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ORIGINAL RESEARCH

IGF2BP1 Enhances Neprilysin mRNA Stability to Promote Proliferation, Invasion, and Angiogenesis in Placental Trophoblasts

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Background and Objective: Preeclampsia (PE) is a severe gestational disorder characterized by sudden hypertension and proteinuria, with substantial risks to both mother and fetus. This study aims to delineate the role of neprilysin, a metalloprotease known for its role in modulating vasoactive peptides, in the pathophysiology of PE.

Methods: We recruited a cohort of 57 participants, comprising 38 patients diagnosed with PE and 19 healthy controls, matched for demographic and clinical characteristics. Neprilysin expression was assessed in serum and placental tissues through quantitative RT-qPCR and Western blot analyses. Functional impacts of neprilysin modulation were explored via siRNA knockdown and overexpression in HTR8/SVneo cells, followed by assessments of oxidative stress, mitochondrial function, apoptosis, and trophoblast invasion using various biochemical assays including CCK-8, DCFH-DA, JC-1 staining, and flow cytometry.

Results: Our results demonstrate a marked overexpression of neprilysin in the serum and placental tissues of PE patients compared to healthy controls. Elevated neprilysin levels were positively correlated with increased systolic and diastolic blood pressures. In functional assays, neprilysin knockdown alleviated H_2O_2-induced oxidative stress, restored mitochondrial function, and improved cell invasion and migration in EVT cells. Conversely, the overexpression of IGF2BP1, a regulator of mRNA stability, exacerbated neprilysin expression and intensified cellular damage under oxidative stress conditions. The reciprocal regulation of neprilysin by IGF2BP1 highlights a critical molecular interplay impacting cellular resilience to oxidative stress in PE.

Conclusion: These findings establish neprilysin as a critical mediator in the pathogenesis of PE, where its aberrant overexpression is linked to exacerbated hypertensive symptoms and impaired trophoblast function. The interaction between neprilysin and IGF2BP1 provides a potential therapeutic target for mitigating the progression of PE, suggesting avenues for future intervention strategies.

Keywords: preeclampsia, neprilysin, IGF2BP1, trophoblast, angiogenesis, proliferation

Introduction

Preeclampsia (PE) is a pregnancy-specific disorder marked by hypertension and proteinuria, typically manifesting after the 20th week of gestation.[1] This condition poses significant risks to maternal health and can also severely impact fetal development.[2] PE has not been able to pinpoint its pathogenesis. However, growing evidence implicates placental dysfunction as central to its pathophysiology, with particular emphasis on trophoblasts, which are essential for placental development and function. When trophoblast function is compromised, it can drive the progression of PE.[3,4]

Dysfunction of placental trophoblasts, especially involving mitochondrial dysfunction and oxidative stress, is considered a key pathological feature of PE.[5,6] Mitochondria, often described as the "powerhouses of the cell", are responsible for energy production. Dysfunctional mitochondria lead to energy deficits that impair cellular physiology.[7] In the placenta of PE patients, trophoblasts frequently exhibit aberrant mitochondrial morphology and compromised function, which contributes to increased cellular stress and apoptosis.[5] Moreover, mitochondrial dysfunction exacerbates

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intracellular oxidative stress, causing further damage to cellular structures and impairing function.[8,9] Oxidative stress, through the excessive generation of reactive oxygen species (ROS), damages cellular membranes and disrupts signaling pathways, thereby promoting inflammation and increasing angiotensin II production, which exacerbates hypertension.[10,11] Thus, mitochondrial dysfunction and oxidative stress in trophoblasts are directly linked to PE progression. These impairments lead to reduced placental perfusion and nutrient exchange, worsening the disorder through mechanisms such as enhanced apoptosis, inflammation, and vascular dysfunction.[12–14] Despite these insights, the molecular mechanisms underlying oxidative stress and mitochondrial dysfunction in trophoblasts remain incompletely understood.

Neprilysin, also known as enkephalinase-24.11, CD10, neutral endopeptidase, or neprilysin enzyme, is a multifunctional zinc metalloprotease.[15] Its role in cardiovascular diseases primarily involves the regulation of vasoactive peptides.[16] These peptides, including natriuretic peptides, angiotensin II, bradykinin, substance P, adrenomedullin, and endothelin-1, are critical mediators of blood pressure, cardiac workload, and vascular homeostasis. Neprilysin maintains cardiovascular balance by degrading these peptides; dysregulation can result in hypertension, impaired cardiac function, and heart failure.[17–19] Recent studies have revealed abnormal neprilysin expression in patients with PE. Women with PE had significantly higher neprilysin levels than those with uncomplicated pregnancies.[20] Serum neprilysin was positively correlated with systolic and diastolic blood pressures, blood urea nitrogen (BUN), uric acid, and creatinine levels. Additionally, a study by Atta et al highlighted that PE involves a delicate balance between trophoblast invasion and fetal-maternal immune tolerance.[21] An imbalance between pro-inflammatory cytokines such as interleukin (IL)-6 and anti-inflammatory cytokines like IL-10 is a hallmark of PE. In patients with PE, neprilysin showed a significant positive correlation with IL-6 and transforming growth factor-beta, while it was negatively correlated with IL-10. However, it remains uncertain whether neprilysin directly contributes to oxidative damage and mitochondrial dysfunction in trophoblasts.

This study aims to investigate the role of neprilysin in regulating mitochondrial dysfunction and oxidative stress in placental trophoblasts and to identify the RNA-binding proteins that modulate neprilysin function in this context. By elucidating neprilysin's role in placental function, particularly its involvement in cellular energy metabolism and oxidative homeostasis, this study not only aims to reveal crucial pathogenic mechanisms underlying PE but also to provide a foundation for the development of novel therapeutic strategies to address pregnancy complications. Specifically, identifying RNA-binding proteins that influence neprilysin activity could offer new drug targets, allowing modulation of specific molecular pathways to improve placental function and maternal-fetal health—a prospect with significant clinical implications.

Materials and Methods

Clinical Sample Collection

This study enrolled 57 participants, consisting of 38 patients with PE (PE group) and 19 normotensive pregnant women (control group). All participants underwent their first cesarean section at Baoying Maternal and Child Health Hospital between March 2021 and May 2023. General clinical characteristics were collected and subjected to statistical analysis, revealing no significant differences between the two groups ($P > 0.05$), thus ensuring comparability. No missing data were present, as participants with incomplete clinical data were excluded based on the predefined exclusion criteria. The study protocol received approval from the Baoying Maternal and Child Health Hospital ethics committee. Written informed consent was obtained from all participants. Inclusion criteria included age between 20 and 40 years, complete pregnancy outcome, comprehensive clinical records, and consistency with the American College of Obstetricians and Gynecologists (ACOG) 2020 diagnostic criteria. Exclusion criteria included severe pre-existing cardiac, hepatic, or renal disease, pregnancy complicated by hypertension or diabetes, multiple gestations, history of smoking, alcohol or drug abuse, language or psychiatric disorders, and incomplete clinical data. Baseline information including age, height, body mass index (BMI), parity, delivery mode, gestational age at delivery, neonatal birth weight, and Apgar scores was collected. Fasting venous blood was drawn, and serum was separated by centrifugation at 4°C and stored at −80°C. Placental samples were collected from the central region near the umbilical cord during cesarean section and immediately frozen at −80°C.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was isolated from both tissues and cells employing TRIzol reagent (Invitrogen, USA), adhering to the guidelines provided by the manufacturer. Utilizing the PrimeScript II First Strand cDNA Synthesis Kit from Takara, Japan, Complementary DNA (cDNA) was produced from mRNA. The qPCR process utilized a Roche 480 Real-Time PCR system, employing SYBR® Premix Ex Taq II (Takara, Japan). The internal reference gene employed was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The comparative expression rates of RNA were determined through the $2^{-\Delta\Delta Ct}$ technique.

