Circular RNA hsa_circ_0026552 inhibits the proliferation, migration and invasion of trophoblast cells via the miR-331-3p/TGF-βR1 axis in pre-eclampsia

LI SHAN[1] and XIAOFEI HOU[2]

[1]Department of Obstetrics, Yantaishan Hospital; [2]Department of Prenatal Screening Laboratory, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, Shandong 264000, P.R. China

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Abstract. Globally, pre-eclampsia (PE) is a gestational disorder that causes increased morbidity of the fetus and mortality induced by pregnancy. Despite various studies, the understanding of the causes or mechanism of the development of PE remains elusive. Thus, the present study aimed to investigate the role of circular (circ)RNA hsa_circ_0026552 (hsa_circ_0026552) in the development of PE and its mechanism of regulation. hsa_circ_0026552 differential expression in PE tissue data and clinical samples were analyzed and it was observed that hsa_circ_0026552 is highly upregulated in PE samples. Furthermore, miR-331-3p was detected as an hsa_circ_0026552 target miRNA and TGF-βR1 gene as a target of miR-331-3p. These results were confirmed using various assays, including dual-luciferase reporter, reverse transcription-quantitative PCR and RNA pull-down assay. It was observed that miR-331-3p expression was negatively correlated to hsa_circ_0026552 relative expression, while TGF-βR1 expression was positively correlated to hsa_circ_0026552 expression evaluated by Pearson's correlation test. The functional experiments, including Cell Counting Kit-8, colony formation and Transwell assay, showed that silencing hsa_circ_0026552 could significantly strengthen the proliferation, migration and invasion of the trophoblastic HTR-8/SVneo cells, but the subsequent overexpression of hsa_circ_0026552 reversed this. Mechanistically, it was concluded that hsa_circ_0026552 acts as a miR-331-3p sponge to upregulate TGF-βR1 expression in trophoblasts and is involved significantly in PE development and progression in pregnant women. The circRNA hsa_circ_0026552 could be a novel therapeutic target and prognostic biomarker for PE.

Correspondence to: Dr Xiaofei Hou, Department of Prenatal Screening Laboratory, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, 20 Yuhuangding Road, Yantai, Shandong 264000, P.R. China
E-mail: xiao2fei68@163.com

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Introduction

PE is a specific gestational disorder characterized by the manifestation of hypertension and proteinuria after 20 weeks of conception (1,2) and is the primary cause of increased fetal morbidity and gestation-induced mortality, affecting ~3 to 8% of pregnant women globally (3-5). PE is defined according to the International Society for the Study of Hypertension in Pregnancy as systolic (≥140 mmHg) and diastolic (≥90 mmHg) blood pressure (6) with proteinuria of ≥30 mg/mol protein or without proteinuria or with austere clinical characteristics after 20 weeks of pregnancy in women that previously had a normal blood pressure (6). Its causes remain mysterious. Dysfunction of the placenta, improper maternal vascular destruction, dysregulation of oxygen and aberrant spiral artery re-modeling are involved in PE pathogenesis (7,8). Notably, the dysfunction of the placenta is now considered as the potentially main cause of PE (7). However, the fundamental course of placental dysfunction in the development and progression of PE remains to be elucidated (9). Furthermore, either shallow or extensive trophoblastic invasion of extravillous trophoblasts (EVTs) at the maternal-fetal line is known as a primary cause of placentation failure, leading to the occurrence of PE (10,11). It has been demonstrated that the migratory activity of restricted EVT cells in maternal decidua may hinder trophoblast function, thus inducing PE (12). As a result, it is important to clarify the pathophysiological mechanisms in the abnormal migration and invasion of the trophoblast and EVTs (13,14).

Circular (circ)RNAs, a subclass of an endogenous form of non-coding RNA, have conserved closed continuous loop structures (without free 3' and 5' ends) and are highly stable and conserved (15-17). CircRNAs constitute a significant part of the mammalian transcriptome and can regulate the expression of specific genes through transcriptional and post-transcriptional modulation and post-translational mechanisms, broadly altering the expression and function of proteins and thus being involved in several cellular mechanisms, for instance, inflammation, apoptosis, cell differentiation, proliferation, migration and invasion (17).

Emerging evidence has suggested that circRNAs are a significant regulator in several diseases (18), including cancers (19,20) and researchers are now giving attention to their regulatory effect in PE, as they have been reported as an important PE biomarkers (21-23). For instance, Jiang *et al* (23) showed that has_circ-0001855 and has_circ_0004904 were markedly raised in individuals with PE and were considered as a possible PE biomarker for subjects by merging with the pregnancy-associated plasma protein A2 (PAPP-A), recognized as homolog of PAPPA in the metzincin superfamily of pappalysins which has attracted considerable attention due to its role in PE pathogenesis. Furthermore, Shen *et al* (24) described that circTRNC18 inhibits the epithelial-mesenchymal transition (EMT) and migration of trophoblast cells by regulating miR-762-mediated expression of Grhl2 in PE. Hsa_circ_0026552 (accession number: NM_002178) is located at chr12: 53494495-53496128 and was found by Salzman *et al* (25) in 2013 in lung cancer cells, human fibroblasts, human normal epidermal keratinocytes and other types of cells with abnormal expressions, suggesting that it may serve a role in regulating cell phenotype. However, no study has reported on the role of hsa_circ_0026552 in PE. Hence, this present study was primarily to determine the role of hsa_circ_0026552 in the progression of PE.

micro (mi)RNAs are involved in the regulation of mRNA expression by targeting them (26). Therefore, they have attracted widespread attention and become a new type of biomarker for the treatment of PE through the mRNA-miRNA-mRNA axis. For example, long non-coding (lnc)RNA MALAT1 regulates trophoblast cell migration and invasion via the miR-206/IGF-1 axis (27), decreased lncRNA ZEB2-AS1 in pre-eclampsia controls the invasive and migratory abilities of the trophoblastic cell line HTR-8/SVneo via the miR-149/PGF axis (28). miR-331-3p serves an important role in inhibiting various disease progressions and is a critical biomarker. For instance, miR-331-3p suppresses cell proliferation in triple-negative breast cancer cells by downregulating neuropilin 2 (NRP2) (29) and suppresses cell invasion and migration in colorectal carcinoma (30). In addition, miR-331-3p inhibits the hepatocellular carcinoma Bel-7402 cell line by downregulation of E2F Transcription Factor 1 (31). However, there are no studies on miR-331-3p in preeclampsia, to the best of the authors' knowledge.

TGF-βs are members of a large superfamily of cytokines, including activins, inhibitors and bone morphogenic proteins (32). In addition, the TGF-β is a multifunctional cytokine that serves an essential role in the pathogenesis of PE (33). For example, miRNAs serve as common regulators of the TGF-β pathway in the preeclamptic placenta and cadmium-treated trophoblasts: Links between the environment, the epigenome and PE (34). Binding of TGF-β to TGF-β receptor which consists of the subunits TGF-βR1, a key receptor of signaling pathway, is a target gene of miR-331-3p (35). Previous studies have shown that TGF-βR1 serves a role in the gene signal axis in a number of diseases, including PE, and can serve as the downstream target gene of multiple mRNAs (36,37). For instance, Kim *et al* (33) indicated that the TGF-βR1 polymorphism may be a genetic risk factor for PE and IUGR-complicated PE. In addition, the serum levels of TGF-βR1 may contribute to the etiopathology of PE (38). Hence the aim of the present study to understand the deep molecular mechanism of PE.

