR-8/Svneo cells were all purchased from ATCC. The cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and incubated at 37 °C in a 5 % CO_2 incubator. The plasmids and siRNAs were transfected into cells using Lipofectamine 3000. Trophoblast cells were treated with 250 μM $CoCl_2$ for 24 h to simulate hypoxia. Trophoblast cells were incubated with LY294002 (Sigma, USA) at a concentration of 0.5 μM for 24 h.

Quantitative PCR assay

Total RNA was extracted from cells using TRIzol reagent (15596-018, Invitrogen). Quantitative PCR was subsequently conducted via SYBR mixture (RR420A, Takara). PRUNE2 mRNA levels were normalized to GAPDH. The primers used in PCR are shown in Table 1.

Table 1: PCR primer sequences

Primer	Sequence (5'-3')
PRUNE2	Forward: GGGTCTTCTGGGATTATGG
PRUNE2	Reverse: CTGGGCTAACAAGGTCTAC
GAPDH	Forward: AGAAGGCTGGGGCTCATTTG
GAPDH	Reverse: AGGGGCCATCCACAGTCTTC

Immunoblot assay

The cell or tissue samples were lysed with radioimmunoprecipitation assay lysis buffer (RIPA) from Beyotime, China, and the protein concentration was determined bty the BCA method. Then the protein samples were separated using a 10 % SDS-PAGE experiment. The total proteins were sequentially transferred onto PVDF membranes (Millipore, USA). Then, nitrocellulose (NC) membranes were blocked by 5 % dry milk with tris-buffered saline with Tween (TBST) buffer and PRUNE2 and GAPDH antibodies. After washing, the membranes were treated with horseradish peroxidase (HRP)-labeled secondary antibodies for 45 min. Each blot was then visualized using an enhanced chemiluminescence (ECL) kit (GE, SA).

Cell viability assays

For CCK-8 assays, the cells were added into 96-well plates (1000 cells per well) and maintained in media (10 % FBS) at 37 °C. Cells were treated with CCK-8 reagent at 37 °C for 4 h. The relative

cell viability was determined with a microplate spectrophotometer at 490 nm wavelength (Bio-Rad, U.S.A.) for 3 days. For colony formation assays, cells were plated into 6-well plates (1,000 cells per well) and maintained for 14 days with the indicated treatment. Cells were fixed with formaldehyde and stained. The number of colonies was then calculated.

Transwell-migration and invasion assays

Cell culture inserts were coated with matrigel or without 100 µL matrigel (invasion, 1:3 diluted with serum-free media), and 10^5 cells were placed in the upper chamber. The invaded cell undersides were fixed with 4 % paraformaldehyde, stained with 0.2 % crystal violet, and imaged.

Determination of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH), and myeloperoxidase (MPO)

After treatment, the cells were collected for the determination of MDA, SOD, GSH, and MPO with appropriate commercial kits (Jiancheng Bioengineering, China). Cells were homogenized and centrifuged (1,000 g) for 20 min, and the supernatant was collected. The cell samples were then added and gently shaken, mixed, and covered for reaction at 37 °C for 2 h. A microplate reader was then employed immediately to determine the OD value of each well at a wavelength of 450 nm by a microplate reader.

Statistics

GraphPad 6.0 was used for statistical analysis. All data are presented as mean ± standard deviation (SD), and $p < 0.05$ was taken as statistically significant.

RESULTS

PRUNE2 suppressed the viability of hypoxia-induced trophoblast cells

Both qPCR and immunoblot assay results indicate $CoCl_2$ treatment increased the expression of PRUNE2 (Figure 1 A and B). siRNA transfection of PRUNE2 significantly decreased its expression in HTR-8/Svneo cells, whereas the transfection of its plasmids increased PRUNE2 expression (Figure 1 A and B). The results of CCK-8 assays showed that $CoCl_2$ treatment suppressed cell viability. Ablation of PRUNE2 contributed to the growth of trophoblast cells, with an increased OD value at 450 nm wave length, whereas overexpression of PRUNE2 suppressed hypoxia-induced trophoblast cell viability (Figure 1 C). Colony formation assays show that $CoCl_2$ treatment increased the number of colonies (Figure 1 D). PRUNE2 knockdown induced an increase of colony numbers (Figure 1 D), and PRUNE2 overexpression suppressed hypoxia-induced trophoblast cell viability. Immunoblot assays show that $CoCl_2$ treatment stimulated cell apoptosis, resulting in increased Bax expression and decreased Bcl-2 expression (Figure 1 E). PRUNE2 knockdown suppressed cell apoptosis (Figure 1 E), and PRUNE2 overexpression promoted hypoxia-induced trophoblast cell apoptosis. The effects of PRUNE2 on the apoptosis of $CoCl_2$-treated HTR-8/Svneo cells were further confirmed through performing flow cytometry (FCM) assays (Figure 1 F). Therefore, PRUNE2 suppresses the viability of hypoxia-induced trophoblast cells.