Cell Culture

The human extravillous trophoblast (EVT) cell line HTR8/SVneo was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (Life Technologies, Shanghai, China) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin (Life Technologies, Shanghai, China). Cells were maintained in a humidified incubator at 37°C with 5% carbon dioxide (CO_2). To induce oxidative stress, cells were treated with hydrogen peroxide for 24 hours.

Cell Transfection

Small interfering RNAs (siRNAs) targeting insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) and neprilysin, as well as overexpression vectors (pcDNA 3.1), were purchased from GenePharma (Shanghai, China). Transfections were performed using Lipofectamine 3000 (Invitrogen). Cells were transfected for 48 hours, after which transfection efficiency was assessed by RT-qPCR and Western blotting.

Cell Counting Kit-8 (CCK-8)

Cell proliferation was assessed using the CCK-8 assay. Briefly, 1×10^3 cells were seeded in 96-well plates, and 10 μL of CCK-8 reagent was added at 0, 24, 48, and 72 hours after seeding. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) following 2 hours of incubation at 37°C.

ROS Detection

Intracellular ROS levels were measured using the fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA). Approximately 6×10^5 cells were seeded per well in 6-well plates. Cells were harvested by centrifugation, resuspended in PBS, and incubated with 20 μM DCFH-DA at 37°C for 30 minutes. After staining, cells were washed twice with PBS and immediately analyzed by flow cytometry using a Beckman Coulter instrument (Beckman Coulter, Atlanta, GA, USA) with an excitation wavelength of 480 nm.

Mitochondrial Membrane Potential (MMP)

MMP was assessed using the JC-1 staining assay (Beyotime, China). The ratio of red (aggregates) to green (monomers) fluorescence was used to indicate changes in MMP, with a decrease in the ratio reflecting mitochondrial depolarization, an early indicator of apoptosis. Cells were incubated with JC-1 working solution for 20 minutes, and fluorescence was observed using a confocal laser scanning microscope (40 × magnification, Leica, Germany). The ratio of aggregates to monomers was quantified using ImageJ software.

Apoptosis Detection

The evaluation of apoptosis utilized the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (Vazyme, China). Fluorescence-activated cell sorting utilized a BD Accuri C6 Plus flow cytometer (BD Biosciences, NJ, USA) and was conducted using FlowJo software. The cells were gathered, cleansed using chilled PBS, and then reconstituted in 100 μL of binding buffer. The cell mixture was supplemented with five microliters of Annexin V-FITC and PI staining solution, followed by a 10-minute incubation period. Ultimately, 400 μL of binding buffer was introduced, followed by cell analysis using flow cytometry within an hour.

Transwell Migration and Invasion Assay

The evaluation of migration and invasion was conducted in 24-well transwell chambers (Corning Inc., Corning, NY, USA). In conducting invasion tests, Matrigel (Corning Inc.) was used to coat transwell inserts. The upper chamber received cell suspensions devoid of serum, while the lower chamber was supplemented with RPMI 1640 medium that included 10% FBS. Following a 24-hour period, cells that had moved or infiltrated the lower chamber were stabilized using 4% paraformaldehyde, colored with crystal violet (Sigma-Aldrich), and tallied using an inverted microscope (Olympus, Tokyo, Japan, × 100).

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng), IL-1β, and IL-6 were measured using ELISA kits. Tumor necrosis factor-alpha (TNF-α), IL-1β, and IL-6 kits were obtained from Thermo Fisher Scientific (USA). Lipopolysaccharide (LPS) and diamine oxidase (DAO) kits were from Cloud-Clone (Wuhan, China).

Western Blot Analysis

Cells and tissue samples were lysed in RIPA buffer containing protease inhibitors (P0013D, Beyotime). Protein concentration was determined using a BCA kit (P0011, Beyotime). Protein samples were loaded onto SDS-PAGE gels and separated by electrophoresis, followed by transfer onto polyvinylidene fluoride membranes. Membranes were blocked in 5% bovine serum albumin for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies diluted in antibody diluent. After incubation with horseradish peroxidase-conjugated secondary antibodies for 1 hour, protein signals were detected using a gel imaging system (Bio-Rad, USA) and analyzed with ImageJ. GAPDH was used as the internal control. Primary antibodies included p-p65 (3033, Cell Signaling Technology), p65 (8242, Cell Signaling Technology), nuclear factor erythroid 2-related factor 2 (Nrf2) (16396-1-AP,Proteintech), heme oxygenase 1 (HO-1) (10701-1-AP, Proteintech), and GAPDH (2118, Cell Signaling Technology).

Actinomycin D Assay

To assess the effect of IGF2BP1 on neprilysin mRNA stability, cells were seeded in 6-well plates and treated with 5 μg/mL actinomycin D to inhibit RNA transcription. Total RNA was extracted at the indicated time points, and neprilysin mRNA expression was analyzed using RT-qPCR.

Co-Immunoprecipitation (Co-IP)

For Co-IP, cells were lysed in RIPA buffer (P0013B, Beyotime, Shanghai, China) according to the manufacturer's protocol. Protein A/G beads were incubated with anti-IGF2BP1 antibody overnight at 4°C, washed three times with lysis buffer, resuspended in RIPA buffer, and boiled for 5 minutes. The samples were then analyzed by Western blotting.

RNA Immunoprecipitation (RIP)

RIP was performed using the EZ-Magna RIP kit (Millipore, Billerica, MA, USA). To analyze the interaction between IGF2BP1 and neprilysin, cell lysates were incubated with magnetic beads conjugated with anti-IGF2BP1 or control immunoglobulin G antibodies. RNA was then extracted from the immunoprecipitates and assessed for enrichment.

Data Analysis

Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA). All data were obtained from at least three biological replicates. The correlation between IGF2BP1 and neprilysin was analyzed using Pearson correlation. Categorical data were analyzed by chi-square tests. Continuous data are presented as mean ± standard deviation (SD), and differences between groups were assessed using Student's *t*-test or one-way analysis of variance.