The present study aimed to explore the role and the potential regulatory mechanism of hsa_circ_0026552 in PE progression. The results demonstrated that hsa_circ_0026552 suppressed cell proliferation and metastasis and invasion by regulating miR-331-3p/TGF-βR1 axis, a critical insight to identify novel molecular targets for the treatment of patients with PE.

Materials and methods

Analysis of pre-eclampsia dataset from gene expression omnibus (GEO) and clinical sample collection. Pre-eclampsia datasets with the accession number GSE96985 were retrieved from the GEO website and analyzed for differential expression using the heatmap method (39) by R project software (r-project.org/; version: 4.0). Placenta tissues obtained from pre-eclampsia whose age distribution was 28.4±3.3 (n=30) and normal pregnant women whose maternal age at delivery is 27.6±2.9 (n=30) at the Yantaishan Hospital between June 2015 and November 2019 were washed in PBS and kept at -80˚C for further experimentation. The diagnosis criteria for severe PE were as follows: Systolic pressure ≥160 mmHg and/or diastolic pressure ≥110 mmHg on at least two occasions with 4 h apart, accompanied by severe proteinuria (>5 g/24 h urine specimen or 3+ on ≥2 random samples collected 4 h apart). All the participants had no vaginal delivery, chronic nephritis, diabetes, heart and autoimmune diseases or cancer. The present study was authorized by the Ethics Committee of Yantaishan Hospital (approval no. KY-E-2020-1-10) and informed written consent was provided by all participants as per the guidelines of the Declaration of Helsinki.

Microarray analysis. Microarray datasets GSE96985 which includes four normal tissues and three PE tissues at platform GPL19978 were used to identify differentially expressed circRNAs through R software of limma package (R-project.org/; version 4.0) (40) with adjusting $P<0.05$ and $|\text{fold change}|>2$. Cluster analysis was performed by using the pHeatmap package in R software and Z-scores (r-project.org/; version: 4.0) was used for normalization. The top 20 most upregulated or downregulated genes were shown in the heatmap and the final value displayed as the units processed by Z-scores.

Cell lines and transfection. The human trophoblast cell line HTR-8/SVneo was acquired from the American Type Culture Collection and cultured in DMEM-F12 medium (HyClone; Cytiva), supplemented with FBS (10%) and penicillin/streptomycin (1%; Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator (at 37˚C and 5% CO_2) and then sub-cultured (ratio of 1:3) at 80-90% confluence to generate a new culture with a lower density of HTR-8/SVneo cells. For cell transfection, hsa-circ-002655 was inserted into an overexpression plasmid pcDNA 3.1(+) (OE-hsa-circ-002655, Sigma-Aldrich; Merck KGaA) and an empty vector [pcDNA3.1(+)]. Circ-002655 and TGF-βR1 knock-down (si-00265521, si-TGF-βR1) procedure was performed by using validated Stealth RNAi siRNA (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

miR-331-3p inhibitor and miR-331-3p mimics, as well as their NC negative controls (NC; mimics-NC, inhibitor-NC) were separately designed by Shanghai GeneChem Co., Ltd. Briefly, $4x10^5$ HTR8/SVneo cells were seeded in a serum medium on the day of operation and 20 nM siRNA and miRNA transfection solution was mixed with Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and serum-free media according to the manufacturer's protocol to produce a final volume of 100 μl transfection reagent mixture at room temperature for 15 min. After 48 h of transfection, the medium was replaced and cells were harvested and at 72 h post-transfection, the transfection efficiency was evaluated before further experiments (39). The sequences were: hsa_circ_0026552, si-#1: 5'-GTCGCTGTTGCAGAGGAGAAT-3'; si-#2: 5'-GGTCGCTGTTGCAGAGGAGAA-3'; si-NC, 5'-CAACAAGATGAAGAGCACCAA-3'; mir-331-3p mimics, 5'-GCCCCUGGGCCUAUCCUAGAA-3'; mimics-NC, 5'-CAGUACUUUUGUGUAGUACAA-3'; miR-331-3p inhibitor, 5'-GUCAUGAAAACACAUCAUGUU-3'; inhibitor-NC, 5'-CGAACGUGUCACGUTT-3'; si-TGF-βR1, 5'-AAAAUUGUCUUUUGUACAGAG-3'; si-NC, 5'-CAACAAGATGAAGAGCACCAA-3'.

Isolation of total RNA and reverse transcription quantitative (RT-q) PCR. Total RNA from HTR-8/SVneo cells and the placental tissues was isolated with TRIzol (Thermo Fisher Scientific, Inc.). cDNA synthesis was performed with the Reverse Transcription kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocols. and detection of mRNA with the SYBR-Green PCR kit (Takara Biotechnology Co., Ltd.) in accordance with the manufacturer's protocols. qPCR was used with an ABI7900 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR amplification involved denaturation at 94˚C for 1 min, 40 cycles of 95˚C for 30 sec and 60˚C for 1 min. GAPDH was used as the internal control for has-circ-0026552 and TGF-βR1, U6 was used as the internal control miR-331-3p (41). The data were processed using a $2^{-\Delta\Delta Cq}$ method (42). The primer sequences were as follows: circ_0026552 forward, 5'-CGCTGGTTGGAAAGAGTGTT-3' and reverse, 5'-GTCTCTGCGGTTCACATAAT-3'; mR-331-3p forward, 5'-TAGCCCCTGGGCCTATCCTAGAGAACT-3' and reverse, 5'-TCAACTGGTGTCGTGGAGTCGGC-3'; TGF-βR1 forward, 5'-TCCAACTACTGGTTTACCATTGC-3' and reverse, 5'-ACAGCAACTTCTTCTCCCCG-3'; GAPDH forward, 5'-TGCACCACCAACTGCTTAGC-3' and reverse, 5'-GGCATGCACTGTGGTCATGAG-3' and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Target gene prediction. StarBase v3.0 (http://starbase.sysu.edu.cn/) was used to predict the possible target miRNA of has-circ-0026552 in pre-eclampsia and also the target mRNA.

Cell proliferation assay. The proliferation of HTR-8/SVneo cells was evaluated using CCK-8 assay in accordance with the manufacturer's guidance. Briefly, after 96 h of transfection, CCK-8 reagent (100 μl; Beyotime Institute of Biotechnology) was added into each well. Incubation was then performed for 2 h at room temperature and the light absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc.). Each group was replicated five times and the assay was performed three times independently.

Colony formation assay. Cells were seeded ($5x10^3$/well) in a six-well plate and transfected with pcDNA3.1-has-circ-0026552 for 96 h. After two weeks, the cells were washed with PBS, fixed with ethanol for 30 min at room temperature and finally stained with 1% crystal violet for 20 min at room temperature (Sigma-Aldrich; Merck KGaA) for counting colony numbers (diameter ≥100 μm). The number of colonies formed was analyzed using a light microscope (magnification, x50; Olympus Corporation). The assay was performed three times independently.

Cell migration and invasion assay. To determine the migration and invasion of the trophoblastic cell lines, the cells were treated or transfected with different oligonucleotides and then suspended, without FBS, in RPMI-1640 medium. Afterward, the cells were seeded in the upper compartment of the Transwell inserts (8 μm-pore size; Sigma-Aldrich; Merck KGaA) coated with or without Matrigel for invasion and migration assay at room temperature for 30 min, respectively, while the lower chamber was occupied with a complete medium. After incubation for 96 h at 37˚C, non-migratory/non-invasive cells attached to the upper portion of the inserts were removed using cotton swabs, while the migrated or invaded cells were fixed with 4% paraformaldehyde (Thermo Fisher Scientific, Inc.) at room temperature and then stained with crystal violet (0.5%) for 10 min at room temperature. Finally, under a light microscope (Leica DMIL-PH1; Leica Microsystems GmbH), the invaded/migrated cells were counted in five random fields of each filter membrane.

