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Original Article

The Inhibition of Protein Kinase C β Contributes to the Pathogenesis of Preeclampsia by Activating Autophagy

Huanqiang Zhao[a,b,1], Lili Gong[a,b,1], Suwen Wu[a,b,1], Tianrui Jing[c], Xirong Xiao[a,b], Yutong Cui[a,b], Huangfang Xu[a,b], Huiqing Lu[a,b], Yao Tang[a,b], Jin Zhang[c,e], Qiongjie Zhou[a,b,*], Duan Ma[c,e,**], Xiaotian Li[a,b,d,e,***]

[a] Obstetrics and Gynaecology Hospital, Fudan University, Shanghai, China
[b] The Shanghai Key Laboratory of Female Reproductive Endocrine-Related Diseases, Shanghai, China
[c] Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, Shanghai, China
[d] The Shanghai Key Laboratory of Birth Defects, Shanghai, China
[e] Institutes of Biochemical Sciences, Fudan University, Shanghai, China

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ABSTRACT

Background: Preeclampsia is a devastating hypertensive disorder of pregnancy with unknown mechanism. Recent studies have considered abnormal autophagy as a new cellular mechanism for this disorder, while little is known about how autophagy is specifically involved and what factors are implicated. Here, we report a previously unrecognized preeclampsia-associated autophagic regulator, PKCβ, that is involved in placental angiogenesis.

Methods: PKCβ levels were evaluated by quantitative real-time PCR, western blotting, immunofluorescence and by the analysis of public data. The autophagy-regulating role of PKCβ inhibition in preeclampsia pathogenesis was studied in a mouse model, and in human umbilical vein endothelial cells (HUVECs) and human choriocarcinoma cells (JEG-3).

Findings: PKCβ was significantly downregulated in human preeclamptic placentas. In a mouse model, the selective inhibition of PKCβ by Ruboxistaurin was sufficient to induce preeclampsia-like symptoms, accompanied by excessive autophagic flux and a disruption in the balance of pro- and anti-angiogenic factors in mouse placentas. In contrast, autophagic inhibition by 3-methyladenine partially normalized hypertension, proteinuria and placental angiogenic imbalance in PKCβ-inhibited mice. Our *in vitro* experiments demonstrated that PKCβ inhibition activated autophagy, thus blocking VEGFA-induced HUVEC tube formation and resulting in the significant upregulation of sFLT1 and downregulation of VEGFA in JEG-3 cells.

Interpretation: These data support a novel model in which autophagic activation due to PKCβ inhibition leads to the impairment of angiogenesis and eventually results in preeclampsia.

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1. Introduction

Preeclampsia (PE), a devastating hypertensive disorder affecting ~8% of pregnancies, involves significant alterations in pathophysiological features, including abnormal autophagy and angiogenesis [1]. This syndrome is pregnancy-specific and characterized by the onset of hypertension and proteinuria after 20 weeks of gestation [1]. Elucidation of the specific mechanism involved has long been considered as one of the toughest challenges in preeclampsia, thereby limiting the establishment of mechanism-based preventative and therapeutic strategies.

* Corresponding author: Dr. Qiongjie Zhou, Obstetrics and Gynaecology Hospital of Fudan University, Fangxie Road 419, Huangpu District, Shanghai 200011, China. Tel: +86-21-63455050.
** Corresponding author: Dr. Duan Ma, Key Laboratory of Metabolism and Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, 130 Dong an Rd, Shanghai 200032, China. Tel: +86-21-54237414.
*** Corresponding author: Dr. Xiaotian Li, Obstetrics and Gynaecology Hospital of Fudan University, Fangxie Road 419, Huangpu District, Shanghai 200011, China. Tel: +86-21-63455050.
E-mail addresses: zhouqiongjie1732@fckyy.org.cn (Q. Zhou), duanma@fudan.edu.cn (D. Ma), xiaotianli555@163.com (X. Li).
[1] These authors contributed equally to this work.

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Research in context

Evidence before this study

Preeclampsia is a devastating hypertensive disorder of pregnancy with unknown mechanism. The current insight into the etiology of preeclampsia is focused on abnormal autophagic flux in placenta, while little is known about how autophagy is involved and what initial factors are related. Evidence showed that Protein kinase C isoform β (PKCβ) played an important role in the regulation of autophagy in HEK293 cells. Besides, hypermethylated PKCβ was identified in human preeclamptic placentas. However, there is no direct evidence to support the fact that PKCβ is involved in the pathogenesis of preeclampsia.

Added value of this study

In this study, we report the causal relationship between selective inhibition of PKCβ and preeclampsia. We found that: (i) PKCβ was significantly downregulated in human preeclamptic placentas; (ii) the selective inhibition of PKCβ was sufficient to induce preeclampsia-like symptoms in mice; (iii) PKCβ inhibition led to excessive autophagic flux and an angiogenic imbalance in mouse placentas; (iv) autophagic inhibition by 3-MA partially normalized hypertension, proteinuria and placental angiogenic imbalance in PKCβ inhibited mice; (v) the inhibition of PKCβ blocked VEGFA-induced HUVEC tube formation and resulted in the upregulation of sFLT1 and downregulation of VEGFA in JEG-3 cells by activating autophagy.

Implications of all the available evidence

This study reveals the autophagy-regulating role of PKCβ in the pathogenesis of preeclampsia. Our results raise the possibility that restoration of PKCβ levels in PKCβ-deficient women may be an efficient means of treating placental diseases, such as, but not limited to, preeclampsia.

The current insight into the etiology of preeclampsia is focused on abnormal autophagic flux in placenta [2-5]. Autophagy is a self-degradative process that produces energy from cytoplasmic contents and subsequently prevents the accumulation of waste products [4]. An appropriate level of autophagy is essential to maintain physiological homeostasis and energetic balance [4,6]. Growing evidence showed autophagy participated physiologically in preimplantation development and placentation [7,8]. Compared with normal pregnancies, excessive levels of autophagic activity have been identified in preeclamptic placentas [3], which is considered to represent a potential cause for inadequate trophoblast invasion in preeclampsia [3,4,9]. Additionally, angiogenesis, an essential but defective process in preeclampsia, has been recently reported to be regulated by autophagy during tumorigenesis [10,11]; however, direct evidence of autophagy's involvement in human placental angiogenesis is scarce, and the initial factors that lead to this process remain poorly understood.

A previous experimental study supported the autophagy-regulating role for protein kinase C isoform β (PKCβ)(12). PKCβ is a predominant conventional isoform of the protein kinase C (PKC) family, and is a serine/threonine protein kinase known to be involved in various cellular signal transductions [13]. Although previous studies linked several members of the PKC family to the pathogenesis of preeclampsia and abnormal placental development [14-17], very little has been carried out on the specific role of PKCβ in preeclampsia. In fact, the regulation of angiogenesis is one of the key roles of these multifunctional isoenzymes in the PKC family, including PKCβ [18-20]. In addition, it has been shown that PKCβ was downregulated in preeclamptic placentas [21]. This observation of aberrant expression and the dual function (i.e., the regulation of autophagy and angiogenesis) of PKCβ led us to hypothesize that this molecule may be involved in the pathogenesis of preeclampsia.

Here, we demonstrated that PKCβ levels in the placenta are reduced in pregnancies complicated by preeclampsia. Furthermore, we provided the first line of *in vivo* and *in vitro* evidence that PKCβ is a novel factor that plays an important role in the pathogenesis of preeclampsia by modulating autophagy. This study provides further understanding of the molecular mechanisms underlying the pathogenesis of preeclampsia.

2. Methods

The data that support the findings of this study are available from the corresponding author upon request.

2.1. Subjects and the Collection of Human Placentas

Placental samples obtained from preeclamptic and control subjects were analysed in a case–control study design at the Obstetrics and Gynaecology Hospital of Fudan University. Eligible subjects were randomly selected from women who delivered by elective caesarean section and had stored placental samples available for analysis. All participants provided written informed consent for the use of their placenta tissues. The study was approved by the Ethics Committee of Obstetrics and Gynaecology Hospital of Fudan University.

Preeclampsia was defined according to the 2013 ACOG (The American College of Obstetricians and Gynaecologists) Hypertension Guidelines [22]. In brief, patients were diagnosed with preeclampsia when blood pressure ≥140 mm Hg systolic, or 90 mm Hg diastolic, on at least two occasions 4 h apart, with (or without) positive urinary protein testing (≥300 mg per 24 h) after 20 weeks of gestation. Severe preeclampsia was defined as a blood pressure ≥160 mm Hg systolic, or 110 mm Hg diastolic, accompanied by organ dysfunction. Controls were pregnant women who did not develop preeclampsia or other complications mentioned in the exclusion criteria below.