Results

Neprilysin Is Aberrantly Overexpressed in PE

To investigate the involvement of neprilysin in PE, we recruited a cohort of 57 participants, comprising 38 patients diagnosed with PE and 19 healthy controls. Demographic and clinical characteristics such as age, Apgar scores, parity, mode of delivery, and gestational age at delivery were comparable between the two groups, with no statistically significant differences. However, systolic and diastolic blood pressures were markedly elevated in the PE group compared to the control group, while both neonatal birth weight and gestational age were significantly reduced in PE patients. To further explore neprilysin expression, we performed RT-qPCR and Western blot analyses on serum and placental tissues. As depicted in Figure 1A and B, neprilysin levels were substantially upregulated in both serum and placental tissues of the PE group compared to healthy controls, highlighting a distinct overexpression profile associated with PE pathology. We next performed Pearson correlation analysis to assess the relationship between neprilysin levels and clinical parameters of PE patients (Figure 1C). The data demonstrated a significant positive correlation between neprilysin expression and both systolic and diastolic blood pressures, suggesting a potential contributory role of neprilysin in the hypertensive phenotype observed in PE. These findings collectively indicate that neprilysin is aberrantly overexpressed in PE, with its heightened expression potentially influencing disease progression through mechanisms related to increased blood pressure.

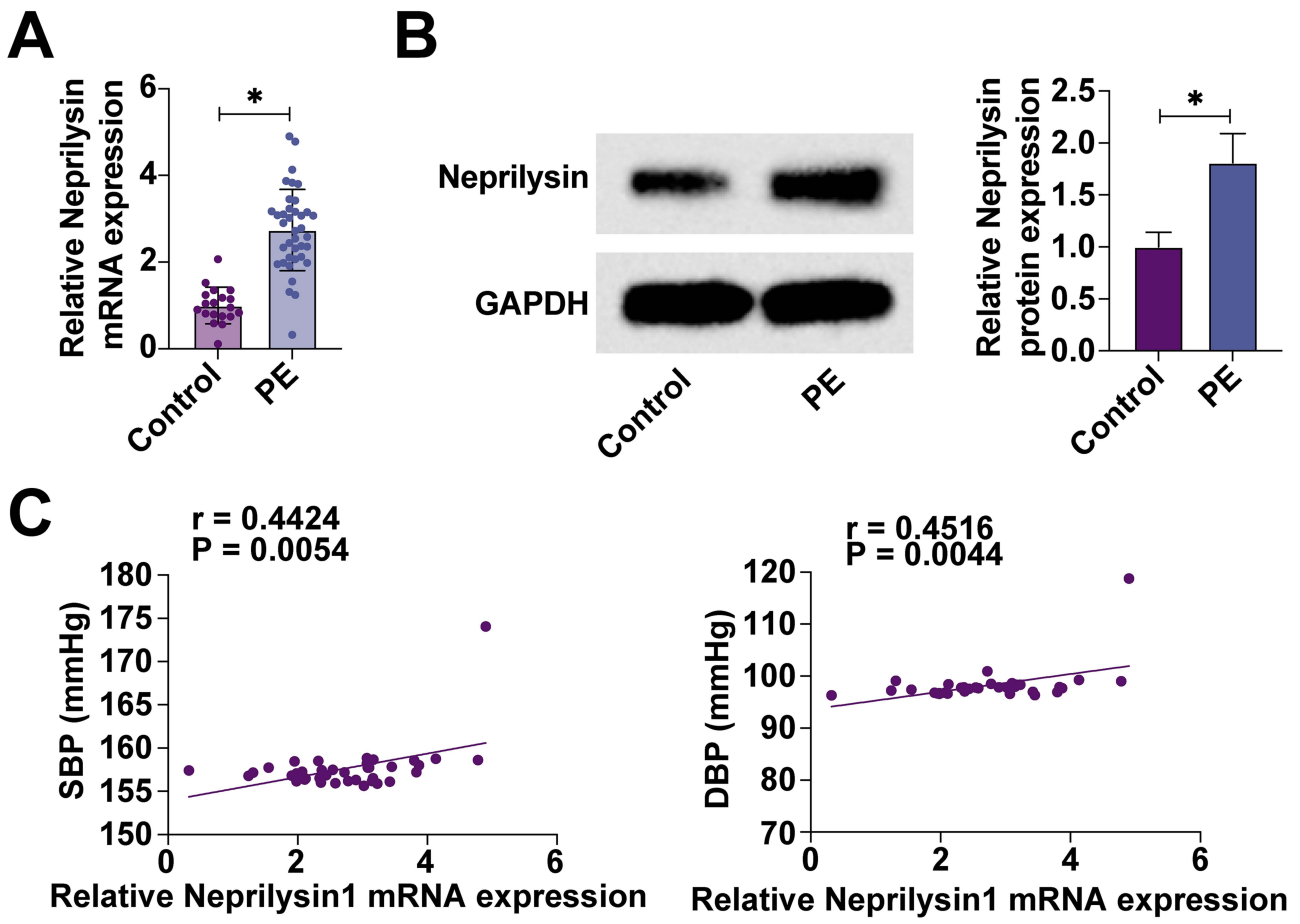

Figure 1 Neprilysin is Upregulated in PE. (**A**) RT-qPCR analysis of neprilysin expression in the serum of PE patients compared to controls. (**B**) Western blot analysis showing neprilysin expression in placental tissues from PE patients and controls. (**C**) Pearson correlation analysis demonstrating the association between neprilysin levels and systolic/diastolic blood pressure in PE patients. Data are shown as mean ± SD. * $P < 0.05$.

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Knockdown of Neprilysin Mitigates H_2O_2-Induced Oxidative Stress, Mitochondrial Dysfunction, and Impaired Invasion in EVT Cells

To determine the functional role of neprilysin in oxidative stress and trophoblast function, we transfected HTR8/SVneo cells with siRNA targeting neprilysin following treatment with H_2O_2. This intervention yielded significant changes in neprilysin expression and cell function. As shown in Figure 2A and B, H_2O_2 treatment significantly upregulated neprilysin mRNA and protein levels, which were effectively reversed by neprilysin knockdown. In a subsequent CCK-8 assay, H_2O_2 exposure markedly inhibited cell proliferation, while neprilysin knockdown restored proliferative capacity (Figure 2C). To further characterize oxidative stress and mitochondrial function, we employed the DCFH-DA fluorescent probe to quantify ROS generation. H_2O_2 treatment significantly increased intracellular ROS levels, but neprilysin silencing markedly reduced ROS accumulation (Figure 2D). MMP was assessed using JC-1 staining. H_2O_2 treatment led to a reduced red/green fluorescence ratio, indicative of mitochondrial depolarization, whereas neprilysin knockdown restored this ratio, suggesting partial recovery of mitochondrial function (Figure 2E). Additionally, flow cytometric analysis revealed that H_2O_2 treatment increased apoptosis, while neprilysin silencing effectively reduced apoptosis rates (Figure 2F). Transwell assays further demonstrated that H_2O_2 treatment impaired trophoblast invasion and migration, an effect that was reversed upon neprilysin knockdown (Figure 2G). In the conditioned media, H_2O_2 exposure increased levels of sFlt-1, sEng, IL-6, and IL-1β, while neprilysin knockdown significantly attenuated these pro-inflammatory factors (Figure 2H). Western blot analysis was used to assess changes in inflammation and antioxidant pathways. H_2O_2 treatment activated the p65 inflammatory pathway and suppressed the Nrf2/HO-1 antioxidant pathway, effects that were inhibited by neprilysin silencing (Figure 2I). Taken together, these findings demonstrate that neprilysin knockdown effectively alleviates H_2O_2-induced oxidative stress, mitochondrial dysfunction, inflammation, and apoptosis in EVT cells.