Dual-luciferase reporter assay. The has_circ_0026552 sequence and TGF-βR1 3'UTR sequence including miR-331-3p-binding sites was cloned into a pGL3 vector (Promega Corporation) creating a pGL3-has_circ_0026552 wild type (WT) plasmid (has-circ-0026552-WT) and pGL3-TGF-βR1 WT plasmid (TGF-βR1-WT), respectively. miR-331-2p binding sites were mutated in both has_circ_0026552 and TGF-βR1 3' UTR sequence and then sub-cloned into the pGL3 vector to generate their mutant type (MUT) vector. To conduct the luciferase reporter assay, the HTR-8/SVneo cells were co-transfected with miR-331-3p mimics or miR-NC and the pGL3-has_circ_0026552 and pGL3-TGF-βR1 reporter construct using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C. After transfection (96 h), the relative luciferase activity of the cells was evaluated with the Dual-Luciferase Reporter Assay System (Promega Corporation) and subsequently normalized to the *Renilla* luciferase reporter activity.

Western blot analysis. Radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology) was used to extract proteins and the concentrations were determined with Bicinchoninic Acid (BCA) assay (Bio-Rad Laboratories, Inc.). Equivalent amounts of proteins (40 μg) were separated by 10% SDS-PAGE electrophoresis and later moved onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked at room temperature

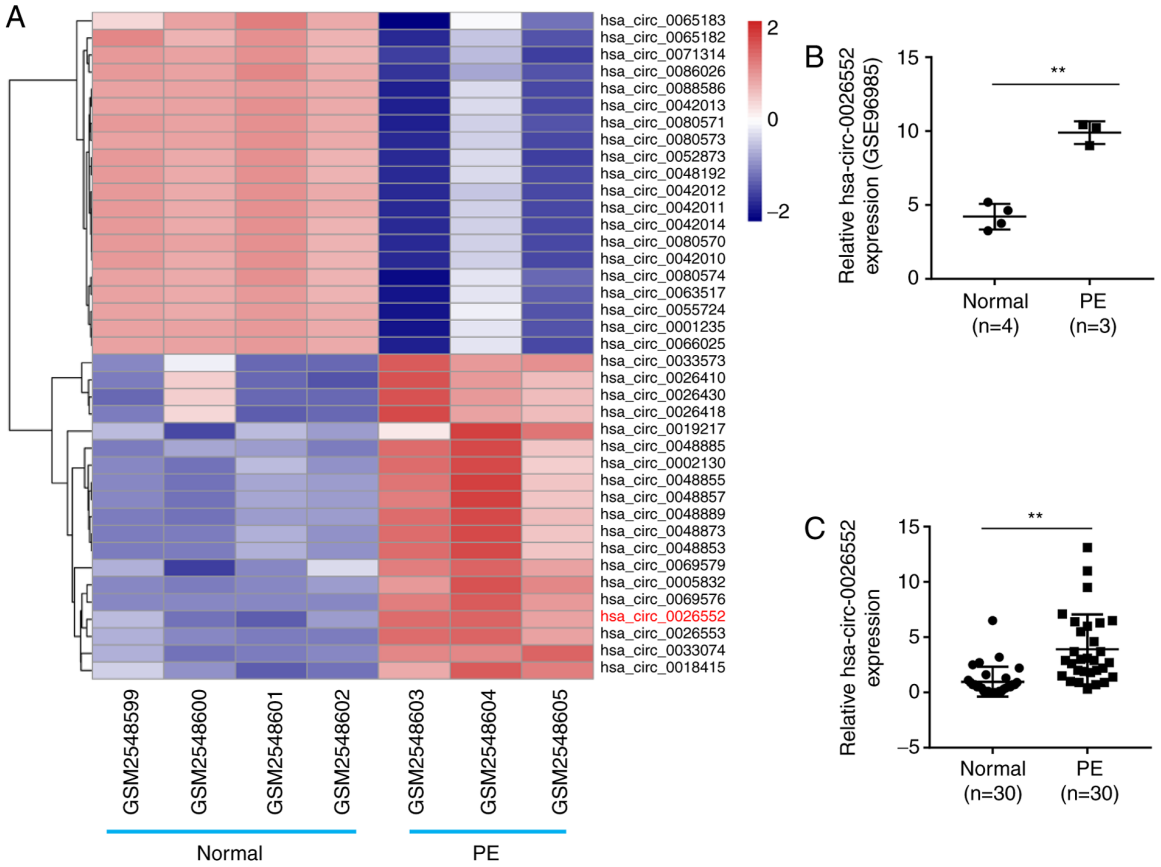

Figure 1. circRNA hsa_circ_0026552 expression in PE placental tissue samples. (A) Heatmap analysis of hsa_circ_0026552 expression in PE tissue samples downloaded from the Gene Expression Omnibus database and normal healthy control tissue samples. (B) circRNA hsa_circ_0026552 was among the top 20 most differentially expressed circRNA in the three PE tissue samples as compared with the normal ones was detected by RT-qPCR. (C) RT-qPCR analysis of hsa_circ_0026552 expression in 30 PE clinical samples. The hsa_circ_0026552 was significantly upregulated in PE samples compared with the normal ones. **P<0.01. circRNA, circular RNA; PE, pre-eclampsia; RT-qPCR, reverse transcription quantitative PCR.

with 5% skimmed milk for 1 h. Incubation was performed overnight using appropriated primary antibodies (rabbit polyclonal antibodies for TGF-βR1 cat. no. 41896S; 1:1,000; Cell Signaling Technology, Inc.) and β-actin (cat. no. ab5694; 1:2,000; Abcam) at 4˚C. Afterward, the PVDF membranes in horseradish peroxidase-conjugated secondary antibodies (cat. no. ab6721, 1:2,000; Abcam) was incubated for 2 h at room temperature. Protein blots were visualized with the ECL-Plus Western Blot Analysis Detection System (Thermo Fisher Scientific, Inc.) and the band densities were quantified using ImageJ software (V1.8.0.112; National Institutes of Health).

Biotinylated RNA pull-down assay. Biotinylation of miR-NC and miR-331-3p to make Bio-miR-NC and Bio-miR-331-3p was performed by Shanghai GenePharma Co., Ltd.. Subsequently, HTR8/SVneo cells were transfected with biotinylated oligonucleotides. Next, the treated HTR8/SVneo cells were lysed by lysis buffer (Thermo Fisher Scientific, Inc.), incubated with streptavidin-coated magnetic beads (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and eluted after 96 h. Finally, the eluted biotin-coupled RNA complex was subsequently washed with wash buffer (Thermo Fisher Scientific, Inc.), collected by centrifugation at 11,100 x g for 10 min at room temperature and assessed by RT-qPCR.

Statistical analysis. SPSS version 20.0 (IBM Corp.) or GraphPad Prism 6 (GraphPad Software, Inc.) was employed for the statistical analysis and all experimental data were presented as mean ± standard deviation. Unpaired t-test was used to identify the significant difference between two groups and the significance among multiple groups was calculated by one way ANOVA with Tukey's correction. Pearson's correlation test was used to evaluate the correlations between different RNA expressions level in PE clinical samples. All the experiments were repeated three times and all data in each histogram were normalized to the mean value of the control group and were presented as fold change, unless otherwise indicated. P<0.05 was considered to indicate a statistically significant difference.