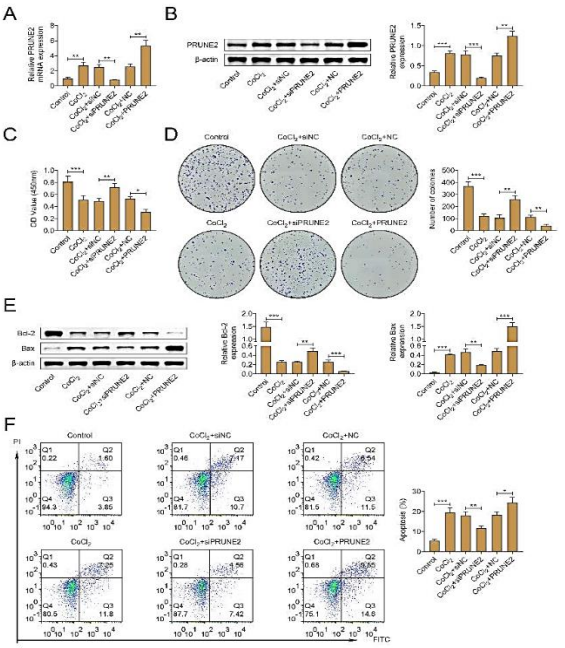

Figure 1: PRUNE2 suppressed the viability of hypoxia-induced trophoblast cells. (A) qPCR showing the mRNA levels of PRUNE2 in HTR-8/Svneo cells upon the indicated treatment. (B) Immunoblot showing the protein levels of PRUNE2 in HTR-8/Svneo cells upon the indicated treatment. (C) The capacity of cell viability of HTR-8/Svneo cells upon the indicated treatment was performed through CCK-8 assays, and the OD value at 450 nm wavelength was measured. (D) The cell viability capacity of HTR-8/Svneo cells upon the indicated treatment was measured through colony formation assays, and the number of colonies was calculated. (E) Immunoblot showing the expressions of Bcl-2 and Bax in HTR-8/Svneo cells upon the indicated treatment. (F) FCM assays showed the effects of PRUNE2 on the apoptosis of HTR-8/Svneo cells upon the indicated treatment. All data are presented as mean ± SD. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control group

PRUNE2 suppressed the motility of hypoxia-induced trophoblast cells

The effects of PRUNE2 on the motility of trophoblast cells were determined using transwell-migration assays. The results show that hypoxia suppressed the motility of trophoblast cells (Figure 2 A and B). PRUNE2 ablation promoted the migration of hypoxia-induced HTR-8/Svneo cells, while PRUNE2 overexpression suppressed cell migration (Figure 2 A). Subsequently transwell-invasion assays were performed, and the results reveal that PRUNE2 knockdown contribute led to the promotion of cell invasion, while overexpression of PRUNE2 suppressed $CoCl_2$-treated cell invasion (Figure 2 B). Therefore, PRUNE2 inhibited the motility of hypoxia-induced trophoblast cells.

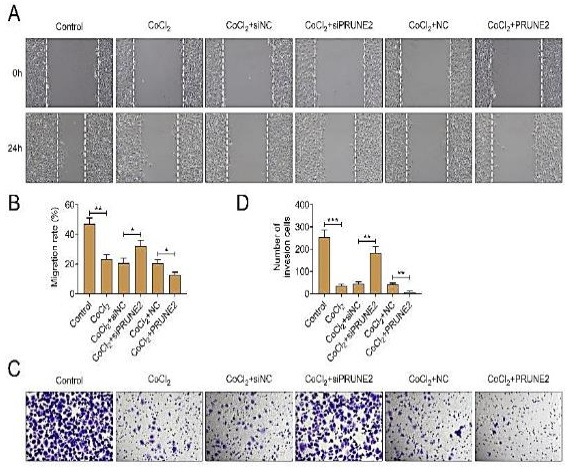

Figure 2: PRUNE2 suppresses the motility of hypoxia-induced trophoblast cells. (A) Wound closure shows the migration capacity of PRUNE2 in HTR-8/Svneo cells upon the indicated treatment. (B). Transwell results showing the invasion degree of PRUNE2 in HTR-8/Svneo cells upon the indicated treatment. The data are represented as mean ± SD. ***P* < 0.01, ****p* < 0.001 compared to control group

PRUNE2 suppressed the oxidative stress of hypoxia-induced trophoblast cells

Hypoxia led to the suppression of the oxidative stress of trophoblast cells with the increase of MDA and MPO and a decrease of SOD and GSH (Figure 3) To further reveal the role of PRUNE2 in oxidative stress, the levels of SOD, MDA, GSH, and MPO were analyzed in hypoxia-induced trophoblast cells. Induction of MDA and MPO, and inhibition of SOD and GSH were found in $CoCl_2$-treated HTR-8/Svneo cells (Figure 3). In addition, PRUNE2 overexpression decreased the levels of SOD and GSH, and increased the levels of MDA and MPO (Figure 3). These results suggest that PRUNE2 suppressed the oxidative stress of hypoxia-induced trophoblast cells.

