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EXPERIMENTAL RESEARCH

Oxidative low-density lipoprotein oxLDL induces pyroptosis in trophoblast cells HTR-8/Svneo by downregulating TET2

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[Abstract] **Objective** To investigate pyroptosis in the trophoblast cells HTR-8/Svneo induced by TET2 in oxidized low-density lipoprotein (oxLDL). **Methods** HTR-8/Svneo cells were treated with vehicle (0 mg/L) and 50 mg/L oxLDL for 24 h. The effects of oxLDL on TET2 expression in HTR-8/Svneo cells were detected by Western blot. Knockdown of TET2 was performed in HTR-8/Svneo cells using si-TET2. CCK8, Transwell, and wound healing assays were used to detect the effect of TET2 knockdown on the proliferation, cell cycle, migration, and invasion of HTR-8/Svneo cells, respectively. Western blot was used to quantify the expression levels of the pyroptosis proteins GSDMD and caspase-1 in si-TET2 treated cells. **Results** oxLDL downregulates TET2 expression in a concentration-dependent manner. TET2 knockdown inhibits HTR-8/Svneo invasion and migration but promotes cell proliferation. Western blot results showed that TET2 knockdown upgregulated the expression of GSDMD and caspase-1. **Conclusion** oxLDL induces pyroptosis of HTR-8/Svneo by downregulating TET2 expression, resulting in decreased trophoblast invasion and migration. These data suggest that TET2-induced pyroptosis play a critical role in gestational pathologies, such as preeclampsia. Further studies can clarify the mechanisms of this process and elucidate potential preventive and therapeutic targets.

[Key words] oxLDL; TET2; HTR-8/Svneo; Pyroptosis

1 Introduction

Trophoblast dysfunction can lead to implantation failure of the embryo, abnormal placental development, and insufficient vasoremodeling at the maternal-fetal interface. These can increase the risk of pregnancy-related diseases such as preeclampsia, preterm birth, and fetal growth restriction. Oxidized low-density lipoprotein (oxLDL) is a major endogenous cytotoxic substance in lipid metabolism disorders and has been shown to induce trophoblast cell dysfunction[1]. However, the mechanism of oxLDL-induced trophoblast dysfunction has not been clarified. The development of the placenta is regulated by DNA methylation, while cellular differentiation in trophoblast cells

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results from differential methylation and non-methylation across the genome[2-3]. As a key enzyme in DNA demethylation, TET2 (ten-eleven translocation oncogene family member 2) modifies the nucleotide methylation state to trigger passive demethylation[4]. In the placental tissues of preeclampsia patients, gene promoter regions with abnormal expression are regulated by DNA methylation and the expression of TET protein is decreased[5-6]. These suggest that TET2-mediated DNA demethylation plays an important role in placental development. The primary aim of this study was to investigate the mechanism of oxLDL-induced pyroptosis in trophoblast cells by observing the effect of oxLDL on TET2 expression in HTR-8/Svneo cells and determining the effects of TET2 knockdown on pyroptosis-related protein expression, cell proliferation, invasion, and migration. This research can serve as a reference for further studies of gestational pathologies related to abnormal function in human chorionic trophoblast cells.

2 Materials and methods

2.1 Materials and reagents

Human trophoblast HTR-8/Svneo cells were purchased from Wuhan Procell Life Technology Co., Ltd. RPMI 1640 cell medium and 1% double antibiotic (penicillin 100 U/mL and streptomycin 100 μg/mL) were purchased from Gibco Co., Ltd. BI fetal bovine serum was purchased from HyClone Co., Ltd. Transwell System was purchased from Corning Co., Ltd. siTET2 was purchased from RiboBio, Guangzhou. Trizol and miRNA reverse transcription kit (Invitrogen), reverse transcription kit (Qiagen), SYBR® Green Master Mix (Med Chem Express), Bio-Rad nitric acid fiber membrane, primary and secondary antibodies for GSDMD and caspase-1 were purchased from Wuhan Sanying Biotechnology Co., Ltd. Lipofectamine 3000 was purchased from Invitrogen Co., Ltd. oxLDL was purchased from Shanghai Yuanye Biotechnology Co., Ltd.