Results

Hsa_circ_0026552 is significantly upregulated in the placental tissues of pre-eclampsia pregnant women. At the beginning of the present study, the pre-eclampsia dataset with the accession number GSE96985 was downloaded from the Gene Expression Omnibus (GEO) website (ncbi.nlm.nih.gov/geo/) and analyzed for differential expression using the heatmap method. As shown in Fig. 1A, hsa_circ_0026552 (in red) was among the top 20 most differentially expressed circRNA in the three PE tissue samples compared with that

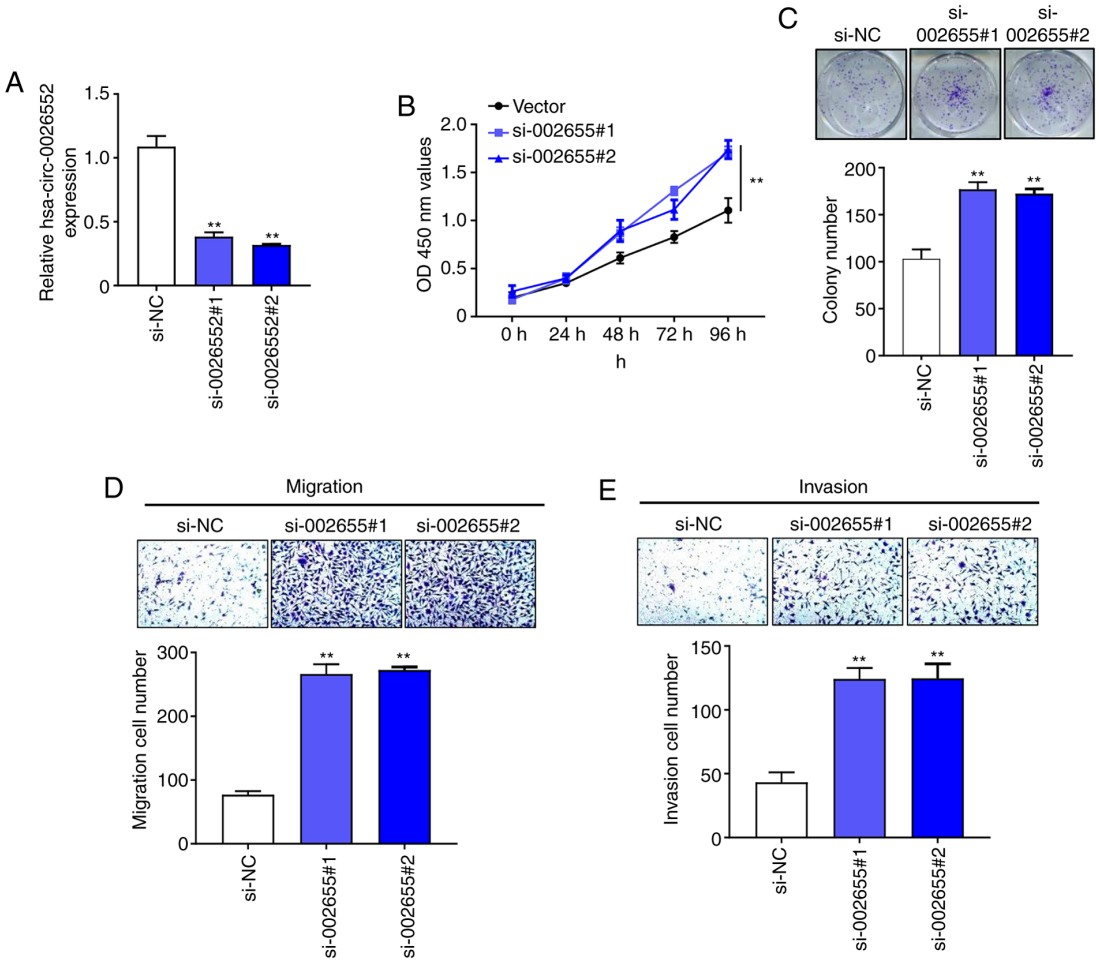

Figure 2. circRNA hsa_circ_0026552 knockdown inhibits proliferation, migration and invasion of trophoblasts. (A) Reverse transcription quantitative PCR showed the hsa_circ_0026552 expression in HTR-8/SVneo cells following knockdown with si-0026552. (B) The proliferative ability of HTR-8/SVneo cell following hsa_circ_0026552 knockdown was evaluated using CCK-8 assay. (C) Colony formation assay was used to analysis proliferative ability of HTR-8/SVneo cells following hsa_circ_0026552 knockdown. Transwell assay for detecting the (D) migration and (E) invasion ability of trophoblasts following hsa_circ_0026552 knockdown. Magnification, x200. All experimental data are shown as mean ± standard deviation of three different experiments **P<0.01. circRNA, circular RNA; PE, pre-eclampsia; si, short interfering.

in the normal tissue samples. Further expression analysis of the hsa_circ_0026552 in the GSE96985 dataset showed that hsa_circ_0026552 is significantly upregulated in PE tissues in comparison to that in the normal tissue samples (P<0.01; Fig. 1B). The expression level of hsa_circ_0026552 was subsequently detected in 30 PE tissues collected from PE pregnant women using the RT-qPCR. It was found that hsa_circ_0026552 was significantly overexpressed in PE tissue samples compared with that in the normal tissue samples collected from pregnant women (P<0.01; Fig. 1C). These data suggested that the circRNA hsa_circ_0026552 might serve a role in the development of PE in pregnant women.

Knockdown of hsa_circ_0026552 suppresses the proliferation, migration and invasion of trophoblasts. To understand the biological function of hsa_circ_0026552 in the development of PE, hsa_circ_0026552 was knocked down in HTR-8/SVneo cells. RT-qPCR and western blot were used to verify whether GAPDH can serve as an internal control (Fig. S1A and B). The results showed that there was no significant difference in the expression of GAPDH under different conditions, indicating that it could be used as an experimental internal control. RT-qPCR analysis showed that hsa_circ_0026552 knockdown (with si-0026552) markedly reduced hsa_circ_0026552 expression in the cells (P<0.01; Fig. 2A). Furthermore, CCK-8 and colony formation assays revealed that silencing hsa_circ_0026552 significantly increased the proliferation of the HTR-8/SVneo cells in a time-dependent manner and the number of colonies formed in the cells (P<0.001; Fig. 2B and C). Transwell assay was performed to understand the function of the hsa_circ_0026552 in the migration and invasion of HTR-8/SVneo cells. The results indicated that hsa_circ_0026552 knockdown significantly increased the migration and invasion ability of the HTR-8/SVneo cells (P<0.001; Fig. 2D and E).

Overexpression of hsa_circ_0026552 inhibits the proliferation, migration and invasion of trophoblasts. The biological effect of the overexpression of hsa_circ_0026552 on cell proliferation, migration and invasion was also determined. To achieve this, pcDNA3.1-hsa_circ_0026552 was transfected to the HTR-8/SVneo cells. As shown in Fig. 3A, the relative expression of hsa_circ_0026552 was significantly upregulated in the HTR-8/SVneo cells transfected with hsa_circ_0026552