Cases involving multiple pregnancies and transplanted organs, or those that were complicated by pre-existing chronic conditions (e.g., chronic hypertension), pregnancy complications other than preeclampsia (e.g., diabetes mellitus), other complications (e.g., autoimmune diseases and oncological diseases), and any known foetal anomalies, were excluded.

The sample size of 26 placental samples in each group was calculated by PASS software based on the PKCβ expression data from the GSE75010 dataset in Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/gds/). Specifically, the calculation was assuming α=0.05, β=0.20, the outcome mean±SD (standard deviation) as 1±0.15 and 0.9±0.10 for control and preeclampsia groups, respectively, and using a 1:1 ratio between groups. Eventually, we selected 30 control and severe preeclamptic samples, respectively, to detect a statistical difference of PKCβ expression.

2.2. Animal Experiments

Adult male and female C57BL/6J mice were purchased from Jiesijie Laboratory (Shanghai, China). All animal protocols were performed in accordance with the guidelines issued by Fudan University for the care and use of laboratory animals. The mice were housed in a temperature- and humidity-regulated environment with free access to standard chow and water. Photoperiod was controlled automatically with 12-h light-dark cycles. Prior to the experiments, all animals were acclimated for one week. For all timed pregnancy experiments, virgin female mice (8–12 weeks old), and stud male mice, were mated overnight at a 2:1 ratio. The day on which a copulation plug appeared was recorded as embryonic day 0.5 (E0.5). Plugged females were then removed from the stud cage and placed into another cage for subsequent experiments. To explore the causal relationship between PKCβ inhibition and

preeclampsia, Ruboxistaurin (RBX, 10 mg/kg/d), a selective and ATP-competitive PKCβ inhibitor [23], was administered to the mice by gavage from E7.5 to E14.5, a period that was approximately equivalent to murine placental development. For the *in vivo* inhibition of autophagy, the mice were intraperitoneally injected with a dose of 20 mg/kg/d of the autophagic inhibitor 3-methyladenine (3-MA, Sigma-Aldrich, MO, USA) from E7.5 to E14.5. We selected DMSO, the solvent of RBX and 3-MA, as the vehicle control.

2.3. Measurements of Blood Pressure and Urinary Protein in Mice

To evaluate the preeclampsia symptoms of animals, blood pressure and urinary protein were measured. We determined the indirect blood pressure in conscious mice by tail cuff plethysmography using the Visitech System BP2000 (Apex, NC, USA). All animals were habituated to the measurement procedure 10 times a day from E4.5–6.5 before blood pressure was formally evaluated. At least 10 consecutive measurements were recorded, but only when the condition of the mice was stable. Seven time points (E7.5, E9.5, E11.5, E13.5, E15.5, E17.5, and after delivery) were selected for assessing blood pressure levels throughout the pregnancy. Twenty four-hour urine samples were collected from the mice in metabolic cages from E16.5 to E17.5, and the volumes were then recorded. Urine was frozen at -80°C until further analysis. Urinary protein concentrations were quantified using a Bradford Protein Assay Kit (Beyotime Biotechnology, Beijing, China), according to the manufacturer's instructions.

2.4. Differential Expression Analysis of mRNA

An mRNA microarray dataset was downloaded from the GEO database and used to confirm the downregulation of PKCβ in human preeclamptic placentas. This placental mRNA microarray data, including 53 placental tissues from normotensive pregnancies and 63 placental tissues from preeclamptic pregnancies, were derived from GSE75010 after excluding 41 pregnancies with chronic hypertension. After normalization and log2-transformation of the raw data, the two-tailed unpaired Student's *t* test was used to evaluate whether the expression of PKCβ was differential between normotensive and preeclamptic pregnancies.

2.5. Histopathology and Immunofluorescence

For histological analysis and assessment of mouse kidney and placenta changes, H&E (haematoxylin & eosin) staining was performed. At E17.5, when urine collection had been completed, the mice were euthanized and samples of kidney and placenta were collected. A small aliquot of each sample was fixed in 4% paraformaldehyde for at least 12 h. Subsequently, these tissues were paraffin-embedded, sectioned to a thickness of 4 μm, and stained using standard H&E

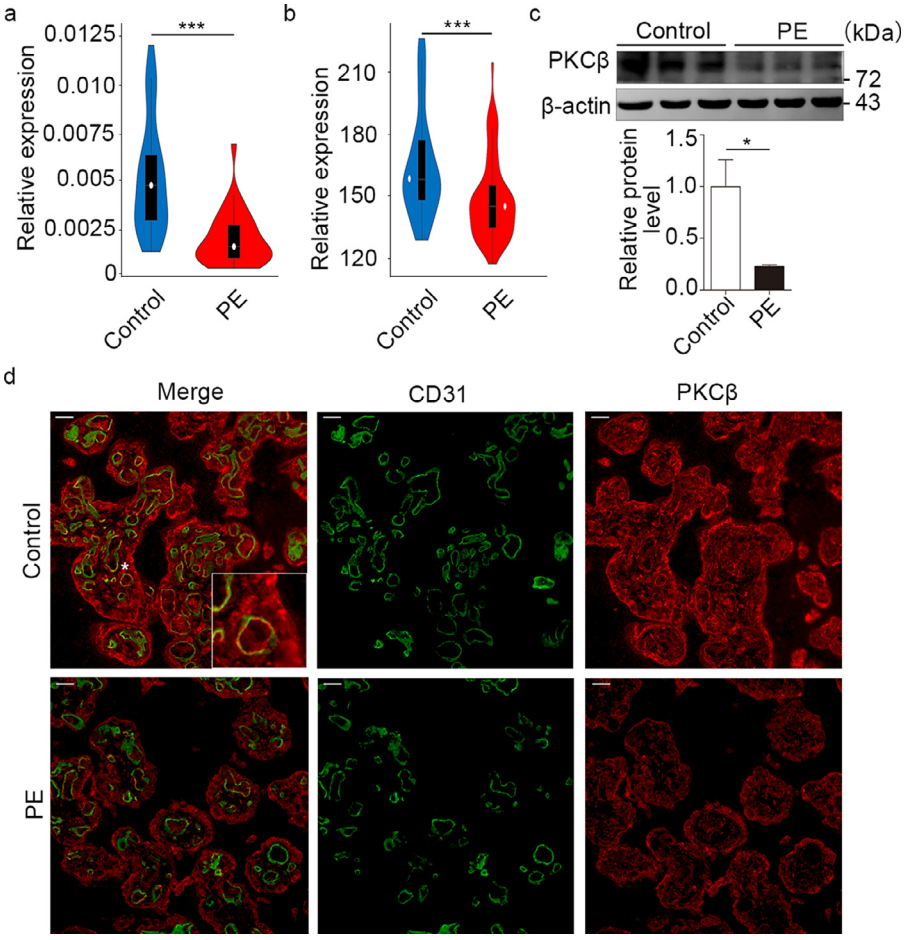

Fig. 1. Decreased PKCβ levels in placentas from pregnant women with preeclampsia. **(a)** Violin plots showing qRT-PCR analysis of placental PKCβ mRNA expression in placentas from normotensive controls (n=30) and preeclampsia patients (n=30), $^{***}P<0.001$ (Mann–Whitney U test). **(b)** Violin plots showing normalized placental PKCβ mRNA expression from preeclampsia (n=63) and normotensive controls (n=53) by analysis of a Gene Expression Omnibus (GEO) dataset (GSE75010), $^{***}P<0.001$ (Mann–Whitney U test). **(c)** Top panel: representative western blot image of PKCβ protein expression in placentas from normotensive and preeclampsia pregnancies. Bottom panel: quantitative results determined by densitometry of the Western blot bands are shown relative to the control (The analysis was based on ≥3 samples per group, $^{*}P<0.05$, Mann–Whitney U test). **(d)** Representative immunofluorescence image of double labelling (PKCβ and CD31) showing PKCβ protein expression and localization in placentas from normotensive pregnancies and preeclampsia patients (n=5 in each group. Scale bars, 20 μm).

techniques. The human and mouse placental immunofluorescence was performed as previously described [24] using an anti-PKCβ antibody (ab189782) from Abcam (Cambridge, UK) or an anti-LC3 antibody (L8918, RRID: AB_1079382) from Sigma-Aldrich (MO, USA). The numbers of LC3 puncta were counted according to previous guidelines [25] and using ImageJ software (RRID: SCR_003070).