IGF2BP1 Enhances Neprilysin mRNA Expression

Next, we sought to explore RNA-binding proteins interacting with neprilysin. Using the Starbase database (https://rnasysu.com/encori/index.php) and integrating previous literature, we focused on IGF2BP1. IGF2BP1 is a well-known RNA-binding protein implicated in stabilizing downstream mRNA, promoting inflammation and pathological damage in multiple diseases. Starbase identified multiple potential binding sites between IGF2BP1 and neprilysin (Figure 3A). We subsequently examined the expression levels of IGF2BP1 in PE tissues compared to controls. As shown in Figure 3B and C, IGF2BP1 expression was significantly elevated in PE tissues. Pearson correlation analysis revealed a positive correlation between IGF2BP1 and neprilysin

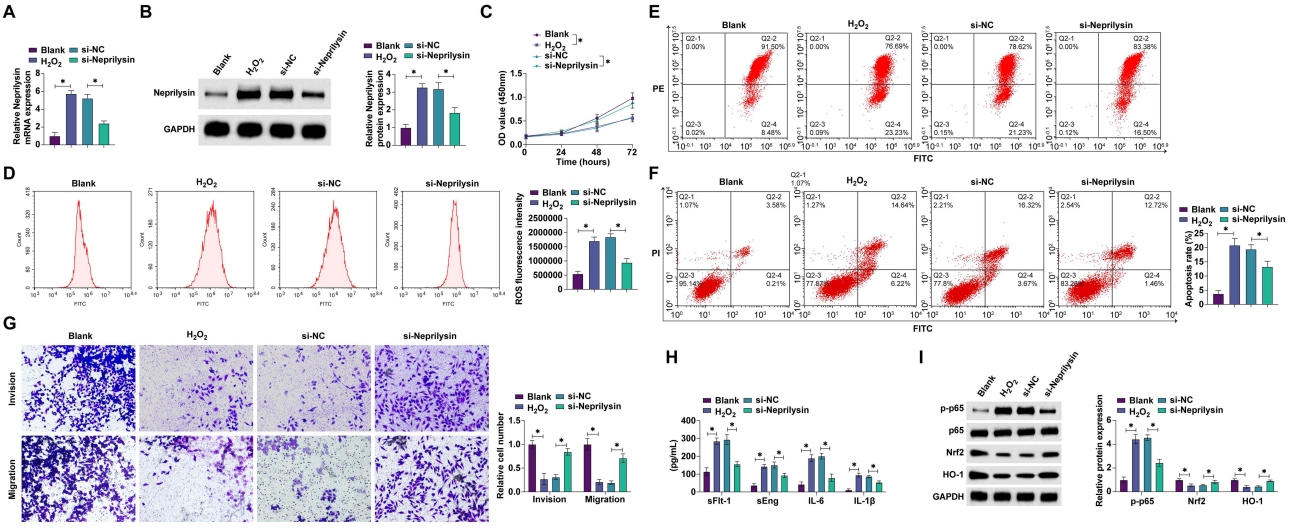

Figure 2 Knockdown of Neprilysin Alleviates H_2O_2-Induced Oxidative Stress and Mitochondrial Dysfunction in EVT Cells. siRNA targeting neprilysin was transfected into H_2O_2-treated HTR8/SVneo cells. (**A**) RT-qPCR showing neprilysin mRNA levels. (**B**) Western blot showing neprilysin protein expression. (**C**) CCK-8 assay measuring cell proliferation rate. (**D**) Flow cytometry for ROS production. (**E**) JC-1 staining for MMP. (**F**) Flow cytometry for apoptosis rates. (**G**) Transwell assays for cell invasion and migration. (**H**) ELISA for sFlt-1, sEng, IL-6, and IL-1β levels in cell supernatant. (**I**) Western blot for p65 and Nrf2/HO-1 pathway activation. Data are shown as mean ± SD (N = 3). * $P < 0.05$.