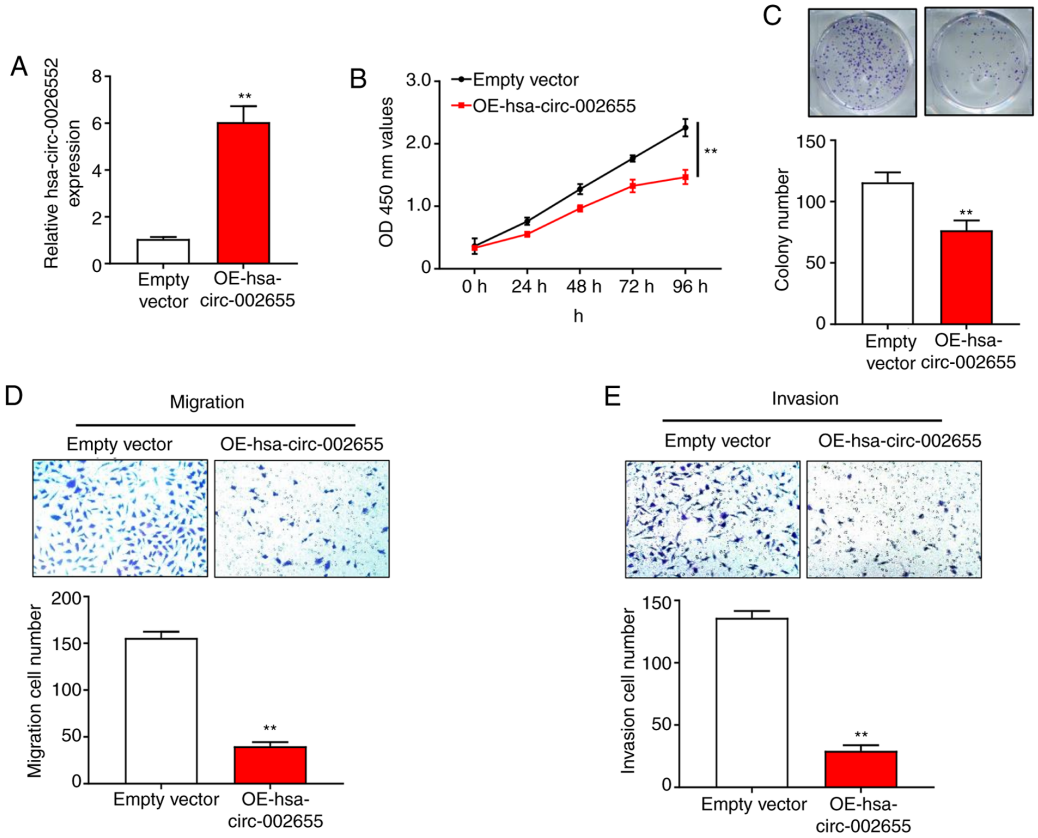

Figure 3. Overexpression of circRNA hsa_circ_0026552 inhibits the proliferation, migration and invasion of trophoblasts. (A) Reverse transcription quantitative PCR detection of hsa_circ_0026552 expression following its overexpression in HTR-8/SVneo cells. (B) CCK-8 and (C) colony formation assays were used to detect HTR-8/Svneo cell proliferation following transfection with hsa_circ_0026552 overexpression plasmid. Detection of (D) migration and (E) invasion ability of the HTR-8/Svneo cells, following transfection with hsa_circ_0026552 overexpression plasmid, using Transwell assay. Magnification, x200. All experimental data are shown as mean ± standard deviation of three different experiments **P<0.01. circRNA, circular RNA; OE, overexpression.

overexpression plasmid (OE-hsa_circ_0026552) compared with those transfected with an empty vector (P<0.01). CCK-8 and colony formation assays showed that the overexpression of hsa_circ_0026552 significantly reduced the proliferative ability of the HTR-8/SVneo cells and also reduced the number of colonies formed in the cells (P<0.01; Fig. 3B and C). Additionally, it was found that overexpressing hsa_circ_0026552 in the HTR-8/SVneo cells significantly reduced the migration and invasion ability of the cells (P<0.01; Fig. 3D and E).

miR-331-3p is a target of hsa_circ_0026552 in trophoblasts. To better understand the hsa_circ_0026552 mechanism of regulation in PE, it was predicted that hsa_circ_0026552 targeted miRNA using the circinteractome and found that hsa_circ_0026552 harbors a binding site for the miR-331-3p seed region (Fig. 4A) and a number of other targeted miRNAs given in Table I, including miR-1184, miR-1225-3p, miR-1233, miR-1277, miR-1307, miR-145 and miR-1825. A dual-luciferase reporter assay and a biotinylated RNA-pull down assay was performed to confirm this result. As shown in Fig. 4B, the overexpression of miR-331-3p significantly inhibited the luciferase activity of cells co-transfected with the WT pGL3-hsa_circ_0026552 plasmid compared with miR-NC. While the mutation of the miR-331-3p binding site on pGL3-hsa_circ_0026552 (i.e., hsa_circ_0026552 MUT) blocked the inhibiting effect of miR-331-3p on the cells (P<0.01; Fig. 4B). The result of biotinylated RNA-pull down assay showed that Bio-miR-331-3p significantly pulled down more hsa_circ_0026552 in the HTR-8/SVneo cells than the Bio-NC (P<0.001; Fig. 4C). Notably, the overexpression of hsa_circ_0026552 significantly inhibited miR-331-3p relative expression (P<0.001; Fig. 4D). In addition, miR-331-3p was found to be significantly downregulated in the PE dataset (GEO number GSE96983) and clinical tissue samples when compared with the normal control samples and its expression was negatively correlated to the hsa_circ_0026552 relative expression level (*P<0.05; Fig. 4E; **P<0.01; Fig. 4F; and P<0.0001; Fig. 4G). The expression of miR-331-3p in HTR-8/SVneo cells transfected with miR-331-3p mimics and miR-331-3p inhibitor (Fig. S2A and B) was then detected using RT-qPCR. The results revealed that in cells transfected with miR-331-3p mimics, the level of miR-331-3p significantly increased but greatly decreased in cells transfected with miR-331-3p inhibitor. It indicated that the transfection was a success.

Hsa_circ_0026552 regulates TGF-βR1 expression in trophoblasts via sponging miR-331-3p. The miR-331-3p target mRNA was also predicted using StarBase and the result showed that miR-331-3p directly targets the 3'UTR region of TGF-βR1 (Fig. 5A). Luciferase reporter assay showed that miR-331-3p could significantly inhibit the luciferase activity of HTR-8/Svneo cells co-transfected with the pGL3-TGF-βR1

