CircTRNC18 inhibits trophoblast cell migration and epithelial-mesenchymal transition by regulating miR-762/Grhl2 pathway in pre-eclampsia

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ABSTRACT

Dysfunctions of epithelial–mesenchymal transition (EMT)-regulated cell migration and invasion have been involved in the pathogenesis of pre-eclampsia (PE). However, the role of circRNAs in EMT of PE has not been widely investigated. In this study, we identified that circTRNC18 was upregulated in PE placentas compared with normal pregnancy placentas. Moreover, circTRNC18 negatively regulated trophoblast cell migration and EMT. Overexpression of circTRNC18 reduced while depletion of circTRNC18 enhanced trophoblast cell migration and EMT. Mechanistically, circTRNC18 sponged miR-762 contributed to inhibit miR-762 activity and elevated EMT-related transcripational factor Grhl2 protein level. miR-762 expression was lower in PE placentas and played a promoting role in trophoblast cell migration and EMT. In contrast, Grhl2 was highly expressed in PE placentas. Furthermore, we confirmed that upregulation of Grhl2 by circ–TRNC18-induced inhibition of miR-762 led to trophoblast cell migration and EMT. In conclusions, circTRNC18/miR-762/Grhl2 axis may be a potential therapeutic target in PE.

INTRODUCTION

Pre-eclampsia (PE) is a clinically significant complication of human pregnancy. It affects about 2–8% of pregnant women and is a major trigger of fetal morbidity and pregnancy-induced mortality [1–3]. The clinical features of PE are new-onset hypertension and proteinuria after 20 weeks of gestation in previously normotensive women [4]. Abnormal spiral artery re-modelling, placental dysfunction, inappropriate maternal vascular damage and oxygen dysregulation have been involved in PE’s pathogenesis [5,6]. Moreover, dysregulation migration and invasion abilities of extra-villous trophoblasts (EVTs) behaviours have been considered important in PE development [7]. Increasing evidence has confirmed that limited EVT cell migratory activity into the maternal decidua can lead to impaired trophoblast function and severe forms of PE [6]. However, the underlying mechanisms of pathogenesis in PE are still unclear.

Epithelial-to-mesenchymal transition (EMT) is a characteristic process by which polarized epithelial cells are transformed into having a mesenchymal phenotype which includes the ability to migrate and invade, which are essential for the development and maintenance of multicellular organisms [8]. EMTs are characterized with a lower level of key epithelial marker (E-cadherin), and a higher expression of mesenchymal marker (β-catenin and vimentin) which are needed for cell migration and invasion [9,10]. Recent studies revealed that EMT functions as a key process of tumour metastasis [11]. Cancer epithelial cells exhibit invasive and metastasis activities which influence cancer progression via EMT. In contrast, downregulation of EMT may reflect the failure of trophoblast cell to acquire the mesenchymal migratory/invasive ability [12]. Dysfunctional trophoblast cell migration and invasion of trophoblasts are critical lesions commonly observed in PE placentas [13]. Therefore, EMT has been identified as an essential physiological process in PE through regulation of its migration and invasion potential.

Circular RNAs (circRNAs) are a novel class of endogenous noncoding RNAs. Unlike linear RNAs, most circRNAs have a covalently closed-loop structure, where 3’ splice donors of the pre-mRNA are covalently linked to 5’ splice acceptors in reverse order [14]. Accumulating evidence has shown that circRNAs play its biological functions in various ways, including sponging miRNA [15], regulating parental genes expression [16], interacting with RNA-binding proteins (RBPs) [17] and rolling circle translation. Recently, increasing numbers of researches have confirmed that circRNAs play an important role in cancer cell invasion, migration and EMT. For example, upregulation of circACAP2 promoted colon cancer cell invasion [18]; Overexpression of circHIPK3 suppressed bladder cancer cell migration, invasion by moderating EMT-related protein expression [19]. Dysregulation of circRNA_100876 was correlated with growth and migration in oesophageal squamous cell carcinoma [20]. However, the role of circRNAs in PE pathogenesis is a still ongoing process.