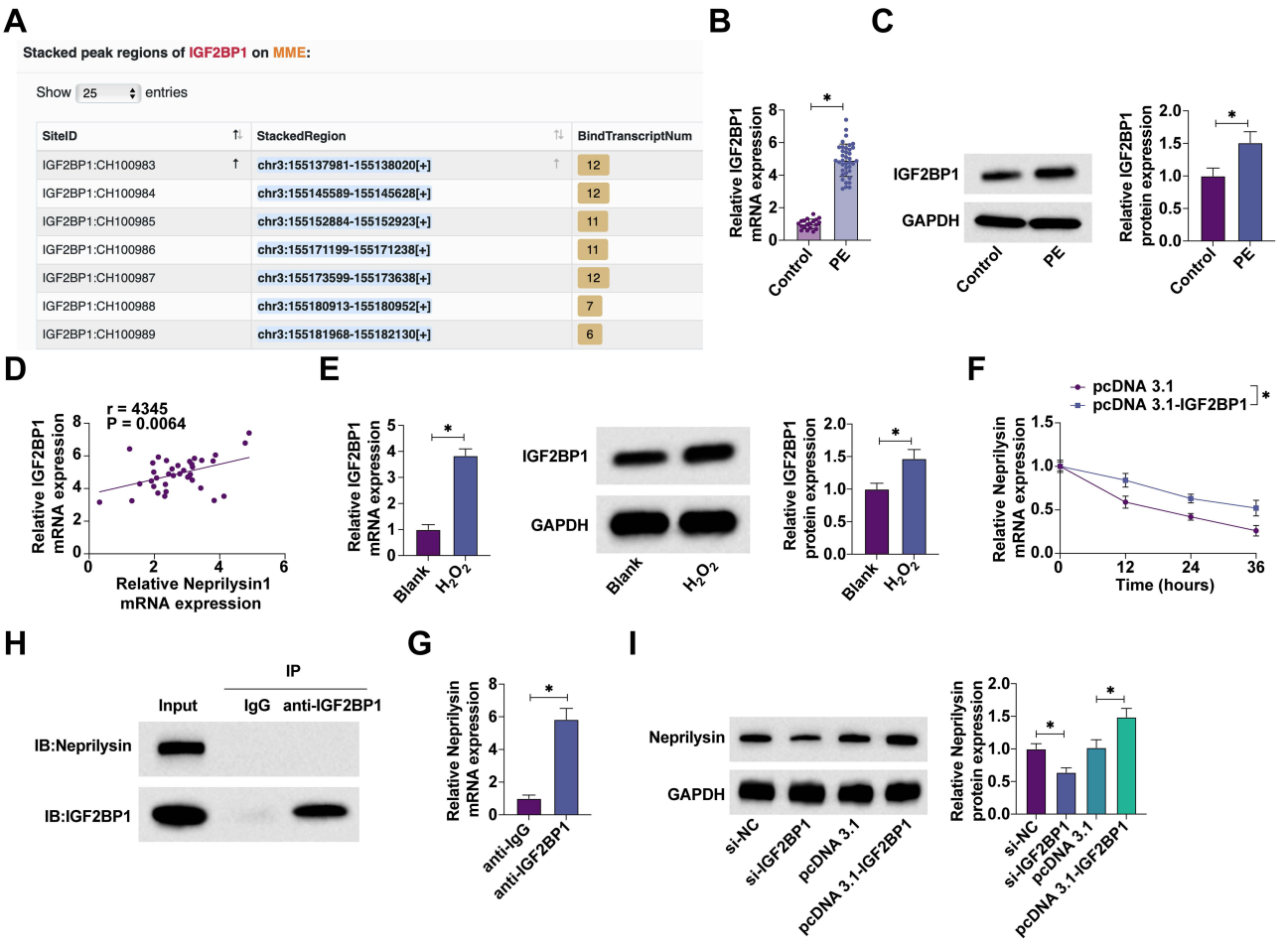

Figure 3 IGF2BP1 Enhances Neprilysin mRNA Expression. (**A**) Starbase analysis revealing binding sites between IGF2BP1 and neprilysin. (**B**) RT-qPCR showing IGF2BP1 levels in PE versus controls. (**C**) Western blot showing IGF2BP1 expression in placental tissues. (**D**) Pearson correlation between IGF2BP1 and neprilysin in PE samples. (**E**) RT-qPCR and Western blot showing IGF2BP1 expression in H_2O_2-treated cells. (**F**) Actinomycin D assay assessing IGF2BP1 effect on neprilysin mRNA stability. (**G**) RIP assay showing IGF2BP1 and neprilysin interaction. (**H**) Co-IP of IGF2BP1 and neprilysin proteins. (**I**) Western blot for neprilysin levels after IGF2BP1 modulation. Data are shown as mean ± SD (N = 3). * $P < 0.05$.

expression in PE samples (Figure 3D). Furthermore, in H_2O_2-treated HTR8/SVneo cells, both mRNA and protein levels of IGF2BP1 were upregulated (Figure 3E). To determine whether IGF2BP1 directly regulates neprilysin, we assessed their interaction. Overexpression of IGF2BP1 enhanced the resistance of neprilysin mRNA to actinomycin D, suggesting increased mRNA stability (Figure 3F). Additionally, RIP assays indicated that IGF2BP1 enriched neprilysin mRNA (Figure 3G). However, CO-IP experiments revealed no direct interaction between IGF2BP1 and neprilysin at the protein level (Figure 3H), suggesting that IGF2BP1 primarily binds neprilysin mRNA rather than the protein itself. Moreover, knockdown or overexpression of IGF2BP1 led to decreased or increased neprilysin protein expression, respectively (Figure 3I). These data collectively suggest that IGF2BP1 enhances neprilysin mRNA stability, thereby promoting neprilysin protein expression.

Knockdown of IGF2BP1 Mitigates H_2O_2-Induced Damage in EVT Cells

To determine IGF2BP1's role in oxidative stress-induced trophoblast dysfunction, IGF2BP1 was silenced in H_2O_2-treated HTR8/SVneo cells. Knockdown of IGF2BP1 significantly decreased both mRNA and protein levels (Figure 4A and B). CCK-8 assays demonstrated that IGF2BP1 knockdown substantially enhanced cell proliferation, reversing the suppressive effects of H_2O_2 (Figure 4C). DCFH-DA fluorescence analysis showed that IGF2BP1 silencing significantly reduced ROS levels (Figure 4D). JC-1 staining demonstrated that knockdown of IGF2BP1 restored MMP, as indicated by an increased red/green fluorescence ratio (Figure 4E). Flow cytometry further revealed that apoptosis rates were markedly reduced upon IGF2BP1 knockdown (Figure 4F). Transwell assays indicated that IGF2BP1 knockdown improved both migration and invasion

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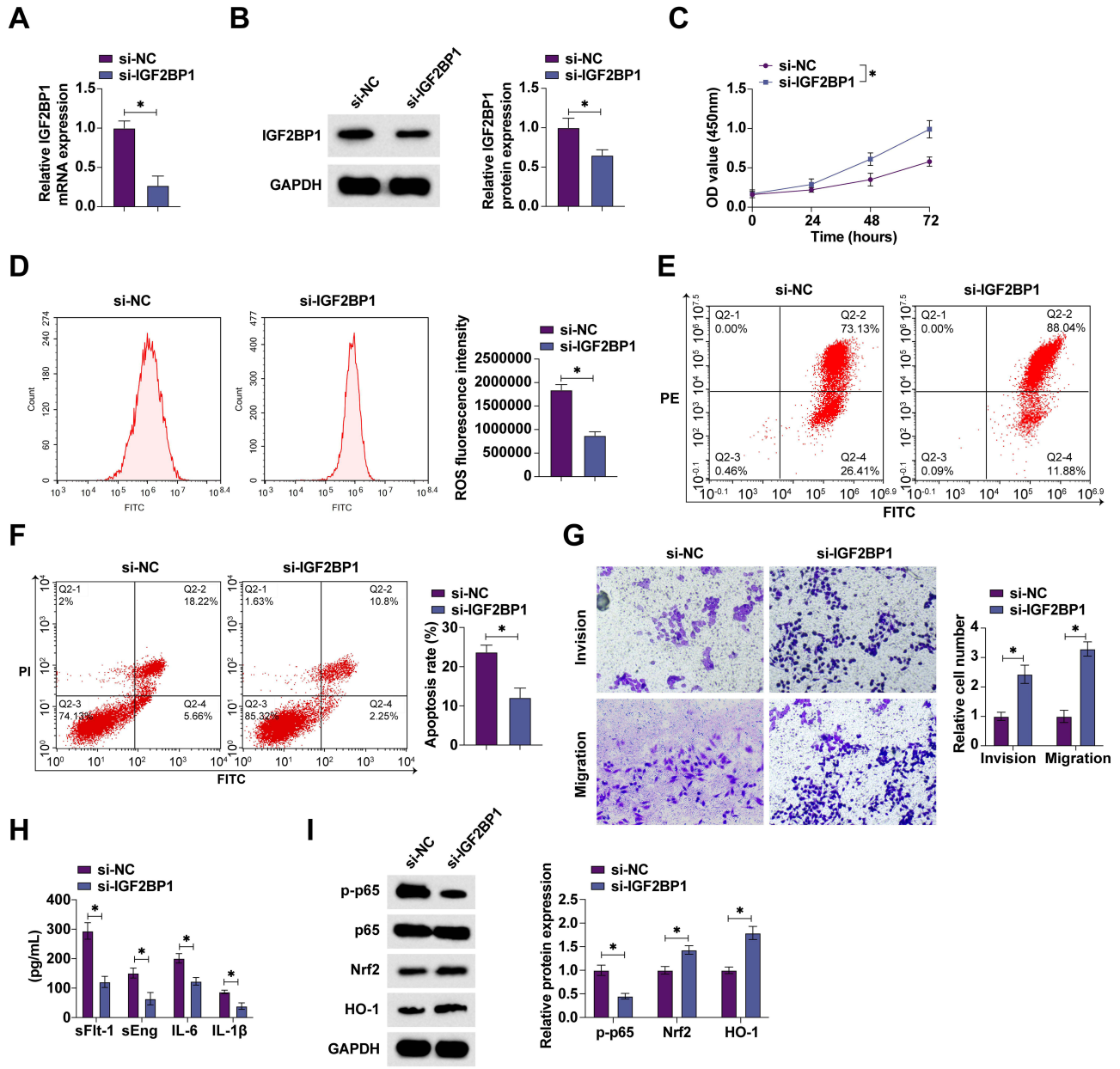