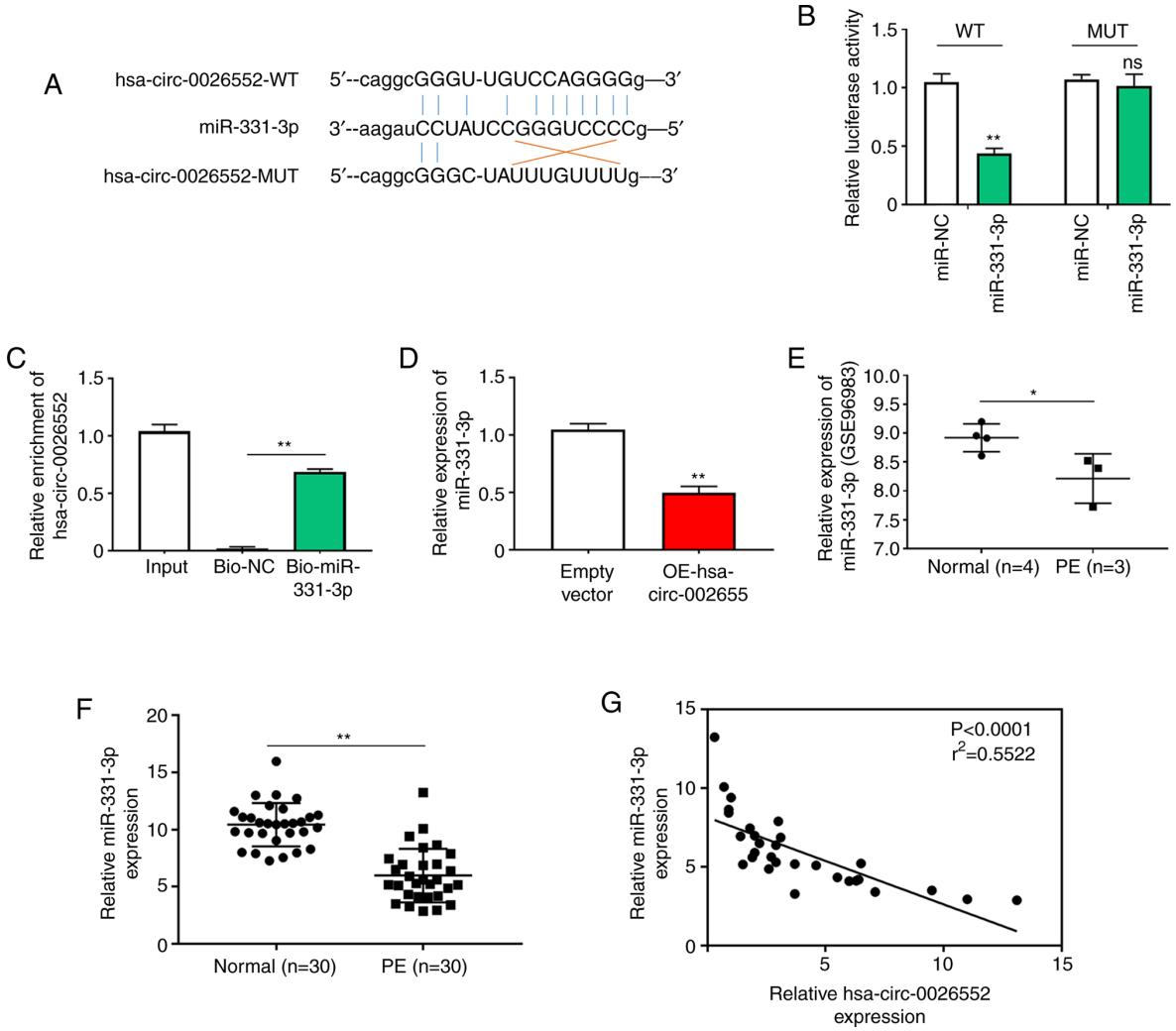

Figure 4. miR-331-3p was a target of hsa_circ_0026552 in trophoblasts. (A) Bioinformatics prediction of hsa_circ_0026552 target miRNA using StarBase database. (B) Dual-luciferase reporter assay for the detection hsa_circ_0026552-miR-331-3p binding ability. (C) Biotinylated RNA pull-down assay was used to conform the interaction between hsa_circ_0026552 and miR-331-3p in the HTR-8/SVneo cells. (D) RT-qPCR detection of miR-331-3p expression after transfecting cells with overexpressed hsa_circ_0026552. (E) Differential expression analysis of miR-331-3p in PE dataset (Gene Expression Omnibus number GSE96983). (F) RT-qPCR detection of miR-331-3p expression in PE clinical tissue samples. (G) Correlation analysis between hsa_circ_0026552 and miR-331-3p expression in PE tissues. The experimental data are shown as the mean ± standard deviation of at least three independent experiments. *P<0.05; **P<0.01; ns, non-significant. miR, microRNA; Bio, biotinylated; RT-qPCR, reverse transcription quantitative PCR; PE, pre-eclampsia; WT, wild type; MUT, mutant; OE, overexpression.

WT plasmid, but it had no significant inhibiting effect on that of the cells co-transfected with the pGL3-TGF-βR1 MUT plasmid (P<0.01; Fig. 5B). RT-qPCR and western blot analysis showed that inhibiting miR-331-3p expression in the HTR-8/Svneo cell significantly upregulated TGF-βR1 relative expression while overexpressing miR-331-3p significantly downregulated TGF-βR1 mRNA and protein expression (P<0.01; Fig. 5C). Similarly, overexpressing hsa_circ_0026552 in the HTR-8/Svneo cell significantly upregulated TGF-βR1 mRNA and protein expression which was later downregulated after co-transfecting the hsa_circ_0026552-overexpressed cell with miR-331-3p mimics (P<0.01; Fig. 5D). It was found that TGF-βR1 was significantly overexpressed in PE tissue samples relative to the normal healthy tissue samples and its expression was positively correlated to hsa_circ_0026552 relative expression, suggesting that hsa_circ_0026552 could upregulate TGF-βR1 expression in trophoblasts by sponging miR-331-3p (P<0.01; Fig. 5E and P<0.0001; Fig. 5F).

hsa_circ_0026552 regulates the proliferation, migration and invasion of trophoblasts by targeting the miR-331-3p/TGF-βR1 axis. To further confirm the hsa_circ_0026552 mechanism of regulation in PE, the HTR-8/Svneo cells were overexpressed with hsa_circ_0026552 and later co-transfected with miR-331-3p mimics or si-TGF-βR1. The results of CCK-8 and colony formation assays showed that the overexpression of hsa_circ_0026552 significantly reduced the proliferation and number of colonies formed in the HTR-8/Svneo cell lines. Co-transfecting the cell with either miR-331-3p mimics or si-TGF-βR1, however, restored the proliferative ability of the HTR-8/Svneo cell (P<0.01; Fig. 6A and B). The migration and invasion ability of the HTR-8/Svneo cells were also significantly reduced after overexpressing hsa_circ_0026552, while co-transfecting the cell with miR-331-3p mimics or silencing TGF-βR1 in the hsa_circ_0026552-overexpressed cell restored the migration and invasion ability of the HTR-8/Svneo cells (P<0.01; Fig. 6C and D). These data indicated that

Table I. Hsa_circ_0026552 targeting miRNAs.

CircRNA	Target miRNA
hsa_circ_0026552	hsa-miR-1184
	hsa-miR-1225-3p
	hsa-miR-1233
	hsa-miR-1277
	hsa-miR-1307
	hsa-miR-145
	hsa-miR-1825
	hsa-miR-198
	hsa-miR-296-5p
	hsa-miR-331-3p
	hsa-miR-361-3p
	hsa-miR-431
	hsa-miR-432
	hsa-miR-486-3p
	hsa-miR-515-3p
	hsa-miR-515-3p
	hsa-miR-519e
	hsa-miR-516b
	hsa-miR-593
	hsa-miR-622
	hsa-miR-629
	hsa-miR-634
	hsa-miR-651
	hsa-miR-653
	hsa-miR-661
	hsa-miR-663b
	hsa-miR-934

circRNA, circular RNA; miR, microRNA.

hsa_circ_0026552 promoted the proliferation, migration and invasion of trophoblastic cell line HTR-8/SVneo by regulating the miR-331-3p/ TGF-βR1 axis. In addition, the TGF-βR1 mRNA expression by RT-qPCR in HTR-8/Svneo cells transfected with si-TGF-βR1 was detected (Fig. S2C). The results demonstrated that following TGF-βR1 knockdown, its expression level decreased significantly, which also indicated that its transfection was successful.