According to a previous microarray analysis of circRNAs, hsa_circ_0006772 is downregulated in PE blood [21]. Hsa_circ_0006772 is an exon transcript from TNRC18 gene. In this study, we confirmed that circTRNC18 (hsa_circ_0006772)
was significantly upregulated in PE placentas and trophoblast cells. Overexpression of circTNRC18 inhibited trophoblast cells EMT by regulating EMT-related protein levels. Mechanistically, circTNRC18 binds to and sponge miR-762 in trophoblast cell, relieving miR-762 repression of transcriptional factor Grhl2 level, thus regulated EMT-related downstream gene expression and affects PE progression.

**Results**

**CircTNRC18 is upregulated in PE placentas**

Previous circRNA profile analysis revealed that hsa_circ_0006772 (circTNRC18) was upregulated in blood of PE [22]. With this, PCR analysis and agarose gel electrophoresis were used to confirm the presence of circTNRC18 in placenta tissues (Fig. 1a). Then, qRT-PCR analysis was performed to detect circTNRC18 expression in PE placentas (n = 30) and normal pregnancy placentas (n = 30). The result showed that the expression level of circTNRC18 was obviously increased in PE placentas compared with that in normal pregnancy placentas. In addition, to examine the expression level of circTNRC18 in different placenta cell lines, results of qRT-PCR revealed that circTNRC18 level was lower in human EVT cell line HTR8/SVneo than in human placenta cell lines Hs 795.Pl (Fig. 1c). These findings suggest circTNRC18 is overexpression in PE placentas. The up-regulation of circTNRC18 may be correlated with PE development.

**CircTNRC18 plays an essential role in trophoblast cell migration**

Because a lower migration ability of trophoblast cell is associated with PE pathogenesis, we then analysed whether circTNRC18 was correlated with the trophoblast cell migration. We transfected HTR8/SVneo cells with the circTNRC18 overexpression vector, pcDNA3.1–circTNRC18, or small interfering RNAs (si-circTNRC18) against circTNRC18, using empty vector or control si-RNA as negative controls, respectively. As shown in Fig. 2a, transfection with pcDNA3.1–circTNRC18 in HTR8/SVneo cells significantly upregulated circTNRC18 level compared with empty vector. On the contrary, si-circTNRC18 (si-circTNRC18-1# and 2#) transfection led to a dramatical downregulation of circTNRC18 level compared with si-control (Fig. 2d). Subsequently, transwell and wound-healing assays were performed to detect the effect of circTNRC18 on HTR8/SVneo cell migration. As shown in Fig. 2b, c, overexpression of circTNRC18 significantly inhibited the migratory ability of HTR-8/SVneo cells. In contrast, the depletion of circTNRC18 level elevated the ability of migration in HTR-8/SVneo cells compared with negative control (Fig. 2e, f). These findings suggest that circTNRC18 may be responsible for trophoblast cell migration.

**CircTNRC18 inhibits trophoblast cell EMT by regulating EMT-related gene**

Because EMT is a key process in cell migration, we then sought to investigate the relationship between circTNRC18 and EMT. Thus, we detected EMT-related proteins, E-cadherin (epithelial marker), vimentin (mesenchymal marker) and EMT-related transcriptional factor grainy head-like 2 (Grhl2) expression. Grhl2, a member of grainy head-like transcription family, has been reported as an EMT-suppressor in several tumour cells [23]. Results of western blot analysis verified that overexpression of circTNRC18 in HTR-8/SVneo cells dramatically increased expression of the E-cadherin and Grhl2 but reduced the expression of Vim level but did not affect Elf5 protein expression (Fig. 3a). Conversely, knockdown of circTNRC18