Figure 4 Silencing IGF2BP1 Alleviates H_2O_2-Induced Damage in EVT Cells. siRNA targeting IGF2BP1 was transfected into H_2O_2-treated HTR8/SVneo cells. (**A**) RT-qPCR for IGF2BP1 mRNA levels. (**B**) Western blot for IGF2BP1 protein expression. (**C**) CCK-8 assay showing cell proliferation. (**D**) Flow cytometry for ROS levels. (**E**) JC-1 staining for MMP. (**F**) Flow cytometry for apoptosis. (**G**) Transwell assay for cell invasion and migration. (**H**) ELISA for sFlt-1, sEng, IL-6, and IL-1β levels. (**I**) Western blot for p65 and Nrf2/HO-1 pathway activity. Data are shown as mean ± SD (N = 3). * $P < 0.05$.

capabilities impaired by H_2O_2 (Figure 4G). Moreover, IGF2BP1 silencing reduced the secretion of inflammatory markers, including sFlt-1, sEng, IL-6, and IL-1β (Figure 4H). Western blot analysis revealed decreased p65 phosphorylation and increased HO-1 and Nrf2 protein levels following IGF2BP1 knockdown (Figure 4I). Collectively, these data demonstrate that IGF2BP1 knockdown mitigates H_2O_2-induced oxidative stress, mitochondrial dysfunction, inflammation, and apoptosis in EVT cells.

Overexpression of IGF2BP1 Exacerbates H_2O_2-Induced Damage in EVT Cells

We further investigated the impact of IGF2BP1 overexpression on EVT cell injury under oxidative stress. HTR8/SVneo cells treated with H_2O_2 were transfected with pcDNA 3.1 constructs to overexpress IGF2BP1. Both mRNA and protein levels of IGF2BP1 were significantly increased following transfection (Figure 5A and B). CCK-8 assays indicated that

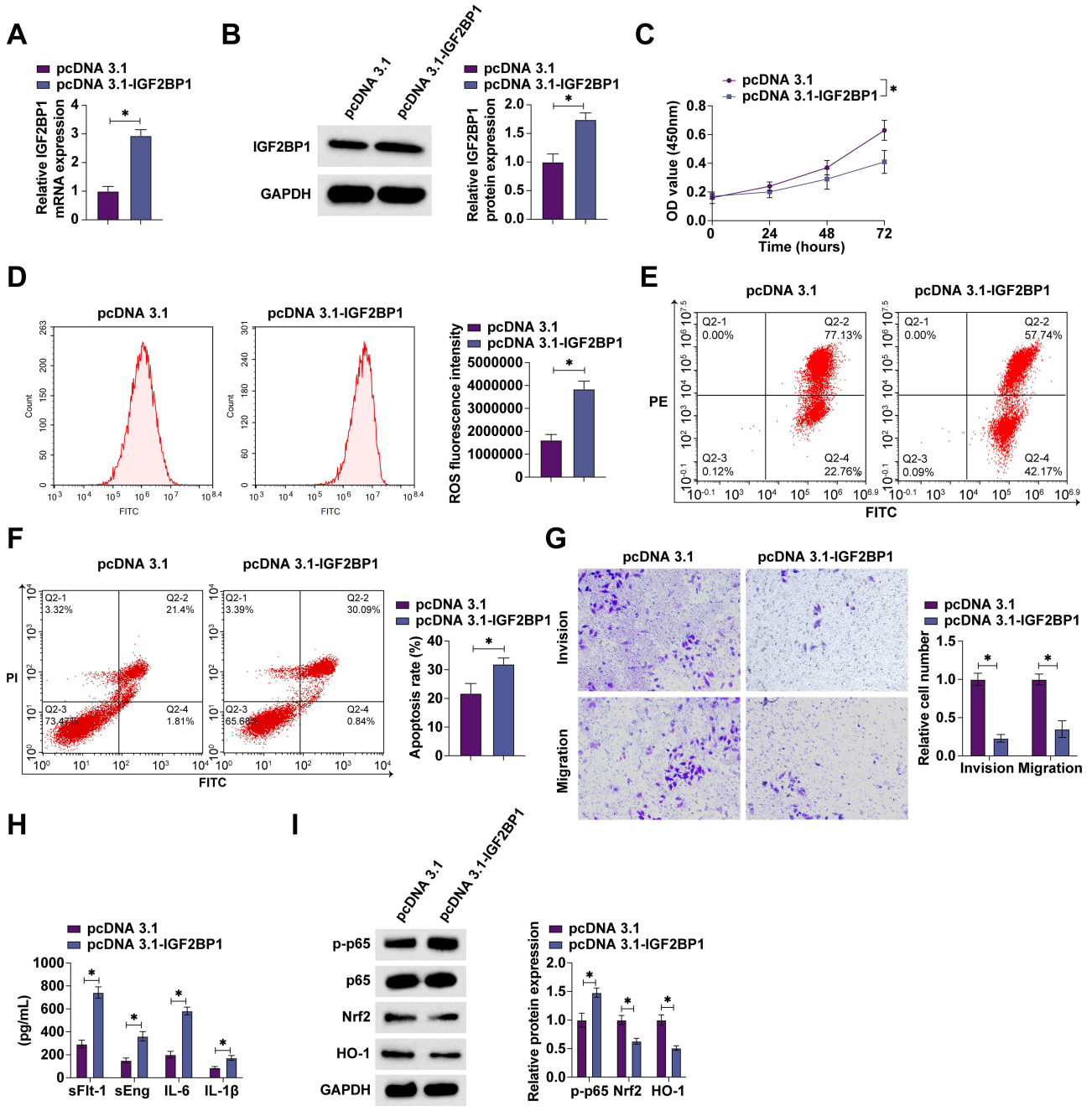

Figure 5 Overexpression of IGF2BP1 Exacerbates H_2O_2-Induced EVT Cell Injury. Cells were transfected with pcDNA 3.1 targeting IGF2BP1 in the presence of H_2O_2. (**A**) RT-qPCR for IGF2BP1 mRNA levels. (**B**) Western blot showing IGF2BP1 protein expression. (**C**) CCK-8 assay showing cell proliferation rate. (**D**) Flow cytometry for ROS levels. (**E**) JC-1 staining showing MMP. (**F**) Flow cytometry for apoptosis rates. (**G**) Transwell assay showing invasion and migration capacity. (**H**) ELISA for sFlt-1, sEng, IL-6, and IL-1β levels in supernatant. (**I**) Western blot analysis for p65 and Nrf2/HO-1 pathway activity. Data are shown as mean ± SD (N = 3). * $P < 0.05$.