2.2 Cell culture

HTR8-Svneo cells were cultured in RPMI 1640 medium containing 10% BI fetal bovine serum and 1% double antibiotic (penicillin 100 U/mL and streptomycin 100 μg/mL). The culture flask was maintained in a 5% CO_2 incubator at 37 ℃. Cell passage was carried out when cell confluence reached about 80%.

2.3 CCK8 measured cell proliferation

When the cell confluence reached 70%–80%, the cell suspension was digested with 0.25% trypsin. HTR-8/Svneo cells at a cell density of 6.0×10^3/mL were inoculated in 96-well culture plates containing si-Ctrl or si-TET2 and incubated at 37 ℃ and 5% CO_2 for 24, 48 and 72 h. At different time points, 10 μL CCK8 reagent was added to each well and incubation was extended for another 2 h. The absorbance was measured at 450 nm with the microplate reader.

2.4 Cell cloning experiment

HTR-8/Svneo with cell density of 6.0×10^3/mL in 24-well culture plates containing si-Ctrl or si-TET2. Plates were cultured at 37 ℃ and 5% CO_2 for 5–7 days. When cells were collected, the culture plates were taken out of the cell chambers and fixed with 10% formalin for 20 min. After washing with PBS 2–3 times and staining with crystal violet for 30 min, the samples were viewed under a microscope and analyzed quantitatively with an enzyme marker.

2.5 Transwell experiment

Transwell chambers with si-Ctrl or si-TET2 were inoculated with 200 μL HTR-8/Svneo cell suspension (without serum) with cell density of 1.0×10^4. A volume of 600 μL complete medium containing 10% BI serum was added into the lower chamber. Chambers were incubated at 37 ℃ and 5%

CO_2 for 36 h. The Transwell chamber was removed and washed with PBS 2–3 times. Cells were formalin-fixed for 20 min, washed with PBS 2–3 times, and stained with 0.1% crystal violet for 30 min. The chamber was washed again with PBS 2–3 times and the upper layers of non-migrated cells were gently wiped off with a cotton swab. After air drying, five visual field cells were randomly observed under the microscope and counted.

2.6 Cell scratch experiment

Cells were digested with trypsin after transfection, followed by treatment with serum-free medium to terminate digestion. After even mixing, the suspension was centrifuged and the cells were counted. Each well of a 6-well plate contained si-Ctrl or si-TET2 and was inoculated with 5.0×10^5 cells. Cells were allowed to firmly attach to the wall for 24 h before a scratch was introduced in each well. Images were taken under a microscope at 0, 24, 48 and 72 h, respectively.

2.7 Plasmid transfection

Cell suspensions with a density of 3.0×10^4/mL were prepared using complete medium and inoculated into 6-well plates. The suspensions were cultured at 37 ℃ for 24 h until cell confluence reached 20%–30%. Plasmid transfection was performed according to the manufacturer's instructions for DNA-Lipofectamine 3000.

2.8 Fluorescence quantitative RT-PCR detects gene expression

Total RNA was extracted from HTR-8/Svneo cells and RNA concentration was determined. RNA was reversely transcribed into cDNA using the mRNA reverse transcription kit with T-RNA as the template. SYBR Green PCR kit was used for real-time quantitative PCR quantification of mRNA expression levels of different genes. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.9 Western blot

The total protein of HTR-8/Svneo cells was extracted and the protein concentration was determined by the BCA method. Equal amounts of protein were added to each SDS-PAGE gel lane. After the protein bands were separated, PVDF membranes with pore size of 0.45 μm were used for protein transfer. Membranes were blocked with skim milk for 1 h and rinsed with TBST six times for 10 min. Incubation with rabbit (rat) anti-human GSDMD, caspase-1, vimentin, *E*-cadherin, *N*-cadherin, and *β*-actin primary antibodies at 1/1 000 concentration was done overnight at 4 ℃, followed by washing of the membrane with TBST for three times at room temperature. Detection was performed using ECL chemiluminescence after incubation of horseradish peroxidase-labeled secondary antibody. Quantification of bands was performed using Image J.

2.10 Statistical analysis

All experiments were independently repeated three times. SPSS 19.0 was used for data analysis and GraphPad Prism 9.0 software was used for statistical mapping. Data were expressed as mean ± standard deviation ($V \pm s$). For normally distributed data, *t*-test and analysis of variance (ANOVA) were used to compare groups, while the non-parametric rank sum test was used for data with non-normal distributions. Categorical variables measured in counts were analyzed using chi-square test. $P<0.05$ was considered statistically significant.