Discussion

Despite the pathological conditions and complications, such as proteinuria and maternal multi-organ dysfunction (including uteroplacental dysfunction, fetal growth restriction and neurological or hematological complications) (7,8), accompanying PE-induced hypertension, the causes and mechanism of the development of PE is remains to be elucidated, reducing the chance of treating or preventing the disease. Only a few effective pharmacological agents have been developed for the treatment of PE and pregnancy termination or early delivery of the fetus and placenta seems to be the most definitive treatment to date (43-46). Thus, recent research has been directed towards identifying regulatory molecules, such as the non-coding RNAs, which have been shown to be involved in the development of PE and their molecular mechanism of regulation (47,48), hoping that identified molecules can serve as biomarkers for non-invasive diagnosis and prognosis of the disease and a therapeutic target for its treatment.

circRNA has been linked to the development of PE and is currently being studied as a potential biomarker and therapeutic target for the treatment PE. For instance, ceRNA expression profiling in pre-eclampsia identifies hsa_circ_0036877 as a potential novel biomarker for early PE (49), circTRNC18 inhibits trophoblast cell migration and EMT by regulating miR-762/Grhl2 pathway in PE (24). hsa_circ_0026552 was reported in 2013 by Salzman (25), but its specific molecular mechanism in PE has not been studied. Therefore, the present study aimed at determining the role of hsa_circ_0026552 in the development of PE. At the beginning of the present study, it was found that the hsa_circ_0026552 was highly upregulated in PE samples. RT-qPCR analysis confirmed that the hsa_circ_0026552 was differentially expressed in PE clinical tissue samples, further implicating the role of hsa_circ_0026552 in the development of PE. In addition, results from the present study revealed that silencing hsa_circ_0026552 markedly improved the proliferative, migrative and invasive ability of the HTR-8/SVneo cell line, suggesting that hsa_circ_0026552 might be an effective therapeutic target for treating PE, for the first time to the best of the authors' knowledge.

circRNAs perform their regulatory role in one of the major ways that affect the regulatory function of miRNAs and they target gene expression through competitively binding to the target miRNA seed region in PE (50). For example, circTRNC18 inhibits trophoblast cell migration and EMT by regulating miR-762/Grhl2 pathway in PE (24). Downregulated circPAPPA suppresses the proliferation and invasion of trophoblast cells via the miR-384/STAT3 pathway (51). The present study predicted all the target miRNAs of hsa_circ_0026552 and selected suitable targets. It was confirmed that hsa_circ_0026552 competitively binds to miR-331-3p. Compared with other target genes of hsa_circ_0026552, the miR-331-3p has been previously reported as an oncogenic miRNA that inhibits the tumor-suppressive ability of the ST7L gene in pancreatic cancer (30). It has also been shown as a tumor suppressor that represses the invasive and migrative capacity of colorectal carcinoma cells through NRP2 post-transcriptional inhibition (30). Xuefang *et al* (52) investigated the miR-331-3p role in the progression of nasopharyngeal carcinoma and found that the miR-331-3p could significantly impede the proliferation of nasopharyngeal carcinoma cell and induce their apoptosis by regulating the F4B-PI3K-AKT signaling pathway. All the above studies have shown that miR-331-3p can have a significant effect on cell phenotype, including inhibiting cell proliferation, migration and invasion and promoting cell apoptosis. Therefore, in PE, it probably promotes the regeneration of trophoblast cells and inhibits the development of PE progression. Other genes may also serve a role in the development of PE, which will be analyzed in future research. The present study found that miR-331-3p is low-expressed in PE and when co-transfected with hsa_circ_0026552 in

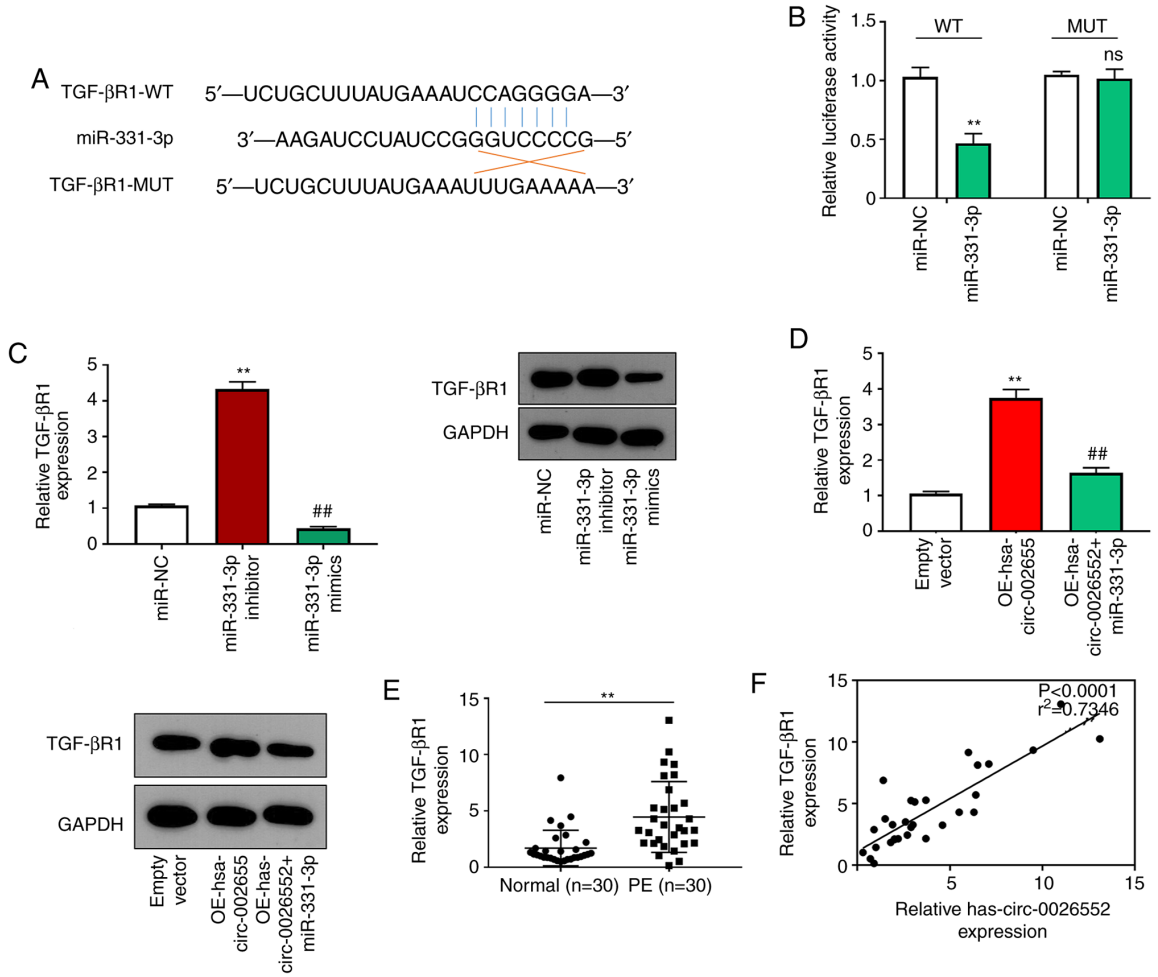

Figure 5. circRNA hsa_circ_0026552 regulates TGF-βR1 expression in trophoblasts via sponging miR-331-3p. (A) Bioinformatics prediction of miR-331-3p target mRNA. (B) Dual-luciferase reporter assay showed the luciferase activity of HTR-8/Svneo cells co-transfected with the TGF-βR1 WT plasmid and TGF-βR1 MUT plasmid. (C) RT-qPCR and western blot analysis of TGF-βR1 mRNA and protein expression following transfection. (D) RT-qPCR and western blot analysis of TGF-βR1 expression in the HTR-8/Svneo cell after overexpressing hsa_circ_0026552 and miR-331-3p. (E) RT-qPCR analysis of TGF-βR1 differential expression in PE clinical tissue samples. (F) Correlation analysis of TGF-βR1 and hsa_circ_0026552 expression in PE tissue samples. The experimental data are shown as mean ± standard deviation of three independent experiments. **P<0.01; $^{\#\#}$P<0.01; ns, non-significant. circRNA, circular RNA; miR, microRNA; WT, wild type; MUT, mutant; RT-qPCR, reverse transcription quantitative PCR; PE, pre-eclampsia; NC, negative control; OE, overexpression.