2.6. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from human placental tissues by Trizol reagent (Invitrogen, CA, USA), in accordance with the manufacturer's instructions. A Nanodrop 2000 (Thermo fisher scientific, MA, USA) was used to measure the amount and purity of the RNA extracted. cDNA was then synthesized from total RNA via reverse transcription using the Primescript RT reagent kit with gDNA Eraser (Takara, Shiga, Japan). Next, qRT-PCR was performed using a TB Green Premix Kit (Takara) on an Eco™ quantitative PCR system (Illumina, CA, USA), in accordance with the manufacturer's protocols. Relative gene expression was calculated by the comparative CT method, with β-actin as the internal control. The following primers were used: PKCβ, 5′-CCCTCAACCCTGAGTGGAAT-3′ and 5′-CTTAAACCAGCCATCAACACTGG-3′, β-actin, 5′-AGAGCTACGAGCTGCCTGAC-3′ and 5′ -AGCACTGTGTTGGCGTACAG -3′.

2.7. Western Blot Analysis

Protein extracts from human and mouse placental tissues, and cell lines, were obtained using RIPA lysis buffer in the presence of 1 mmol/L PMSF (Beyotime Biotechnology). The protein concentration was then determined by a Bradford protein assay kit (Beyotime Biotechnology). Proteins were separated on SDS-PAGE gels of an appropriate concentration, and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat milk, the membranes were probed with primary antibodies against PKCβ (Abcam, ab189782), LC3 (L8918, RRID: AB_1079382, Sigma-Aldrich), p62 (sc-28359, RRID: AB_628279, Santa Cruz Biotechnology), VEGFA (19003-1-AP, RRID: AB_2212657, Proteintech), sFlt-1 (ab32152, RRID: AB_778798, Abcam), mTOR (2983, RRID: AB_2105622, Cell Signaling Technology), and Phospho-mTOR (Ser2448) (5536, RRID: AB_10691552, Cell Signaling Technology), followed by incubation with goat anti-rabbit (7074, RRID: AB_2099233, Cell Signaling Technology) /mouse (CW0102, RRID: AB_2814710, CWBio) IgG, horseradish peroxidase (HRP)-linked secondary antibody, as required. Visualization of the blots was achieved by enhanced chemiluminescence (ECL) using NcmECL Ultra (New Cell & Molecular Biotech, Suzhou, China). β-actin (60008-1-Ig, RRID: AB_2289225, Proteintech) was used as the internal standard.

2.8. Tube Formation Assay

Forty-eight-well plates were precoated with 100 μl of Matrigel (BD Biosciences, CA, USA) and incubated at 37°C for 2 h. VEGFA (40 ng/ml) was used to induce Human umbilical vein endothelial cells (HUVECs) tube formation with reference to previous reports [26,27]. To investigate the effects of PKCβ- and autophagy-inhibition on angiogenesis, HUVECs were treated with VEGFA (40 ng/ml), RBX (100 nmol/L) and 3-MA (5 mmol/L), alone, or in combination, as required, in ECM media supplemented with 3% FBS for 24 h in 6-well plates (Hangzhou Xinyou Biotechnology Co., Ltd, China). DMSO, the solvent used for RBX and 3-MA, was selected as the vehicle control. Then,

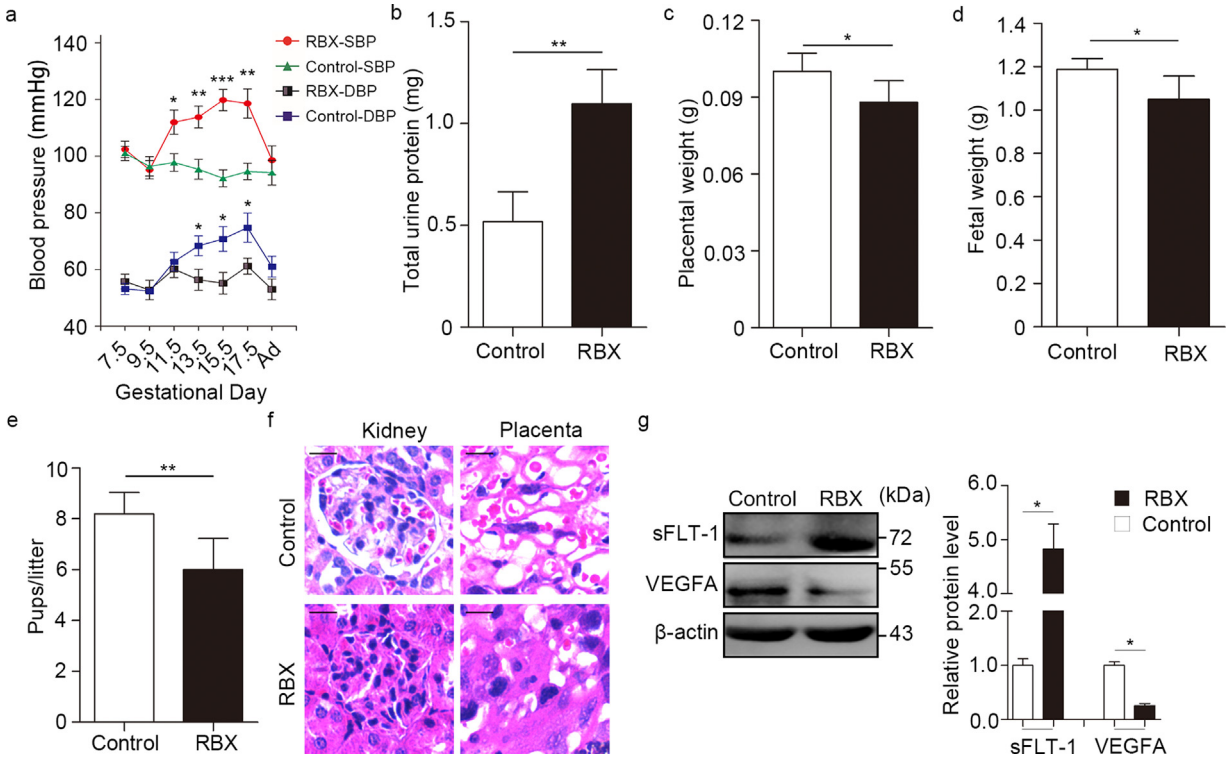

Fig. 2. PKCβ-inhibited pregnant mice exhibited preeclampsia-like features. **(a)** Systolic blood pressure (SBP) and diastolic blood pressure (DBP) measurements of control and RBX-treated pregnant mice at the indicated gestational day (n=5 mice per group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs Control group at the same time points, Repeated measures analysis of variance). **(b-f)** PKCβ inhibition significantly increased the **(b)** 24 h total urinary protein level measured at E17.5 (n=5 mice per group, $^{**}P<0.01$, Mann–Whitney U test) and decreased the **(c)** placenta weight (n=41 and 30 placentas in control and RBX groups, respectively, $^{*}P<0.05$, Student's *t* test), **(d)** foetal weight (n=41 and 30 foetuses in control and RBX groups, respectively, $^{*}P<0.05$, Student's *t* test) and **(e)** number of pups per litter (n=5 mice per group, $^{**}P<0.01$, Mann–Whitney U test). **(f)** Glomerular endotheliosis and abnormal stromal collagen deposition were evident in the kidney and placental labyrinth layer, respectively. Scale bars, 20 μm. (**g**) Left panel: placental samples from PKCβ-inhibited and control mice were analysed for sFlt-1 and VEGFA expression by immunoblot, and a representative blot is presented. Right panel: quantitative results determined by densitometry of the Western blot bands are shown relative to the control (The analysis was based on 5 placental samples per group, $^{*}P<0.05$, Mann–Whitney U test).

HUVECs were seeded at a density of 10^5 cells per well in 48-well plates. After 4 h, the tube-like structures were labelled with a green tracer (Thermo Fisher Scientific) for 2 min at 37°C. Appropriate images were acquired by fluorescence microscopy. Three key parameters were then quantified by Image J Software (RRID:SCR_003070): total tube length, branching point count, and tube count.

2.9. Cell Culture and Lentiviral Transduction

JEG-3, HUVEC, and HTR-8/SVneo cells were routinely cultured in DMEM, ECM, and RPMI-1640 medium, respectively, with 10% foetal bovine serum (Gibco, Carlsbad, CA, USA). In order to prepare the lentivirus encoding the short hairpin RNA (shRNA) targeting PKCβ, a unique sequence of the PKCβ open reading frame (5'-CCTGTCAGATCCCTACG-TAAA-3'), was cloned into the pGreen−CMV−puro−vector plasmid. Scrambled shRNA (5'-TTCTCCGAACGTGTCACGTC-3') was used as a negative control. Viral particles were produced by lipofectamine 3000 transfection into human embryonic kidney fibroblasts. Cells were infected with lentiviruses in the presence of 8 μg/ml polybrene (Genomiditech, Shanghai, China) for 24 h. Then cells were selected by 1μg/ml of puromycin. Knockdown of PKCβ was confirmed by western blotting.