3 Results

3.1 oxLDL downregulates TET2 expression in HTR-8/Svneo cells

HTR-8/Svneo cells were treated with 50 mg/L oxLDL and vehicle for 24 h. Western blot showed that oxLDL in HTR-8/Svneo inhibited TET2 expression in a concentration-dependent manner

(Fig. 1A–1B) and upregulated GSDMD and caspase-1 expression (Fig. 1C–1D).

3.2 Downregulation of TET2 promotes proliferation and clonal formation of HTR-8/Svneo cells

After transient TET2 knockdown in HTR-8/Svneo, TET2 knockdown promoted HTR-8/Svneo cell proliferation (A) and clonal formation (B) compared to controls. These differences between the two groups were statistically significant ($^{*}P<0.05$, Fig. 2C).

3.3 Downregulation of TET2 inhibits HTR-8/Svneo cell migration and invasion

The results of the Transwell migration and invasion experiment showed that compared with the si-Ctrl control group, the migration ability and the number of invasive cells in the si-TET2 group were significantly lower at 24 h and 48 h ($^{*}P<0.05$), as shown in Fig. 3.

3.4 TET2 knockdown upregulates GSDMD and caspase-1 expression in HTR-8/Svneo cells

TET2 knockdown upregulated the expression of GSDMD and caspase-1 in HTR-8/Svneo cells, indicating that TET2 downregulation could induce pyroptosis, as shown in Fig. 4.

3.5 Downregulation of TET2 inhibits epithelial-mesenchymal transformation in HTR-8/Svneo cells

Western Blot and RT-PCR showed that compared with the si-Ctrl control group, the relative expressions of *N*-cadherin and vimentin mRNA and protein in the si-TET2 group were significantly reduced ($P<0.05$), while *E*-cadherin mRNA and protein expression levels increased, as shown in Fig. 5.