IGF2BP1 overexpression led to a further reduction in cell proliferation rates (Figure 5C). To assess oxidative stress and mitochondrial function, DCFH-DA staining revealed a marked increase in ROS levels upon IGF2BP1 overexpression (Figure 5D). JC-1 staining demonstrated that overexpression of IGF2BP1 caused a further decrease in the red/green fluorescence ratio, indicating reduced MMP (Figure 5E). Flow cytometric analysis showed that IGF2BP1 overexpression significantly increased apoptosis rates compared to control cells (Figure 5F). Transwell assays revealed that IGF2BP1 overexpression impaired both invasion and migration capacities of EVT cells (Figure 5G). Additionally, overexpression of IGF2BP1 resulted in elevated levels of sFlt-1, sEng, IL-6, and IL-1β in the cell supernatant, highlighting enhanced

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inflammatory activity (Figure 5H). Western blot analysis further showed that IGF2BP1 overexpression promoted phosphorylation of p65 while suppressing the expression of HO-1 and Nrf2 (Figure 5I). These data suggest that IGF2BP1 overexpression exacerbates H_2O_2-induced apoptosis, mitochondrial dysfunction, inflammatory responses, and oxidative stress in EVT cells, starkly contrasting with the protective effects observed upon IGF2BP1 knockdown.

IGF2BP1 Drives H_2O_2-Induced Cellular Injury by Stabilizing Neprilysin

To better understand how IGF2BP1 contributes to H_2O_2-induced cellular injury and to assess whether its effects hinge on neprilysin, we simultaneously knocked down IGF2BP1 and overexpressed neprilysin in H_2O_2-treated HTR8/SVneo cells. As illustrated in Figure 6A and B, neprilysin overexpression restored neprilysin levels diminished by IGF2BP1 knockdown. CCK-8 proliferation assays revealed that reducing IGF2BP1 significantly boosted cell proliferation, but neprilysin overexpression negated this beneficial effect (Figure 6C). To further explore oxidative stress and mitochondrial function, DCFH-DA staining demonstrated that knocking down IGF2BP1 led to a substantial reduction in ROS accumulation, while neprilysin overexpression reversed this trend, increasing ROS levels once again (Figure 6D). JC-1 staining showed an increase in MMP with IGF2BP1 knockdown, indicated by a higher red/green fluorescence ratio. However, neprilysin overexpression countered this mitochondrial recovery (Figure 6E). Flow cytometry analysis also confirmed that IGF2BP1 knockdown significantly reduced apoptosis rates, an effect that neprilysin overexpression largely negated (Figure 6F). Transwell assays provided further evidence that IGF2BP1 knockdown enhanced the migratory and invasive capabilities of EVT cells. However, neprilysin overexpression suppressed these positive effects, again driving the cells back toward an impaired state (Figure 6G). Additionally, the reduction in inflammatory markers—sFlt-1, sEng, IL-6, and IL-1β—seen with IGF2BP1 knockdown was reversed by neprilysin overexpression, restoring elevated levels of these markers and highlighting a pro-inflammatory shift (Figure 6H). Western blot analysis showed that IGF2BP1 knockdown suppressed p65 phosphorylation and boosted the expression of HO-1 and Nrf2, two crucial antioxidant proteins.

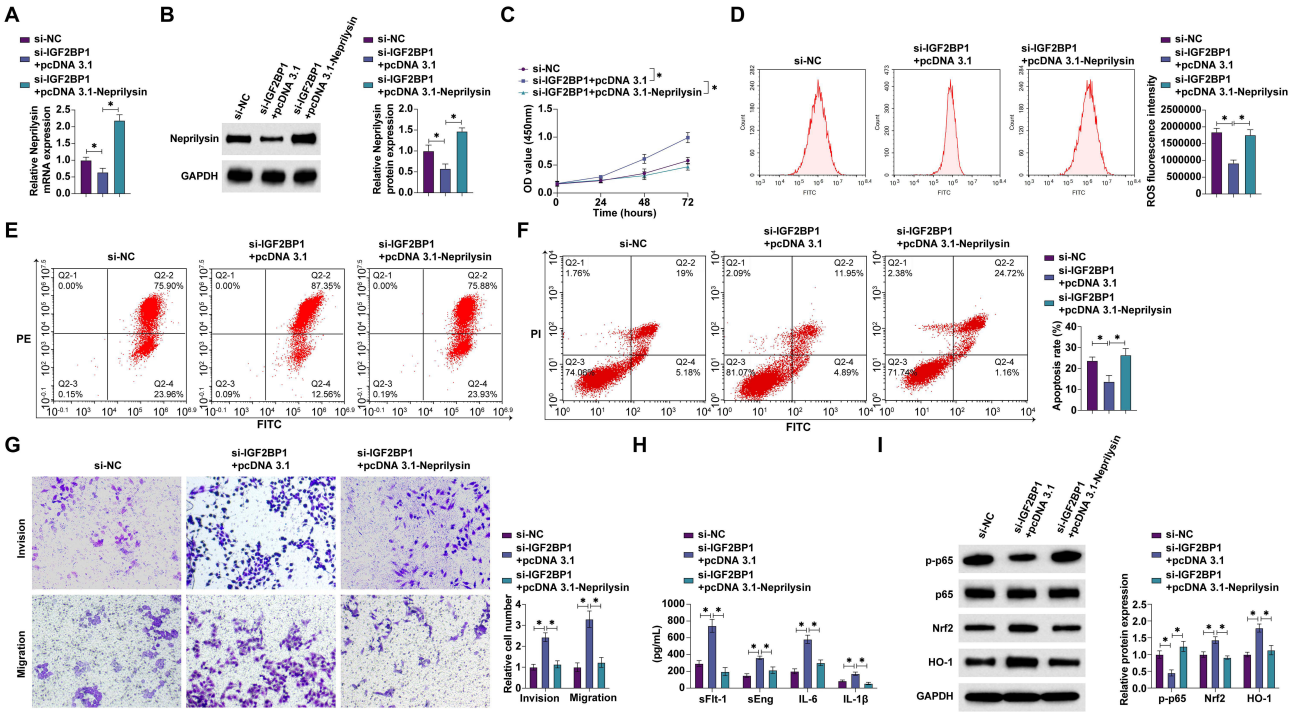

Figure 6 IGF2BP1 Promotes H_2O_2-Induced Cell Damage via Stabilization of Neprilysin. HTR8/SVneo cells subjected to H_2O_2 treatment were co-transfected with si-IGF2BP1 and pcDNA 3.1-Neprilysin to assess the functional role of neprilysin under IGF2BP1 knockdown conditions. (**A**) RT-qPCR analysis showing neprilysin mRNA levels following transfection. (**B**) Western blot analysis illustrating neprilysin protein expression changes. (**C**) CCK-8 assay evaluating the impact on cell proliferation rate. (**D**) Flow cytometry detecting changes in intracellular ROS levels. (**E**) JC-1 staining used to assess MMP. (**F**) Flow cytometry analysis for quantifying apoptotic cells. (**G**) Transwell assay evaluating cell invasion and migration capabilities. (**H**) ELISA measuring the levels of sFlt-1, sEng, IL-6, and IL-1β in cell supernatants. (**I**) Western blot analysis for p65 and Nrf2/HO-1 signaling pathway activation. Data are presented as mean ± SD (N = 3). * $P < 0.05$.

However, neprilysin overexpression once again reverted these changes, favoring a pro-inflammatory and oxidative state (Figure 6I). These findings reveal a complex interplay where IGF2BP1 exacerbates H_2O_2-induced apoptosis, mitochondrial dysfunction, oxidative stress, and inflammation by stabilizing neprilysin.