2.10. Statistical Analysis

Statistical analyses were performed using SPSS version 20.0 (SPSS Inc., IL, USA). The normality of data was analysed by the Kolmogorov-Smirnov normality test. The differences between groups were analysed by the Student's *t* test for continuous variables that were distributed normally and by the Mann−Whitney U test for continuous variables with a non-normal distribution or a sample size ≤5. The Chi-square test or Fisher' exact test was used for categorical variables. One-way analysis of variance (ANOVA) followed by a Tukey's post hoc test was used to analyse normally distributed baseline variables across multiple groups. The Kruskal-Wallis test, with Bonferroni correction, was used if the data were not normally distributed. Binary logistic regression was used to examine the association between placental PKCβ levels and the risk of developing preeclampsia by calculating unadjusted and adjusted odds ratios (ORs). Maternal age and pre-gestational body mass index (BMI) were included in the analysis of qRT-PCR results, and the GSE75010 dataset analysis, as potential confounders regardless of statistical significance, since these factors are associated with preeclampsia [28]. Gestational age was regarded as a potential confounder only when it altered the association between PKCβ level and preeclampsia by >10%. Repeated measures analysis of variance was used for blood pressure analysis followed by the Bonferroni post hoc test as required. A *P* value less than 0.05 was regarded as statistically significant. All the exact *P* values calculated in this study are shown in **Supplementary Table 1.**

3. Results

3.1. Clinical Characteristics of the Study Population

The clinical characteristics of preeclampsia patients (n=30) and normotensive controls (n=30) are shown in **Supplementary Table 2**. Maternal age and pre-gestational body mass index (BMI) were comparable between the two groups. However, there were significant differences between the normotensive and preeclampsia groups in terms of the gestational age at delivery, highest systolic and diastolic blood pressures, and newborn birth weight.

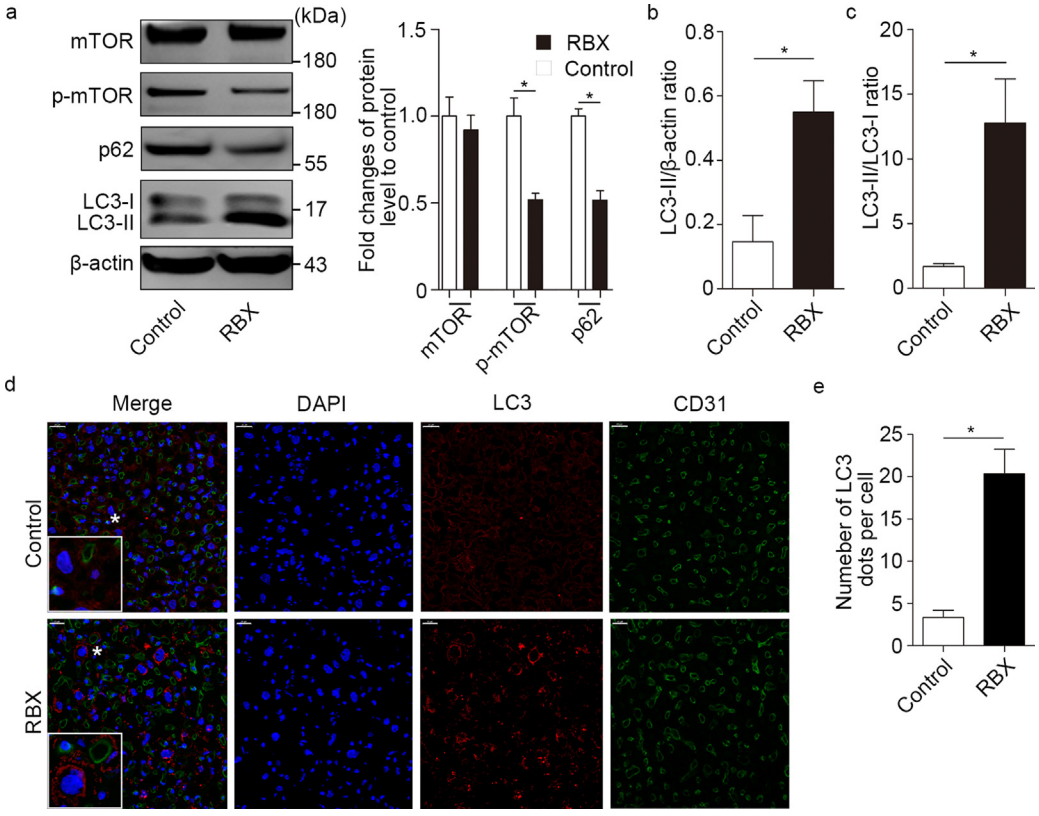

Fig. 3. The inhibition of PKCβ augments autophagy in mouse placentas. **(a)** Left panel: Western blot analysis of murine placental mTOR, p-mTOR, p62, and the conversion of LC3-I to LC3-II with PKCβ inhibition. Right panel: quantitative results determined by densitometry of the Western blot bands (mTOR, p-mTOR and p62) are shown relative to the control. Quantification of LC3-II levels was performed by densitometry of **(b)** LC3-II/β-actin and **(c)** LC3-II/LC3-I ratio. **(d)** Visualization of LC3 puncta in the placentas of mice. Placental sections of RBX-treated pregnant mice and controls were triple stained with anti-LC3 (red), anti-CD31 antibody (green) and DAPI (blue). Representative images are shown. Scale bars: 20 μm. **(e)** Quantitative results of the data are depicted in (d) (All the analyses were based on 5 placental samples per group, $^*P<0.05$, Mann−Whitney U test).

3.2. Aberrant Expression of PKCβ in Preeclamptic Placentas

To investigate the potential association between PKCβ and preeclampsia, we first evaluated PKCβ mRNA and protein levels in placentas from pregnant women with and without preeclampsia. qRT-PCR revealed a significant reduction of PKCβ mRNA expression in preeclamptic placentas compared with normal placentas (Fig. 1a). After adjusting this difference for maternal age, BMI and gestational age, PKCβ mRNA expression remained significantly lower in the preeclampsia group (**Supplementary Table 3**). This downregulation of PKCβ in preeclampsia was confirmed by the subsequent analysis of PKCβ mRNA expression in 63 preeclamptic and 53 control placentas from a GEO dataset (GSE75010) (Fig. 1b and **Supplementary Fig. 1a**); this dataset is the largest one, thus far, containing the gene expression data of normal and preeclamptic placental samples. After adjusting for maternal age and BMI, PKCβ mRNA expression in GSE75010 remained downregulated in preeclampsia (**Supplementary Table 3**). Western blotting further showed a reduction in PKCβ protein levels in three representative controls in comparison with three preeclamptic placentas (Fig. 1**c**). The immunostaining of placental sections revealed the downregulation of PKCβ in preeclamptic placentas, and also showed that PKCβ was predominantly expressed in syncytiotrophoblasts and vascular endothelial cells in normal placentas at term (Fig. 1**d and Supplementary Fig. 1b**). Collectively, these data suggest that PKCβ was downregulated in preeclamptic placentas and highlight the likelihood that PKCβ exerts certain biological functions in the pathogenesis of preeclampsia.

3.3. The Selective Inhibition of PKCβ by RBX Induces Preeclampsia-like Symptoms in Mice

To confirm whether PKCβ is a causal factor of preeclampsia, we administered RBX (10 mg/kg/d), a specific inhibitor of PKCβ, into pregnant and non-pregnant mice by gavage to determine whether the inhibition of PKCβ induces preeclampsia-like features. The PKCβ activity in mouse placentas was significantly decreased with RBX treatment (**Supplementary Fig. 2a**). The inhibition of PKCβ by RBX induced preeclampsia-like symptoms in pregnant mice as evidenced by hypertension (Fig. 2**a and Supplementary Table 4**) and proteinuria (Fig. 2**b**). The overall systolic and diastolic blood pressure changes at the indicated gestational day can be seen in Figure 2**a**. Specifically, RBX treated pregnant mice exhibited an increase in blood pressure, which began to rise at E11.5, reached a peak at E15.5, and continued until delivery. In a manner similar to human recovery after delivery, the blood pressure eventually returned to normal postpartum. The variation of blood pressure from E11.5 to postpartum were comparable to those of other mouse models of preeclampsia [29-31]. In non-pregnant mice, it should be noted that RBX did not affect the systolic blood pressure (**Supplementary Fig. 2b**), further simulating the pregnancy-specific feature of preeclampsia. In addition, severe proteinuria was observed in RBX-treated pregnant mice at E17.5 (Fig. 2**b**), whereas the 24 h total urinary protein levels showed no significant difference when compared between RBX treated non-pregnant mice and controls (non-pregnant mice) (data not shown).