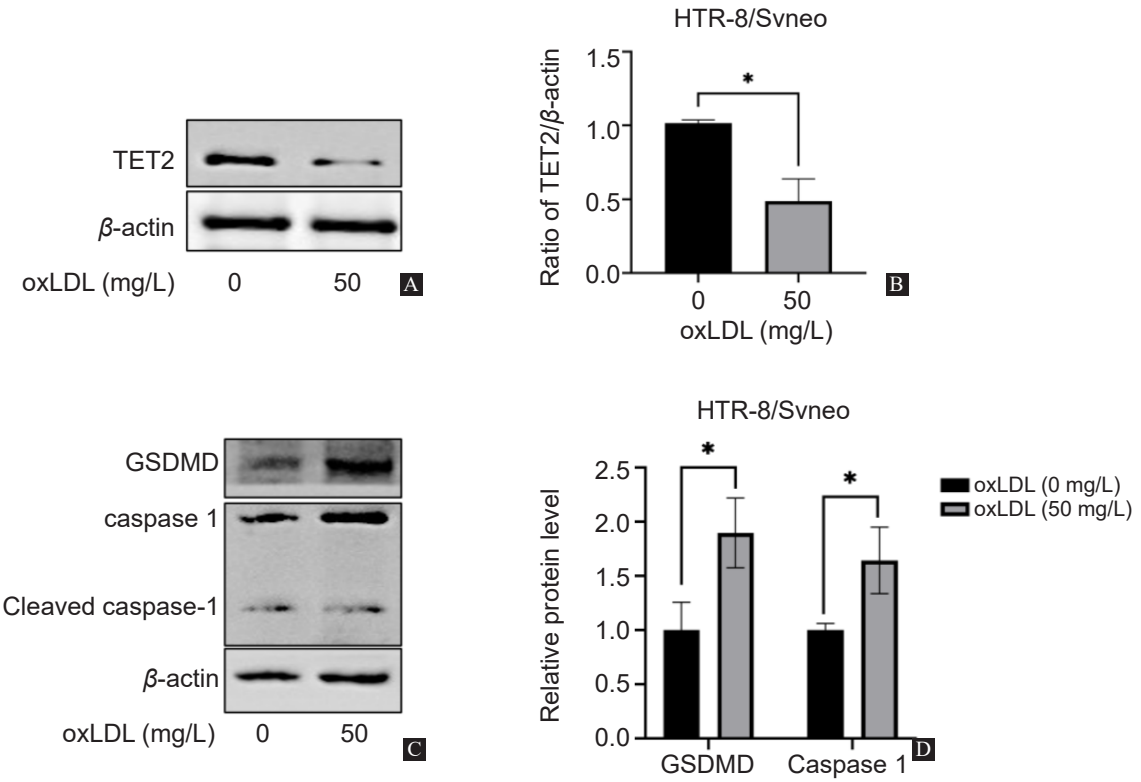

Fig. 1 Expression of TET2, GSDMD, and caspase-1 proteins in HTR-8/Svneo cells treated with oxLDL.
A–B. Comparison of TET2 protein relative expression levels after oxLDL and vehicle treatment; C–D. Comparison of the relative expression levels of GSDMD and caspase-1 proteins in cells treated with oxLDL.

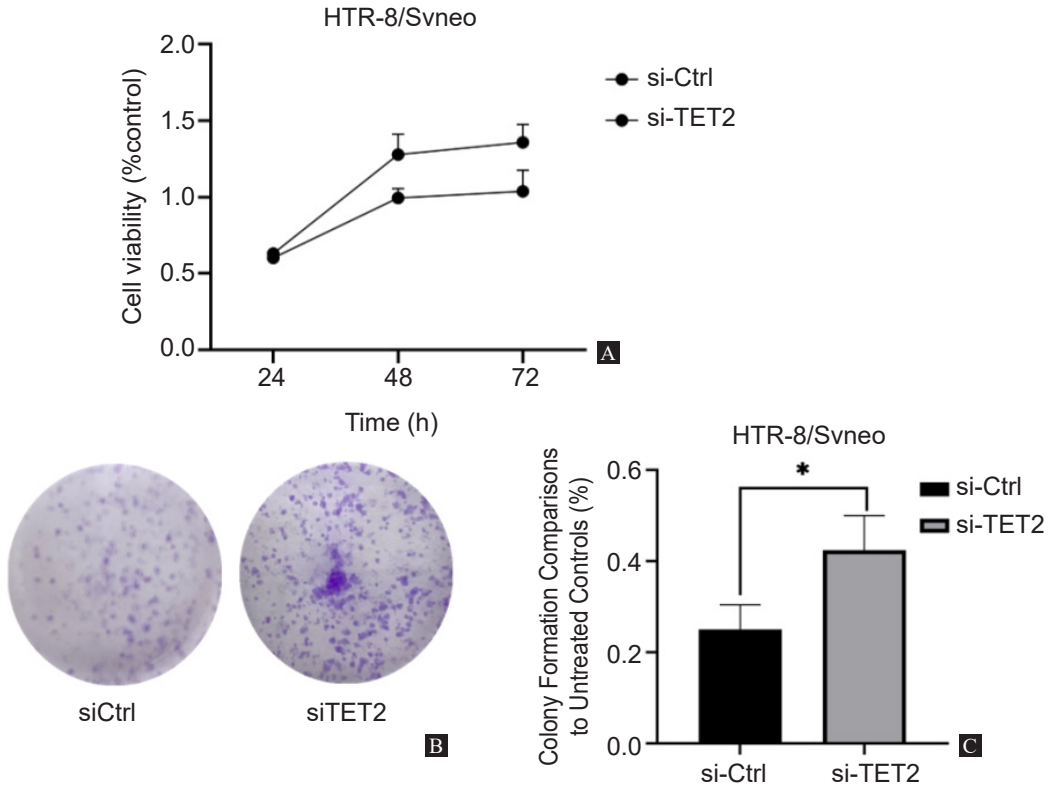

Fig. 2 Effects of TET2 knockdown on proliferation and clonogenesis of HTR-8/Svneo cells.
A. Comparison of cell proliferation capacity between the two groups after 24, 48, and 72 h; B–C. Comparison of cell cloning ability between the two groups after 5 days.