Discussion

Oxidative stress and mitochondrial dysfunction within placental trophoblasts play pivotal roles in the pathogenesis of pregnancy-related complications, including PE.[22] These cells are essential for nutrient exchange and endocrine regulation in the placenta, and their impairment directly compromises placental function and overall maternal-fetal health.[23,24] However, the molecular pathways that allow trophoblasts to mitigate oxidative stress and preserve mitochondrial integrity remain incompletely understood. Notably, the roles of neprilysin and IGF2BP1 in these processes within the context of PE have not been thoroughly investigated, representing a significant gap in the current studies. In this study, we uncovered a novel mechanism whereby IGF2BP1, an RNA-binding protein, modulates neprilysin mRNA stability. Our findings indicate that IGF2BP1 directly interacts with neprilysin mRNA, enhancing its transcription and translation, and ultimately influencing oxidative damage in EVT cells under H_2O_2-induced stress.

Neprilysin, a zinc-dependent metalloprotease, is broadly expressed in mammalian tissues and primarily functions to degrade various bioactive peptides, including neuropeptides, angiotensin I, enkephalins, and pro-inflammatory peptides, thereby regulating their bioavailability and physiological activity.[25] In the cardiovascular system, neprilysin plays a critical role in modulating blood pressure and cardiac function by breaking down vasoactive peptides such as angiotensin I.[19,26] Despite the well-characterized physiological and pathological roles of neprilysin in the cardiovascular system, its specific functions and regulatory mechanisms in PE remain inadequately explored. Existing studies primarily focus on its role in blood pressure regulation, with limited insights into its involvement in placental biology and trophoblast function. Although the physiological roles of neprilysin have been well characterized, its contribution to specific disease contexts, such as PE, requires further exploration. Recent studies have shown neprilysin is overexpressed in PE and positively correlates with clinical markers of disease severity.[21] Our findings not only confirm these associations but also demonstrate, for the first time, that neprilysin is directly involved in the pathogenesis of PE.

Significantly, our data suggest that targeting neprilysin may offer therapeutic potential in alleviating oxidative stress and inflammation in several pathological settings. Neprilysin inhibition has been shown to disrupt oxidative stress, inflammation, and caspase-3-mediated apoptosis, thus reducing cardiotoxicity in doxorubicin-treated models.[27] Moreover, neprilysin inhibitors like LCZ696 have been reported to mitigate diabetic cardiomyopathy by reducing inflammation, oxidative stress, and apoptosis.[28] However, the application of neprilysin inhibitors in the context of PE has not been extensively studied, particularly regarding their effects on placental trophoblasts and mitochondrial function. In this study, we observed that neprilysin inhibition in H_2O_2-treated EVT cells ameliorated oxidative damage, improved mitochondrial function, and reduced apoptosis and inflammation. These findings position neprilysin as a promising therapeutic target in PE, and future development of neprilysin inhibitors or RNAi-based approaches could hold substantial clinical promise. However, these findings necessitate further validation in in vivo models to determine their translational potential.

Our results also establish IGF2BP1 as a key regulator of neprilysin at both transcriptional and translational levels. While IGF2BP1 has been extensively studied in the context of cancer and cellular stress responses, its specific interaction with neprilysin in PE has not been previously reported, underscoring a novel aspect of PE pathophysiology. IGF2BP1, a member of the insulin-like growth factor 2 mRNA-binding protein family, has been implicated in regulating mRNA stability and translation, impacting cellular processes such as proliferation, migration, survival, and metabolism.[29,30] Although its role in PE had not been previously characterized, previous studies indicate that IGF2BP1 can promote oxidative stress and inflammatory pathways. For example, IGF2BP1 has been shown to regulate MDM2 mRNA stability in an m(6)A-dependent manner, mediating oxidative stress-induced dysfunction in granulosa cells.[31] Additionally, silencing IGF2BP1 has been reported to reduce macrophage RUNX1 expression and enhance autophagy, thereby attenuating ox-LDL-induced lipid accumulation and inflammation.[32] In line with these studies, our findings demonstrate that IGF2BP1 exacerbates oxidative stress and inflammation in PE by influencing mitochondrial dysfunction in EVT cells and enhancing ROS production, which in turn activates downstream apoptotic and inflammatory pathways. Crucially, our study reveals that IGF2BP1 directly stabilizes neprilysin mRNA, thereby increasing neprilysin protein levels and

amplifying its pathological effects in PE. This novel interaction highlights a previously unrecognized regulatory axis that contributes to the oxidative and inflammatory milieu observed in PE.

Despite these advancements, several gaps remain in our understanding of the interplay between neprilysin and IGF2BP1 in PE. Our study uncovers that IGF2BP1 enhances neprilysin mRNA stability, promoting its protein expression, which is associated with increased neprilysin levels during H_2O_2-induced EVT cell injury. This interaction uncovers a complex regulatory network that drives oxidative stress and inflammation in PE, highlighting IGF2BP1 and neprilysin as key contributors to disease pathology. However, the specific downstream signaling pathways through which neprilysin and IGF2BP1 exert their effects on trophoblast function and mitochondrial integrity require further investigation. Additionally, the potential involvement of other molecular players in this regulatory network remains to be explored. Targeting this regulatory axis may present a novel therapeutic strategy for mitigating the progression of PE and its associated complications.

Moreover, while our in vitro findings provide significant insights, the in vivo relevance of neprilysin and IGF2BP1 interactions in PE needs to be validated using animal models and clinical samples. Future research should focus on elucidating the detailed molecular mechanisms by which neprilysin and IGF2BP1 interact and regulate oxidative stress and mitochondrial function in the placenta. Investigating the expression and functional roles of neprilysin and IGF2BP1 across different PE subtypes could also provide a more nuanced understanding of their contributions to the disease's heterogeneity. Additionally, exploring the therapeutic potential of targeting neprilysin and IGF2BP1 in combination with other molecular pathways involved in PE may lead to more effective treatment strategies.

Furthermore, this study opens avenues for exploring other molecular factors that may interact with neprilysin and IGF2BP1, thereby enhancing our understanding of the regulatory networks involved in PE. For instance, examining potential post-translational modifications of neprilysin or identifying additional RNA-binding proteins that may collaborate with IGF2BP1 could uncover new layers of regulation. Integrating multi-omics approaches, such as transcriptomics and proteomics, in future studies could facilitate the identification of novel interactions and pathways relevant to PE pathogenesis, providing a comprehensive view of the molecular landscape of PE.

In conclusion, our study reveals that IGF2BP1 enhances neprilysin mRNA stability, promoting its protein expression, which is associated with increased neprilysin levels during H_2O_2-induced EVT cell injury. This interaction uncovers a complex regulatory network that drives oxidative stress and inflammation in PE, highlighting IGF2BP1 and neprilysin as key contributors to disease pathology. Targeting this regulatory axis may present a novel therapeutic strategy for mitigating the progression of PE and its associated complications.

Data Sharing Statement

Data is available from the corresponding author on request.

Ethics Statement

The present study was approved by the Ethics Committee of Baoying Maternal and Child Health Hospital (No.202011004) and written informed consent was provided by all patients prior to the study start. All procedures were performed in accordance with the ethical standards of the Institutional Review Board and The Declaration of Helsinki, and its later amendments or comparable ethical standards.

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Disclosure

The authors have no conflicts of interest to declare for this work.

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