Symptoms of preeclampsia in PKCβ-inhibited mice were accompanied by a significant reduction in placental weight (Fig. 2**c**), foetal growth restriction (Fig. 2**d**), and a reduced number of viable foetuses (Fig. 2**e**), as expected, compared to control mice. The morphological changes in preeclampsia are mainly reflected in the kidney and placenta [32,33]. Inhibition of PKCβ induced typical preeclampsia-related renal morphological characteristics including glomerular endotheliosis and capillary occlusion in pregnant mice (Fig. 2**f**). Moreover, placentas from PKCβ inhibited mice isolated at E17.5 exhibited obvious abnormal stromal collagen deposition in the labyrinth layer (Fig. 2**f**).

Abnormal placental angiogenesis resulting from failing to maintain the balance of proangiogenic (e.g. soluble vascular endothelial growth factor receptor 1, sFLT1) and antiangiogenic (e.g. vascular endothelial growth factor A, VEGFA) factors has been implicated in preeclampsia pathogenesis. Analogous to preeclampsia patients [34], the PKCβ inhibition induced an angiogenic imbalance as evidenced by the significant downregulation of VEGFA and upregulation of sFLT1 in RBX-treated murine placentas (Fig. 2**g**).

Collectively, these data suggest that the PKCβ inhibition is a potential cause for preeclampsia.

3.4. The Inhibition of PKCβ Augments Autophagy in Mouse placentas

Impaired autophagy is known to induce poor placentation in preeclampsia [4,6]. Considering that PKCβ serves as a key regulator of autophagy by modulating the mitochondrial energy status in HEK293 cells [12], we attempted to determine whether the inhibition of PKCβ reproduces this process *in vivo*. As shown in Fig. 3**a,** the activity of mammalian target of rapamycin (mTOR), an essential inhibitor of autophagy, was significantly inhibited in the placentas of RBX-treated mice in comparison with control mice. The placentas of RBX-treated mice also exhibited enhanced conversion rates of LC3 I to LC3 II

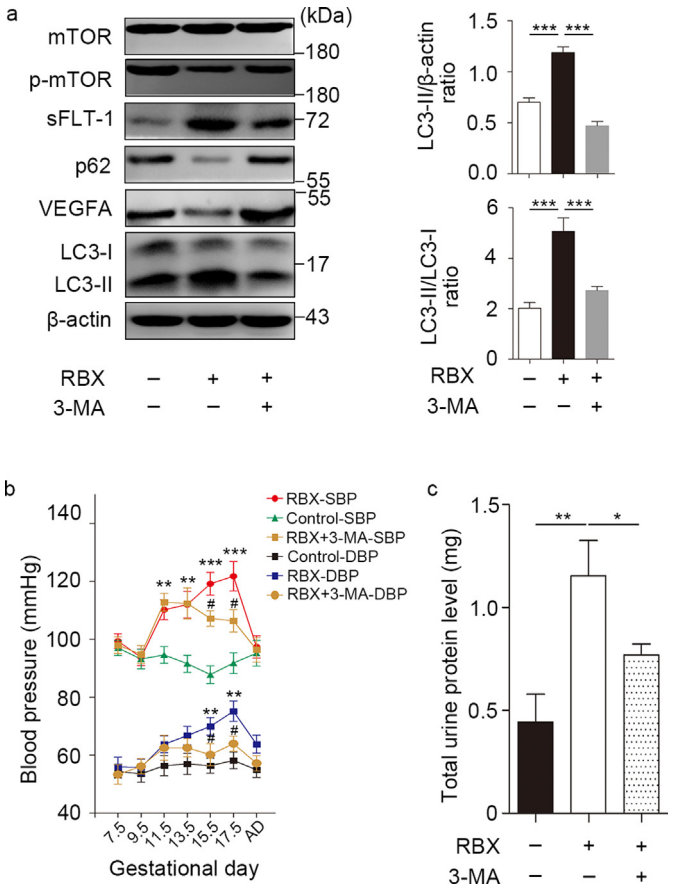

Fig. 4. The blockade of 3-MA-mediated autophagy attenuates preeclampsia-like symptoms in PKCβ-inhibited mice. RBX-treated pregnant mice were injected with 3-MA from E7.5−E14.5 and placentas were collected on E17.5. **(a)** Left panel: the placental protein levels of mTOR, p-mTOR, sFLT-1, VEGFA, p62 and LC3, were detected by immunoblotting. Right panel: Quantification of LC3-II levels was performed by densitometry of LC3-II/β-actin and LC3-II/LC3-I ratio (The analyses were based on 5 placental samples per group. ***P<0.001, One-way ANOVA). **(b)** Systolic and diastolic blood pressures of mice were measured on the indicated gestational day (n=5 mice per group, **P<0.01, ***P<0.001 vs Control groups; #P<0.05 vs RBX group at the same time points, Repeated measures analysis of variance). **(c)** The 24 h total urinary protein level was determined at E17.5 (n=5 mice per group, *P<0.05, **P<0.01, One-way ANOVA). RBX, Ruboxistaurin; 3-MA, 3-methyladenine; SBP, Systolic blood pressure; DBP, Diastolic blood pressure.

(Fig. 3**a-c**) and decreased expression of p62 (Fig. 3**a**); these are regarded as being specific markers of autophagy. There were considerably higher levels of LC3 protein in the placenta of RBX-treated mice than in controls, as visualized by the increased accumulation of LC3-positive puncta (Fig. 3**d-e and Supplementary Fig. 3**). However, no significant differences in the levels of BECN1 were observed (data not shown). Together, these data suggested that the inhibition of PKCβ caused activation of autophagic signals in murine placentas.

3.5. *The Blockade of 3-MA-Mediated Autophagy Attenuates Preeclampsia-like Symptoms in PKC*β*-inhibited Mice*

Since PKCβ activates autophagy during pregnancy, we decided to investigate whether the administration of 3-MA (20 mg/kg/d), an inhibitor of autophagy, alleviates preeclampsia-like symptoms induced by PKCβ inhibition. As shown in Fig. 4**a**, a reduction of autophagy activity was observed after the administration of 3-MA, as evidenced by the reduced levels of LC3-II, and the increased levels of p62 and p-mTOR, in the placentas of 3-MA+RBX-treated mice, when compared with RBX-treated mice. Most importantly, 3-MA treatment reduced the severity of preeclampsia-like symptoms in mice, including the reduction in blood pressure (Fig. 4**b**) and catabatic proteinuria (Fig. 4**c**), although the mice did not fully recover. These results suggested that the inhibition of PKCβ exerts its impact on preeclampsia in mice by regulating autophagy, at least in part.

3.6. *The Suppression of PKC*β *Exerts an Anti-Angiogenesis Effect* in vitro

Considering that the alteration of angiogenesis has been revealed to play an important role in the pathogenesis of preeclampsia [35], we decided to examine whether the inhibition of PKCβ disrupts the action of VEGFA in endothelial cells. To do this, we performed the tube formation assay. Our data showed that HUVECs were organized into complex capillary-like structures with the addition of VEGFA (40 ng/ml), and that this effect was suppressed by RBX-mediated PKCβ inhibition (Fig. 5**a**). Three parameters were used to evaluate the ability to form tubes, including tube number (Fig. 5**b**), total tube length (Fig. 5**c**), and branching point (Fig. 5**d**). In addition, the anti-angiogenesis effect of PKCβ inhibition was validated by detecting (anti) angiogenic factor expression in human choriocarcinoma cells (JEG-3). After 24 h of RBX treatment, we found that VEGFA was significantly downregulated, while sFLT1 was markedly upregulated (Fig. 5**e**). These data convincingly demonstrate that selective PKCβ inhibition exerts its anti-angiogenesis effects by affecting endothelial function and by disrupting the balance of proangiogenic and antiangiogenic factor expression.

3.7. *The Inhibition of PKC*β *Promotes Autophagy* in vitro

To further confirm the role of PKCβ as an autophagic regulator, we quantified the levels of autophagic signals in HUVEC and JEG-3 cells treated with PKCβ inhibitor, RBX. RBX, at a concentration of 100 nmol/L, suppressed the expression of p62 and increased the conversion of LC3 I to LC3 II, both in HUVEC (Fig. 6**a**) and JEG-3 cells (Fig. 6**b**). We also measured autophagic flux using tandem fluorescent mRFP-GFP-LC3. With the RFP-GFP-LC3 assay, autophagosomes emit both GFP and RFP markers, while autolysosomes emit only an RFP signal because the GFP signal is lost in the acidic lysosomal environment. As detailed in Fig. 6**c-d**, the selective inhibition of PKCβ increased the formation of total autophagosomes and autolysosomes, both in HUVEC and JEG-3 cells. Taken together, these results corroborate the fact that the inhibition of PKCβ promotes autophagy *in vitro*.