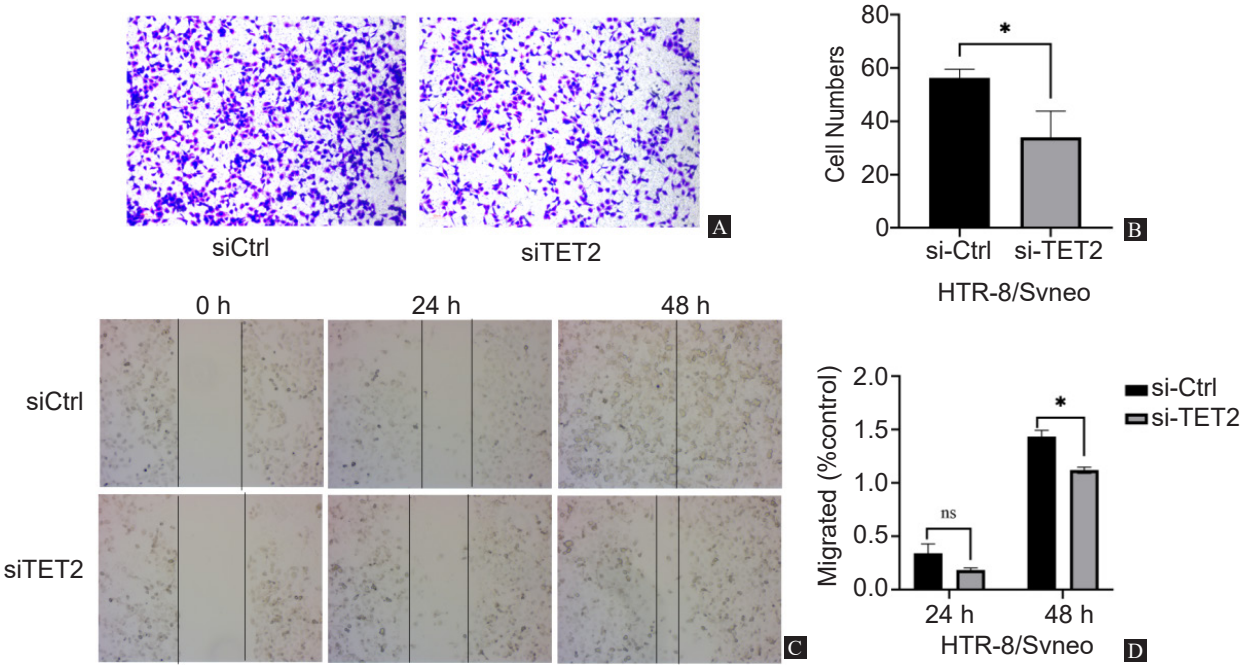

Fig. 3 Effects of TET2 knockdown on invasion and migration of HTR-8/Svneo cells.
A–B. Comparison of cell invasion ability between the two groups after 36 h culture; C–D. Comparison of cell migration ability between the two groups after 24 h and 48 h culture.