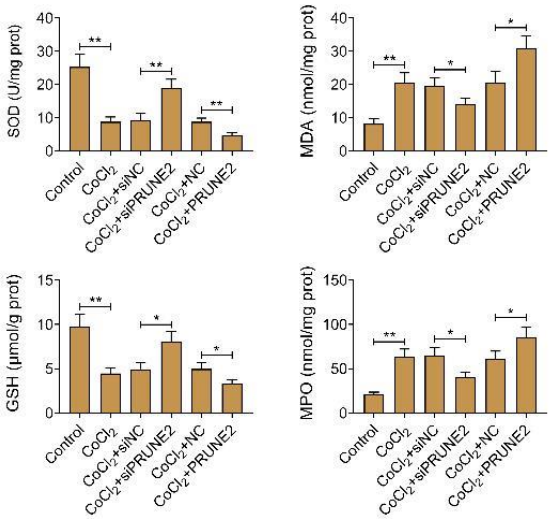

Figure 3: PRUNE2 increased the oxidative stress of hypoxia-induced trophoblast cells. The SOD, GSH, MDA, and MPO levels in control, CoCl2, and the indicated siRNA or plasmid transfection HTR-8/Svneo cells are shown. The data are represented as mean ± SD. **P* < 0.05, ***p* < 0.01 compared to the control group

PRUNE2 mediated PI3K/AKT pathway in PE

Previous research has shown the effects of the PI3K/AKT pathway in PE progression. Using immunoblot assays, $CoCl_2$ treatment was shown to suppress the PI3K/AKT pathway with decreased phosphorylation levels of AKT and mTOR (Figure 4). PRUNE2 depletion increased the phosphorylation levels of AKT and mTOR in $CoCl_2$-induced HTR-8/Svneo cells (Figure 4). In contrast, overexpression of PRUNE2 decreased the phosphorylation levels of AKT and mTOR (Figure 4). Therefore PRUNE2 mediates the PI3K/AKT pathway in PE.

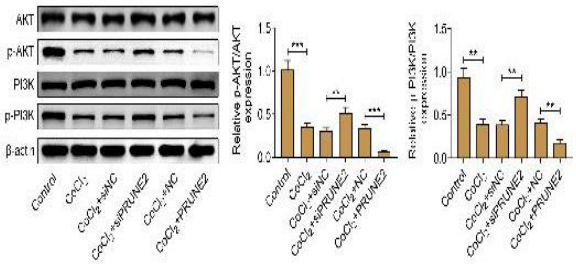

Figure 4: PRUNE2 mediated PI3K/AKT pathway in PE. The level of p-AKT, p-mTOR, AKT, and mTOR in control, $CoCl_2$, and the indicated siRNA or plasmid transfection HTR-8/Svneo cells. The data are presented as mean ± SD. ***P* < 0.01, ****p* < 0.001 compared to control

Knockdown of PRUNE2 reversed PI3K/AKT signaling and trophoblast proliferation inhibition induced by LY294002

The PI3K/AKT pathway inhibitor, LY294002, was used to treat trophoblast cells, and the proliferation capacity was explored. Immunoblot assay results showed that PRUNE2 depletion reversed PI3K/AKT signaling pathway inhibition. These results were confirmed using the reversed phosphorylation levels of PI3K and AKT (Figure 5A). Colony formation assay results show that PRUNE2 also reversed trophoblast proliferation inhibition induced by LY294002 (Figure 5B). Therefore, knockdown of PRUNE2 reversed PI3K/AKT signaling and trophoblast proliferation inhibition induced by LY294002.