3.8. *The Autophagy Inhibition Partially Abrogates PKC*β *Inhibition-Mediated Anti-Angiogenesis Effects*

The angiogenetic behaviour of endothelial cells has been found to be dictated by autophagy signals [36]. Given the dual functions of

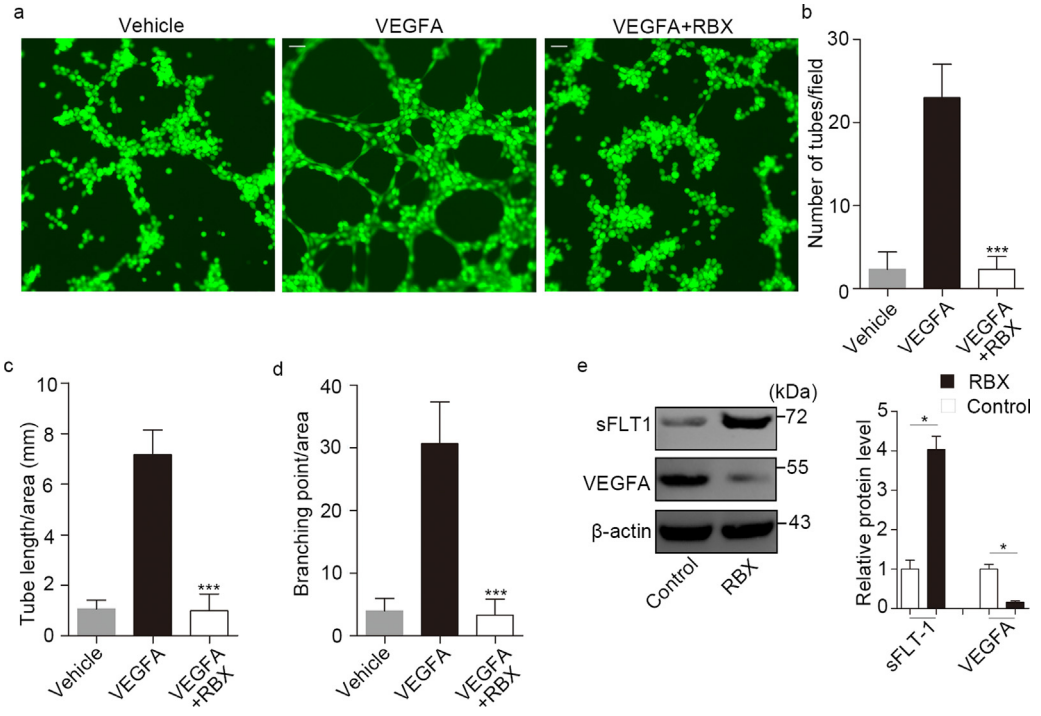

Fig. 5. The suppression of PKCβ exerts anti-angiogenesis effects *in vitro*. **(a)** Representative images showing the suppression of PKCβ by RBX inhibited VEGFA-induced tube formation in HUVECs. Scale bars, 50 μm. **(b-d)** The capacity of tube formation was quantified by **(b)** tube number, **(c)** tube length, and **(d)** branching point (***P<0.001 vs VEGFA group, One-way ANOVA). **(e)** Left panel: validation of the expression of representative proangiogenic and antiangiogenic factors. The protein levels of VEGFA and sFLT1 were detected by immunoblotting in RBX-treated human choriocarcinoma cells (JEG-3). Right panel: quantitative results determined by densitometry of the western blot bands (sFLT-1 and VEGFA) are shown relative to the control (*P<0.05, Mann−Whitney U test). All experiments were performed at least three times independently.

PKCβ on the regulation of autophagic flux and angiogenesis, we next investigated whether the anti-angiogenic effects of PKCβ inhibition were mediated by autophagic dysfunction. Results from western blotting experiments showed that 3-MA efficiently inhibited autophagic flux in HUVEC and JEG3 cells in which PKCβ had been inhibited (Fig. 7**a and Supplementary Fig. 4a-b)**. Under this condition, the PKCβ inhibition-mediated downregulation of VEGFA, and the upregulation of sFLT1, were partially reversed in JEG3 cells (Fig. 7**b and Supplementary Fig. 4c**). Moreover, autophagic inhibition by 3-MA partially rescued the tube formation potential of HUVECs treated with RBX (Fig. 7**c**), as evidenced by the recovery of tube count (Fig. 7**d)**, total tube length (Fig. 7**e)**, and branching point count (**Fig. 7f**). Therefore, these results proved that autophagic activation may be responsible, at least in part, for the anti-angiogenesis effect of PKCβ inhibition.

3.9. The Contribution of PKCβ Inhibition to Preeclampsia is Independent of Inadequate Trophoblast Invasion

Considering that the impairment of spiral artery remodelling caused by inadequate trophoblast invasion results in poor placental perfusion and the consequent pregnancy-related diseases [37], we next sought to determine whether the contribution of PKCβ inhibition to preeclampsia is mediated by inadequate trophoblast invasion. Surprisingly, the genetic or pharmacological inhibition of PKCβ promoted both the migration and invasion of human trophoblast HTR8/SVneo cells (Fig. 8**a and Supplementary Fig. 5**), thus indicating that the inhibition of PKCβ is beneficial to spiral artery remodelling and placental perfusion. Therefore, we propose that the contribution of PKCβ inhibition to preeclampsia is independent of inadequate trophoblast invasion. This phenomenon may be interpreted as a

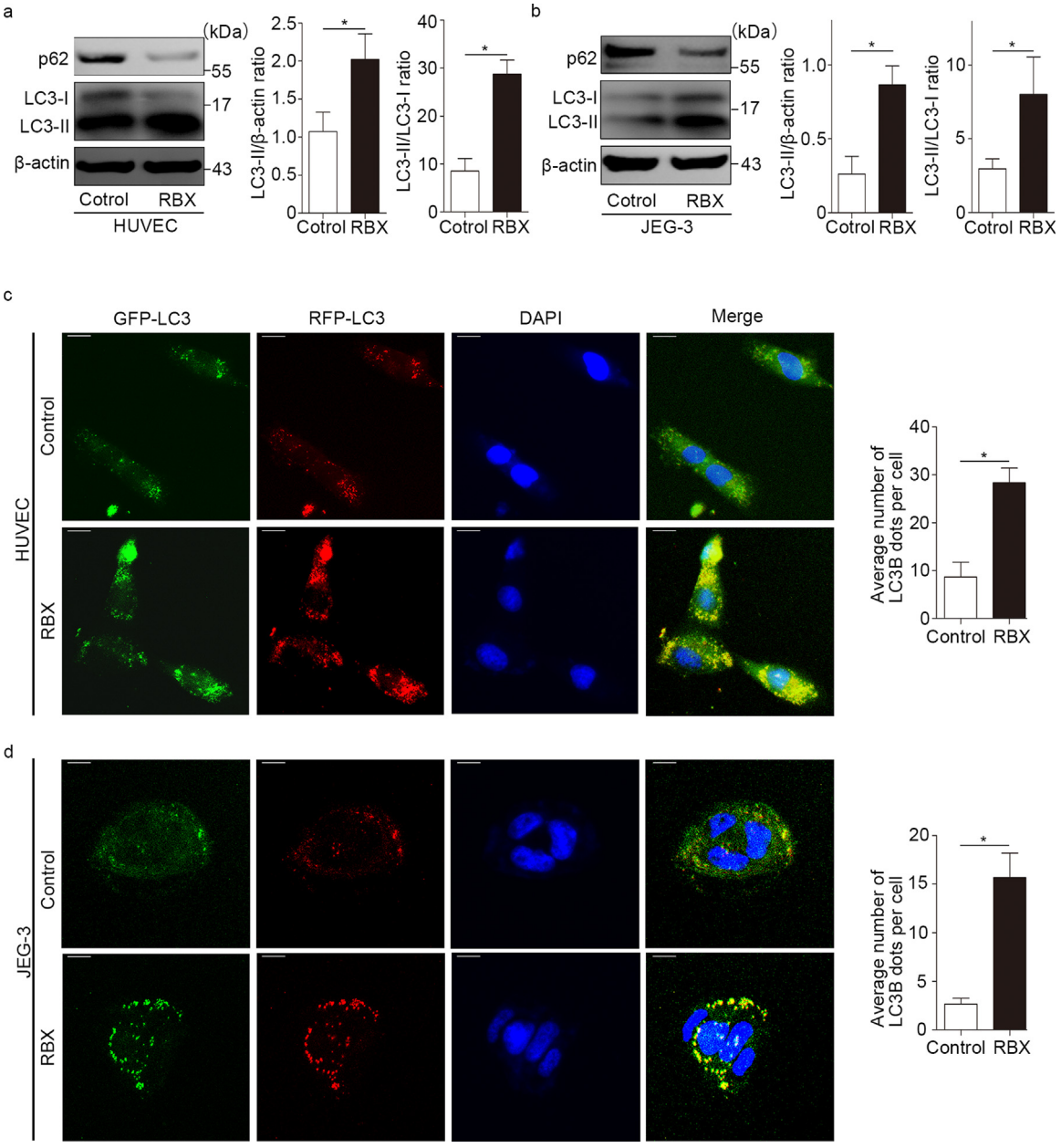

Fig. 6. The inhibition of PKCβ induces autophagy *in vitro*. **(a-b)** The inhibition of PKCβ reduced p62 expression and increased the conversion of LC3 I to LC3 II, both in HUVECs (a) and JEG-3 cells (b). LC3-II levels were quantified by densitometry of LC3-II/β-actin and LC3-II/LC3-I ratio. **(c-d)** Formation and quantitative analysis of RFP-GFP-LC3 puncta before or after RBX treatment for 2 h in HUVECs (c) and JEG-3 (d) cells. Scale bars, 20 μm. All experiments were performed at least three times independently. $^{*}P<0.05$ (Mann−Whitney U test).