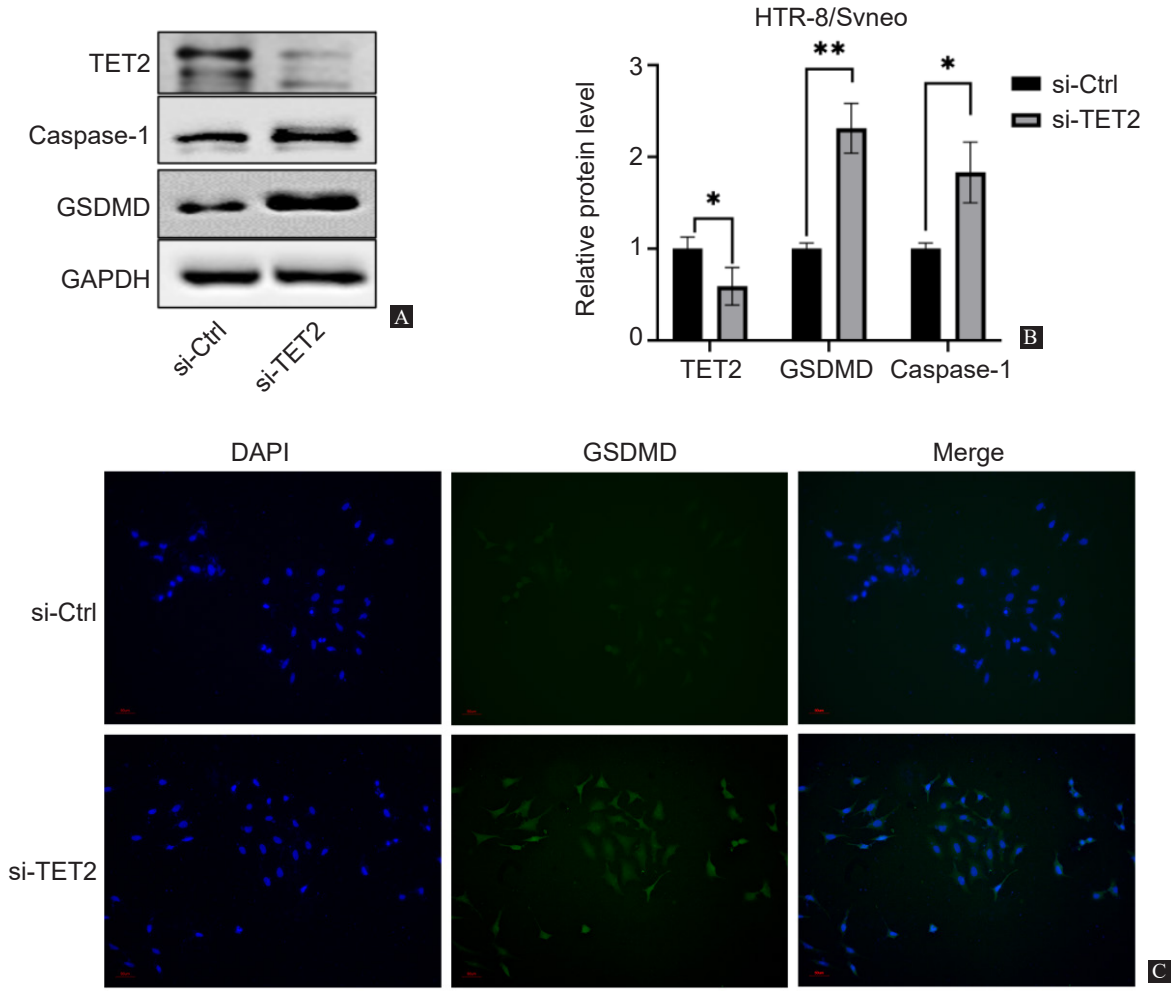

Fig. 4 Effects of TET2 knockdown on pyrogenic protein and immunofluorescence in HTR-8/Svneo cells.
A-B. Comparison of the relative expression levels of GSDMD and caspase-1 proteins in the two groups; C. Comparison of GSDMD fluorescence intensity in the two groups of cells.

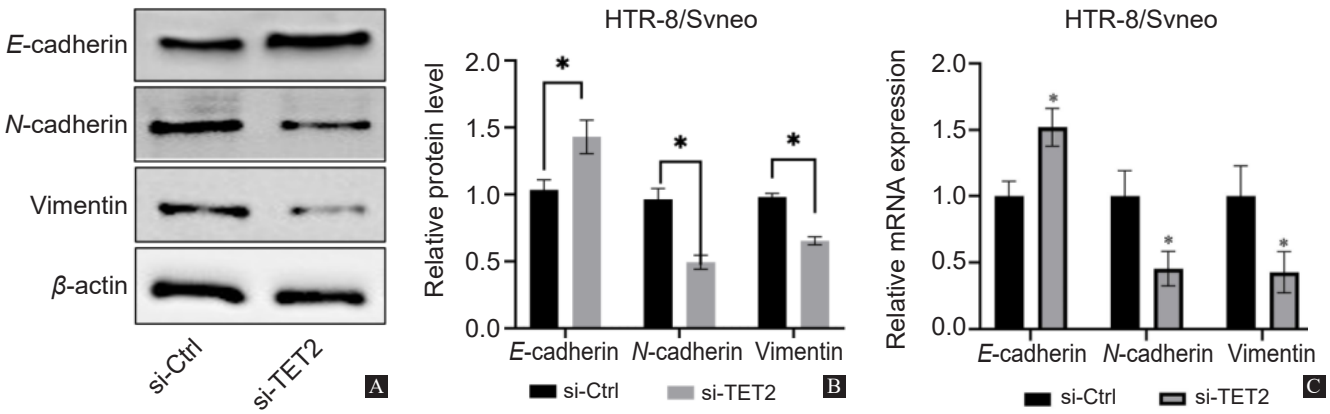

Fig. 5 TET2 knockdown inhibited epithelial-mesenchymal transformation in HTR-8/Svneo cells.
A-B. Comparison of the relative expression levels of *E*-cadherin, *N*-cadherin, and vimentin in two groups of cells; C. Comparison of the relative expression levels of *E*-cadherin, *N*-cadherin, and vimentin mRNA in the two groups.