HTR-8/SVneo cells, overexpression of miR-331-3p effectively reverses the effect of hsa_circ_0026552 on cell proliferation. This molecular regulation mechanism in PE is consistent with other studies (43,52,53). In addition, the TGF-βR1 gene was indicated as miR-331-3p target in the present study. The inhibition of this gene together with the TGF-β/Smad signaling pathway has been shown to rescue the decline in the migrative and invasive property of trophoblast cells following DNMT3A knockdown (54). A previous study demonstrated that the aberrant expression of TGF-βR1 is strongly associated with a poor prognosis of patients diagnosed with pancreatic cancer and that TGF-βR1 might be a potential biomarker for the prognosis and treatment of pancreatic cancer (55). Notably, TGF-βR1 has been studied in PE; Kim *et al* (33) indicated that the TGF-βR1 polymorphism may be a genetic risk factor for PE and IUGR-complicated PE. The serum levels of TGF-βR1 may contribute to the etiopathology of PE (38). However, the molecular mechanism in PE of TGF-βR1 has not been deeply studied. Therefore, the present study found for the first time to the best of the authors' knowledge, that TGF-βR1 relative expression positively correlated with the expression of hsa_circ_0026552 and its upregulation might have inhibited the proliferative, migrative and invasive ability of trophoblasts.

The EMT is a key part of the embryonic development where epithelial cells lose their property and take on the migratory and invasive phenotype of mesenchymal cells (24). Studies have shown that a disruption in EMT-regulated migration and invasion contribute to PE (24). The present study noted that the overexpression of hsa_circ_0026552 significantly inhibited proliferation, migration and invasion of trophoblasts while miR-331-3p overexpression or TGF-βR1 knockdown reversed this condition, suggesting that the overexpression of hsa_circ_0026552 could induce a dysfunctional EMT-regulated cell migration and invasion in trophoblasts which might result in the occurrence of PE. The overexpressed hsa_circ_0026552 inhibited the regulatory ability of miR-331-3p on TGF-βR1 and consequently upregulated TGF-βR1 expression to promote PE. Taken together, the present study confirmed that the circRNA hsa_circ_0026552 was upregulated in PE and inhibited the proliferation, migration and invasion of trophoblast cells via the miR-331-3p/TGF-βR1 axis. The hsa_circ_0026552 might be involved in the development of PE and could be a possible therapeutic target for treating PE.

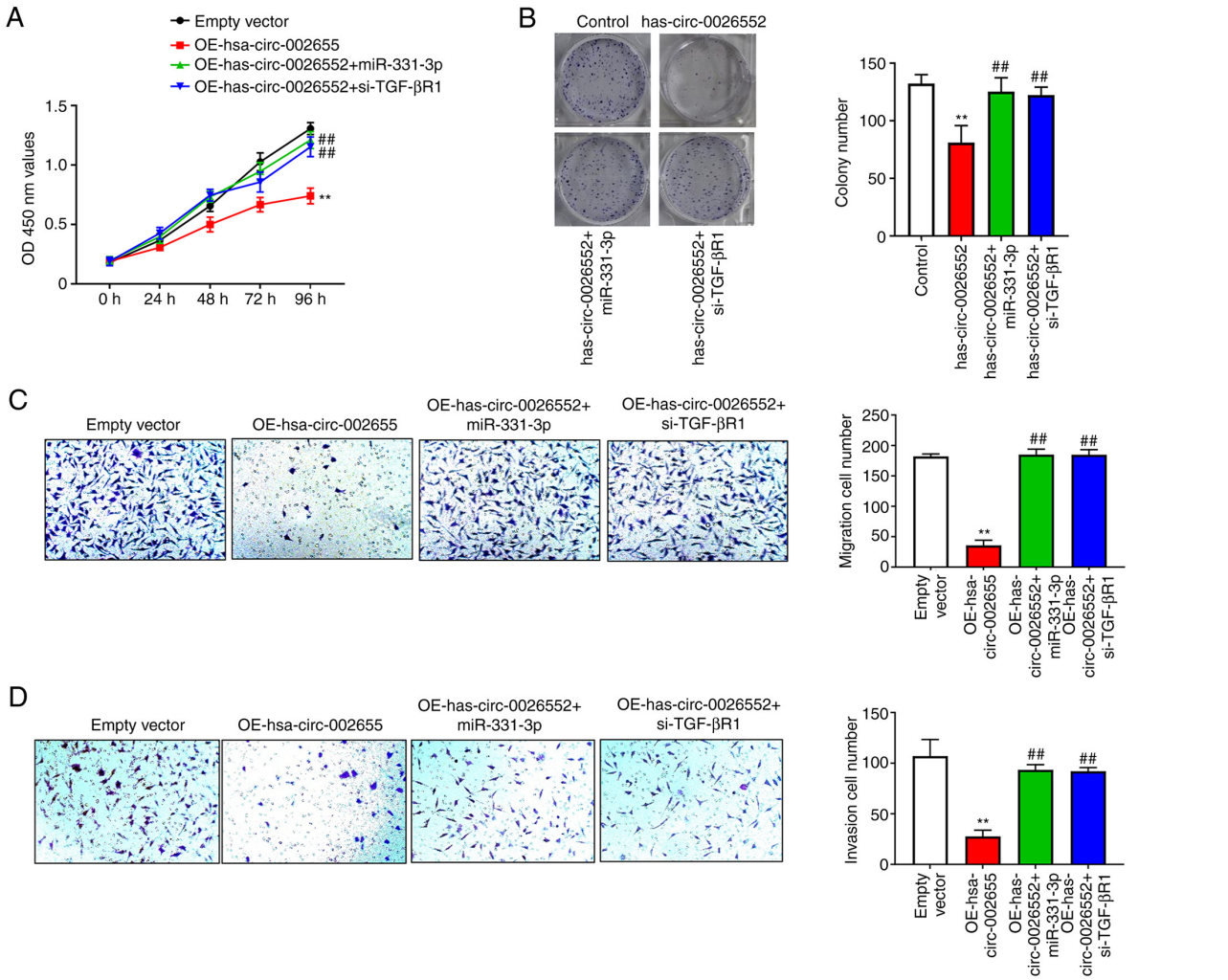

Figure 6. CircRNA hsa_circ_0026552 regulates the proliferation, migration and invasion of trophoblasts by targeting the miR-331-3p/ TGF-βR1 axis. (A) CCK-8 and (B) colony formation assay analysis of the proliferation of HTR-8/Svneo cell lines following transfection. Transwell assay analysis of the (C) migration and (D) invasion of the HTR-8/Svneo cells following transfection. Magnification, x200. **P<0.01; $^{\#\#}$P<0.01. circRNA, circular RNA; miR, microRNA; OE, overexpression.

The present study reported that hsa_circ_0026552 exhibited a significant upregulated expression in PE patients and promotes the migration, invasion and proliferation of HTR-8/SVneo cells by regulating the miR-331-3P/TGF-βR1 axis. The hsa_circ_0026552 could be a new therapeutic target for PE treatment. In addition, the proliferation, migration and invasion of trophoblast cells may be involved in PE development.

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Availability of data and materials

All supporting data of this work, which are not available in public because of the ethical restrictions, are available from the corresponding author upon request.

Authors' contributions

LS designed and directed the experiments; LS and XH performed the experiments and data analysis. Both authors read and approved the final manuscript. LS and XH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was authorized by the Ethics Committee of Yantaishan Hospital (approval no. KY-E-2020-1-10) and informed written consent was provided by all participants as per the guidelines of the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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