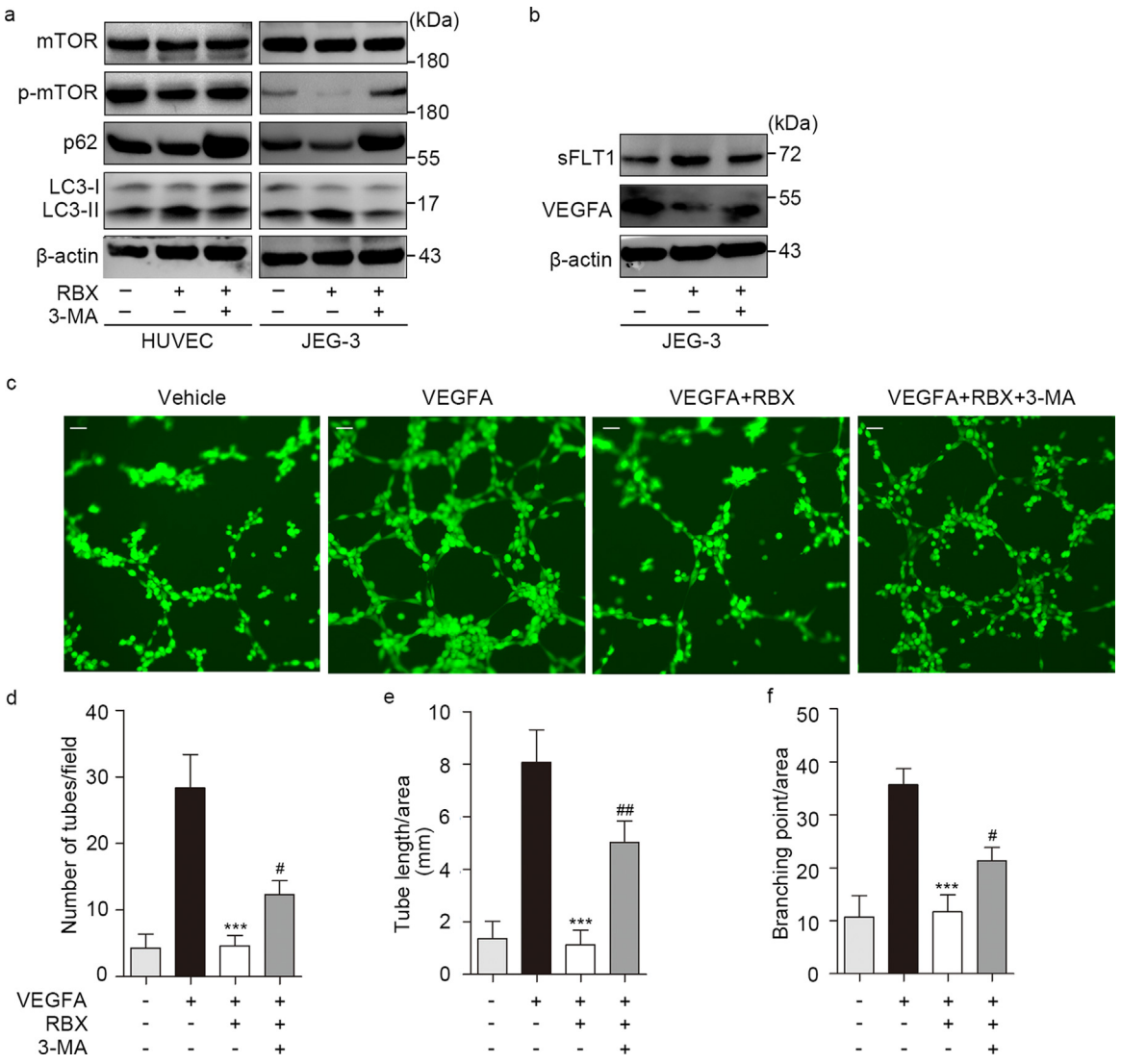

Fig. 7. Autophagic inhibition by 3-MA partially abrogates the PKCβ inhibition-mediated anti-angiogenesis effect. HUVECs and JEG-3 cells were exposed to RBX (100 nmol/L) and/or 3-MA (5 mmol/L), as indicated. **(a)** Immunoblot analysis of mTOR, p-mTOR, p62, and the conversion of LC3 I to LC3 II in HUVECs and JEG-3 cells. **(b)** VEGFA and sFLT1 protein levels were determined by immunoblotting. **(c)** The capacity for tube formation was quantified by **(d)** tube number, **(e)** tube length, and **(f)** branching point. Scale bars, 50 μm. All experiments were performed at least three times independently. ***$P<0.001$ vs VEGFA groups; #$P<0.05$, ##$P<0.01$ vs VEGFA+RBX group (One-way ANOVA).

compensatory mechanism to limit the adverse effects of PKCβ inhibition in preeclampsia.

4. Discussion

In this article, we report a previously unrecognized preeclampsia-associated autophagic regulator, PKCβ, that is involved in placental angiogenesis. First, in preeclamptic placentas, we found that PKCβ was downregulated. Second, in an animal model, the selective inhibition of PKCβ was sufficient to induce preeclampsia-like symptoms, and result in excessive autophagic flux, and a disruption in the balance of pro- and anti-angiogenic factors in mouse placentas, all of which could be partially normalized by 3-MA, an autophagic inhibitor. These findings suggest that autophagy mediated, at least in part, the impairment of angiogenesis in PKCβ inhibition-induced preeclampsia. Third, our *in vitro* experiments demonstrated that the inhibition of PKCβ activated autophagy, thus blocking VEGFA-induced HUVEC tube formation and resulting in the significant upregulation of sFLT1 and the downregulation of VEGFA. These data support a model in which autophagic activation, due to PKCβ inhibition, leads to the impairment of angiogenesis and eventually results in preeclampsia in pregnancy (Fig. 8**b**). Ameliorating the toxic effects of PKCβ inhibition by the restoration of PKCβ or autophagic inhibition may alleviate the preeclamptic morbidity of pregnant women.

A previous study has documented hyper-methylation of the PKCβ gene in the preeclamptic placentas [21]; however, no causal link was established between PKCβ and preeclampsia. RBX is a selective and ATP-competitive PKCβ inhibitor [23]. Because of its ability to inhibit retinal neovascularization, the inhibition of PKCβ by RBX is currently used as a strategy for the treatment of diabetic retinopathy [38]. Given that angiogenesis is an indispensable process for placental development [35], we speculate that the harmful effects of PKCβ inhibition may be generated in pregnant women by a mechanism involving placental neovascularization suppression. In this study, the placental levels of PKCβ were evaluated by various biological experiments and by analysing a dataset in the public database. We obtained findings to indicate that PKCβ was downregulated in preeclamptic placentas; this was in general agreement with the results described in a previous report [21]. Furthermore, we administered RBX into pregnant mice by gavage to inhibit the function of PKCβ. As a result, almost all of the characteristic features of preeclampsia appeared, including hypertension, proteinuria, imbalance in angiogenic factors,