4 Conclusion and discussion

Abnormal function of trophoblast cells is an important link leading to pregnancy-related diseases such as intrauterine growth restriction, gestational hypertension, spontaneous abortion, premature delivery, and chorionic carcinoma. oxLDL is a lipid free radical, which is formed by a large amount of unsaturated fatty acids in natural LDL after reacting with oxygen free radicals and other oxidants. It causes chain reactions that lead to further lipid peroxidation[7]. Previous studies have shown that plasma oxLDL levels in pregnant women with preeclampsia are significantly higher than those in normal pregnant women[8]. Leatitia et al[9]. studied trophoblast cells cultured *in vitro* and found that oxLDL was involved in the pathogenesis of preeclampsia by inhibiting trophoblast infiltration. It is suggested that oxLDL may be an important factor in the development of pregnancy-related diseases by affecting the function of trophoblast cells and causing placental dysfunction. In this study, oxLDL downregulates TET2 expression in human trophoblast cells HTR-8/Svneo.

As a key enzyme in DNA demethylation, TET2 can change the state of DNA methylation, thus triggering passive DNA demethylation[10]. TET2 levels were decreased in endometrial adenocarcinoma and correlated with 5-hmC levels. Downregulation of TET2 may be a potential prognostic indicator of endometrial adenocarcinoma. It has been shown that TET protein expression is also reduced in the placenta of preeclampsia patients[5,10]. It is suggested that TET2-mediated DNA demethylation plays an important role in placental development. In this study, TET2 knockdown promoted the proliferation of HTR-8/Svneo and inhibited its migration and invasion. Moreover, TET2 downregulation resulted in the abnormal proliferation ability of trophoblast cells and increased apoptosis, leading to insufficient cell infiltration ability. These results indicate that the abnormal proliferation, migration, and invasion of trophoblast cells caused by TET2 downregulation are closely related to placental dysfunction.

Pyroptosis is an inflammatory programmed cell death process characterized by the release of inflammatory mediators and has been implicated in the pathogenesis of preeclampsia[11]. During pyroptosis, the precursor protein of cysteinyl aspartate specific proteinase-1 (caspase-1) is activated, which in turn, activates gasdermin D (GSDMD). It forms porous structures on the cell membrane and releases multiple inflammatory factors and cellular contents, triggering local inflammatory responses[12]. Our experimental results showed that TET2 downregulation could upregulate the pyroptosis marker GSDMD and the expression of caspase-1, suggesting that TET2 downregulation could induce pyroptosis in HTR-8/Svneo cells. Low expression levels of TET2 can upregulate the expression of *E*-cadherin and inhibit the expression of *N*-cadherin and vimentin, suggesting that downregulation of TET2 can inhibit the epithelial-mesenchymal transformation epithelial-mesenchymal transition (EMT) in trophoblast cells and, thus, inhibit the migration and invasion ability of HTR-8/Svneo cells. Invasion and migration of trophoblast cells play important physiological functions in the implantation of the fertilized egg, placenta formation, and establishment of the maternal-fetal connection. Both these cellular movement processes depend on moderate epithelial-mesenchymal transition (EMT)[13-14]. In addition, EMT is an important molecular mechanism for the differentiation of human cell proliferative cells (CTB) into abnormal trophoblasts (EVT)[15].

In conclusion, we propose that oxLDL induces pyroptosis in trophoblast cells and inhibits EMT by downregulating TET2 expression in trophoblast cells. These inhibit the migration and invasion of vascular endothelial cells. Future in-depth studies on the molecular mechanisms of

TET2-induced pyroptosis in trophoblast cells will provide experimental basis for further clarification of the function of trophoblast cells damaged by oxLDL. Importantly, these data can contribute to the development of effective interventions for the prevention and treatment of pregnancy diseases, including preeclampsia.

5 Conflicts of interest

These authors have no conflict of interest to declare.

6 Acknowledgments

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