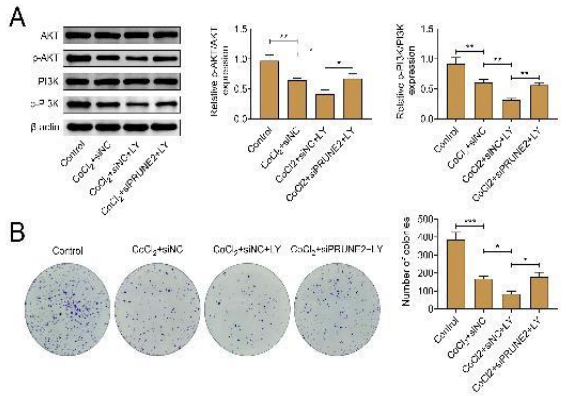

Figure 5: Knockdown of PRUNE2 reversed PI3K/AKT signaling and inhibition of trophoblast proliferation induced by LY294002. (A) Immunoblot showing the protein levels of PRUNE2 in HTR-8/Svneo cells upon the indicated treatment. (B) viability of HTR-8/Svneo cells upon the indicated treatment, was measured through colony formation assays, and the number of colonies was calculated. The data are presented as mean ± SD. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared to control group.

DISCUSSION

Preeclampsia is characterized by elevated blood pressure and proteinuria after 20 weeks of gestation, with symptoms such as headache and dizziness [11]. Eclampsia progresses from PE to a more serious condition that causes convulsions or coma and can lead to severe maternal and infant complications [12]. The etiology of PE is still unknown, and there is no effective treatment other than termination of pregnancy. Existing treatments are aimed at controlling the disease and prolonging the gestational age [13]. Pregnancy should be terminated as soon as PE occurs and the condition is under control [14]. Therefore, it is important to identify new targets of PE and uncover the molecular mechanisms. In this study, PRUNE2 was overexpressed in $CoCl_2$ treated trophoblast cells, and ablation of PRUNE2 stimulated the proliferation and motility of $CoCl_2$ treated trophoblast cells. Depletion of PRUNE2 reduced oxidative stress in the cells. PRUNE2 could serve as a target of PE.

In this study, a $CoCl_2$ treated trophoblast cell model was used to simulate PE. Trophoblast cells were treated with 250 μM $CoCl_2$ for 24 h to simulate hypoxia. Subsequently, using a series of *in vitro* assays (e.g., CCK-8, colony formation, immunoblot, and ELISA), the effects of PRUNE2 on the viability, motility, and oxidative stress of hypoxia-induced trophoblast cells were revealed. PRUNE2 played a critical role in PE progression. In fact, the multiple functions of PRUNE2 in different diseases have been widely revealed, and the role of PRUNE2 in a variety of tumor types has been reported. For example, overexpression of PRUNE2 is associated with a favorable prognosis in neuroblastoma and leiomyosarcoma, PRUNE2 inhibits the progression of colorectal cancer and has been shown to be an inhibitor of human prostate cancer [15]. In addition, PRUNE2 reduce the phosphorylation of AKT and its upstream kinase PDK1 and induced cell apoptosis. PRUNE2 was a prostate cancer suppressor mediated by the lncRNA PCA3 [16]. All these studies confirm PRUNE2 is critical in different diseases.

In a previous study, microarrays were used to analyze gene expression profiles in placenta tissues of pregnant women with PE and those with normal pregnancies [7]. Previous studies found that PRUNE2 was up-regulated in the placenta of pregnant women with PE [17]. Importantly, PRUNE2 was overexpressed in $CoCl_2$ treated trophoblast cells, which is consistent with the previous study. The precise molecular mechanisms should be investigated in the near future.

The C-terminal of PRUNE2 has a conserved BCH scaffold domain. In fact, proteins with BCH domains regulate morphogenesis, differentiation, motility, and apoptosis by binding to components of the signaling network, such as Rho, Ras, and MAPK signaling[18]. Therefore, PRUNE2 may regulate PE progression by affecting related signaling pathways. Interestingly, PRUNE2 regulated the PI3K/AKT signaling pathway, thus affecting PE. Knockdown of PRUNE2 reversed PI3K/AKT signaling and trophoblast proliferation inhibition induced by the PI3K/AKT inhibitor LY294002. These findings further confirm the conclusion that the role of this signaling pathway in PE has been widely revealed and can be used as a potential therapeutic target for PE.

CONCLUSION

A $CoCl_2$-treated trophoblast cell model has been successfully constructed to simulate PE. The findings of this study shows that PRUNE2 is overexpressed in hypoxia-induced trophoblast cells, that ablation of PRUNE2 stimulates proliferation and motility of $CoCl_2$-treated trophoblast cells, and depletion of PRUNE2 reduce oxidative stress in the cells. PRUNE2 also mediates PI3K/AKT pathway and, thus affects the proliferation, motility, and oxidative stress of trophoblast cells. Therefore, PRUNE2 is a potential target for the treatment of PE.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jing Wang and Yanping Zhao designed and carried out the experiments, as well as analyzed and interpreted the data, and prepared the manuscript.

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