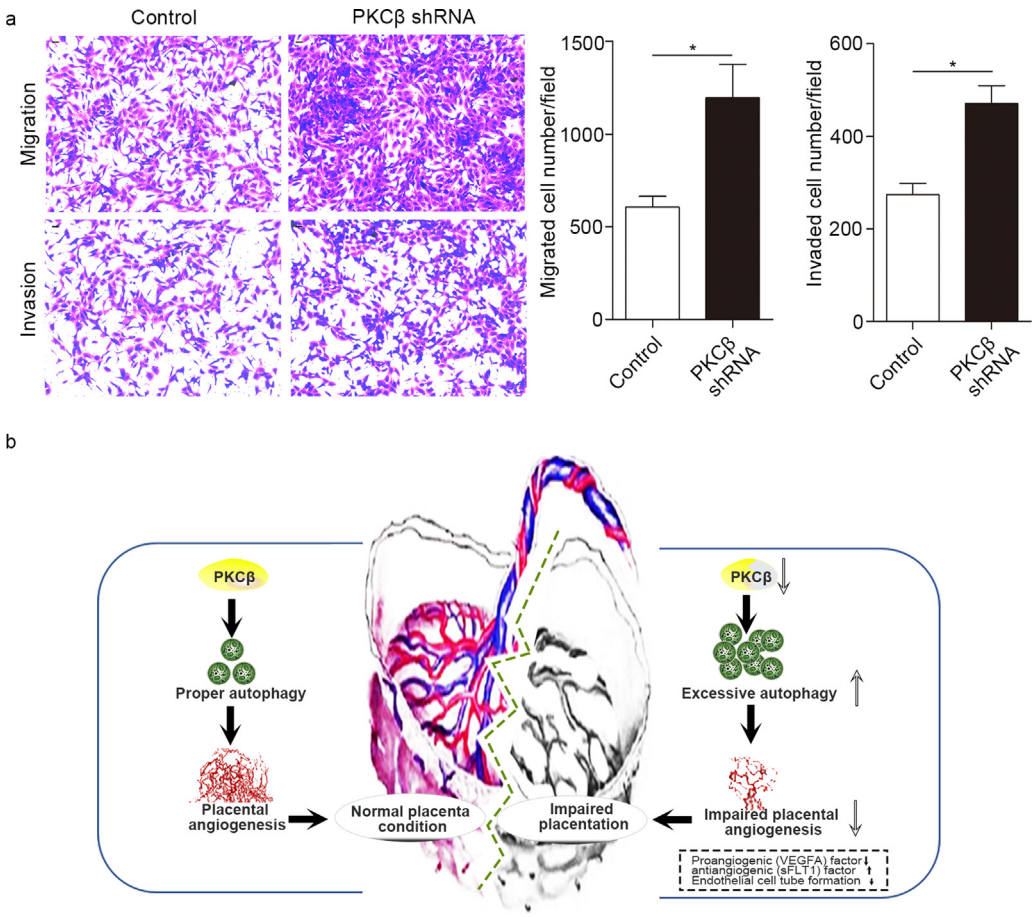

Fig. 8. The contribution of PKCβ inhibition to preeclampsia is independent of inadequate trophoblast invasion. **(a)** Representative images of cell migration and invasion for PKCβ- and control-shRNA infected HTR8/SVneo cells. Quantitative results are depicted in the graph on the right. Scale bars, 30 μm. This experiment was performed three times independently. $^{*}P<0.05$ (Mann−Whitney U test). **(b)** Working model: autophagic activation due to PKCβ inhibition leads to the impairment of angiogenesis and eventually results in preeclampsia.

and morphological changes in kidney and placenta. These data suggest that the inhibition of PKCβ contributes to the pathogenesis of preeclampsia, which therefore indicates that the restoration of PKCβ in PKCβ-deficient women may provide a new paradigm for preeclampsia treatment. Our results also suggest that side effects, including pregnancy-associated diseases (e.g. preeclampsia), should be noted when RBX is marketed.

Recent studies support the hypothesis that autophagic dysfunction is associated with preeclampsia [2,4,6]. The dysregulation of autophagy may induce placental dysfunction by failing to maintain homeostasis [3,4,9] and consequently influencing multiple cell behaviours including trophoblast invasion in the process of human placentation [3-5,8]. Akitoshi *et al.* found that the impairment of autophagy occurred in preeclamptic extra villous trophoblasts (EVTs), and resulted in the failure of EVT invasion and vascular remodelling [4,5]. However, there is no direct evidence that autophagy is involved in human placental angiogenesis, which is considered to be an essential process in placentation, but defective in preeclampsia. In the present study, *in vivo* and *in vitro* methods were used to validate our hypothesis that autophagy partially mediates the PKCβ-inhibition induced impairment of angiogenesis in preeclampsia. In this scenario, we may reconstruct, at least in part, the pathological status of the placenta in preeclampsia.

Impaired angiogenesis and inadequate trophoblast invasion are the two most important pathophysiological features of preeclampsia [37]. Angiogenesis generally depends on maintaining the balance of both pro- and anti-angiogenic factors. VEGFA and sFLT1 have been recognized as potential key candidates [39-41]. The upregulation of sFLT1, and the downregulation of VEGFA, play major roles in the pathology of preeclampsia by leading to long-lasting systemic vascular dysfunction [34,42]. Our current data show that the inhibition of PKCβ exerts an inhibitory effect on VEGFA and facilitates the expression of sFLT1 both *in vivo* and *in vitro*. This finding is in accordance with some pathogenicity-related factors in preeclampsia, such as Toll-Like receptor 9 [29] and retinoic acid [43]. Since inadequate trophoblast invasion is another essential factor that leads to the development of preeclampsia [37], we also investigated the effect of PKCβ inhibition to trophoblast invasion in this study. Our data showed that the suppression of PKCβ by RBX subsequently activated autophagy and promoted the migration and invasion of HTR8/SVneo cells. We speculate that this phenomenon occurred in the trophoblast as a compensatory mechanism to limit the adverse effects (e.g. impaired angiogenesis as mentioned above) of PKCβ inhibition in preeclampsia. This can be explained by the fact that intracorporal compensation usually occurs under extreme circumstances. Another intracorporal compensation is that PKCβ is able to interrupt the stimulation of endothelial nitric oxide synthase (eNOS), which reduces NO production, and causes endothelial dysfunction [44,45]. The downregulation of PKCβ in preeclamptic placenta may attempt to activate eNOS to restore vascular function. These findings therefore suggest that the contribution of PKCβ inhibition to preeclampsia is achieved by the impairment of angiogenesis, and that this might also involve the activation of a compensatory response.

Our study leaves several unanswered questions for further exploration. Firstly, a major limitation of this study was that the global inhibition of PKCβ might cause unrelated signs from other

organs besides placenta in the mouse model. However, considering the facts that no signs of hypertension were found in PKCβ-inhibited non-pregnant mice, and the blood pressure of PKCβ-inhibited pregnant mice returned to normal postpartum, we hypothesized that non-placental response is limited in this pre-eclampsia model. Despite of this, PKCβ activity should be evaluated in other mouse tissues and the results should be validated in placenta-specific PKCβ-knockout mice. Secondly, the gestational age between normal controls and preeclampsia is significantly different in the case-control study, which was likely to be a confounder when analysing the changes of placental PKCβ expression. Indeed, it is not practical to make preeclampsia and normal controls matched for gestation at delivery, since pre-eclamptic patients have a high risk of preterm labour, while the seemingly ideal candidate controls, preterm labour, are not generally considered normal. Considering this kind of concerns, the difference of PKCβ mRNA expression was adjusted for some potential confounders including gestational age. The results showed the difference remained significant, suggesting the confounders was likely to have limited effects. Thirdly, we do not know whether other mechanisms, such as processes besides autophagy that reversely induced by PKCβ inhibition and 3-MA induction, maternal-foetal immune tolerance and epigenetic regulation, are involved in the PKCβ inhibition-mediated impairment of angiogenesis [46,47]. Further studies are now warranted to complement the roles of PKCβ in preeclampsia. Despite of these limitations, our findings provided a novel target in term of uncovering the mechanism of preeclampsia.

In conclusion, we have proposed that PKCβ is a previously unrecognized preeclampsia-associated autophagic regulator, and that the selective inhibition of PKCβ contributes to the pathogenesis of pre-eclampsia by autophagy-mediated impairment of placental angiogenesis. These findings may have important implications in the pathogenesis of autophagy dysfunction−associated preeclampsia. In addition, our results raise the possibility that restoration of PKCβ levels in PKCβ-deficient women may be an efficient means of treating placental diseases, such as, but not limited to, preeclampsia.

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Data availability

The article with related data have been deposited in the Mendeley (DOI: 10.2139/ssrn.3532516).

Author contributions

X.L., D.M. and Q.Z. designed the study. H.Z., S.W. and L.G. conducted experiments. H.Z., L.G., T.J., X.X., Y.C. and H.X. collected and analysed data. H.Z., L.G., H.L., Y.T. and J.Z. interpreted experimental results. X.L. and Y.T. acquired the funding. X.L. and H.Z. drafted the manuscript. X.L., D.M., Q.Z. and H.Z. reviewed and edited the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102813.

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