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**Figure 1. circTNRC18 is upregulated in PE placentas. (a) qRT-PCR analysis was used to examine circTNRC18 in PE placentas using different primers (convergent or divergent primers) circTNRC18 can be amplified in cDNA but not in genomic DNA (gDNA) by using divergent primers. GAPDH was used as the linear control. (b) qRT-PCR analysis examined circTNRC18 expression in PE placentas (n = 30) and normal pregnancy placentas (n = 30). Normalized to GAPDH. *P < 0.05, vs. normal pregnancy placentas. (c) qRT-PCR analysis examined the level of circTNRC18 in human choriocarcinoma cell lines (JAR and 3A-subband) and EVTs cell line (HTR8/ SVneo). *P < 0.05 vs. JAR cells.**
expression in HTR-8/SVneo cells markedly reduced E-cadherin and Grhl2 level and increased Vim level (Fig. 3b). Similarly, immunofluorescence staining further confirmed the above results. As shown in Fig. 3c, overexpression of circTNRC18 in HTR-8/SVneo cells obviously increased E-cadherin protein expression and decreased Vim protein...
expression. However, depletion of circTNRC18 reduced E-cadherin levels in the cell membrane and promoted Vim expression (Fig. 3d). Taken together, these findings demonstrate that circTNRC18 inhibits trophoblast cell EMT by regulating of Grhl2, E-cadherin and Vim protein expressions.

**CircTNRC18 sponges miR-762 in trophoblast cell**

Because circRNAs function as miRNAs sponges to inhibit their effects, we wanted to predict the potential binding miRNA of circTNRC18. Firstly, we identified potential miRNAs targeting circTNRC18 by using three target prediction programs, miRanda, RNA22 and RNAhydrid and found 10 putative circTNRC18-binding miRNAs (Fig. 4a). Secondly, pull-down assay using a biotin-labelled circTNRC18 probe in HTR-8/SVneo was performed to examine the interaction of miRNAs in circTNRC18-overexpressed cells. qRT-PCR was used to examine pull-down efficiency. The result showed that pull-down efficiency of circTNRC18 was obviously enhanced in circTNRC18-overexpressed cells compared with the vector and control probe groups (Fig. 4b). Thirdly, we detected the level of the potential miRNAs in the precipitates that were pulled down with circTNRC18 biotin-labelled probe by qRT-PCR analysis. As shown in Fig. 4c, the expression levels of miR-762 and miR-1250-5p were dramatically enriched in the precipitates. Fourthly, we sub-cloned the circTNRC18 region containing miR-762/miR-1250-5p binding sites (wild type/mutant) into psiCHECK-2 luciferase vector-sand transiently co-transfected the reporter construct with miR-762/miR-1250-5p mimics into HTR-8/SVneo cells. As shown in Fig. 4d, miR-762 significantly reduced the luciferase activity by 47% but not miR-1250-5p, which suggested that miR-762 but not miR-1250-5p bound to circTNRC18. Furthermore, qRT-PCR analysis result showed that the expression of miR-762 was lower in PE placentas compared with normal tissues (Fig. 4e). In addition, results of the correlation analysis revealed a significant negative correlation between miR-762 and circTNRC18 expression level in PE placentas (P = 0.0211)(Fig. 4f). These results indicate that circTNRC18 binds to miR-762.

**MiR-762 promotes trophoblast cell EMT by targeting directly Grhl2 gene 3’UTR**

Because our finding showed that miR-762 was downregulated in PE placentas, we wanted to investigate whether miR-762 played a role in trophoblast cell EMT. First, miR-762 was overexpressed or depleted in HTR-8/SVneo and EMT-related gene were detected by western blot analysis. As Fig. 5a showed, miR-762 positively regulated Vim protein expression and negatively regulated E-cadherin and Grhl2 protein levels. Next, we used computer-based sequence analysis (TargetScan) to search for the potential target gene. The results showed that Grhl2 3’-UTR exists a putative miR-762 binding site (Fig. 5b).

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**Figure 4. circTNRC18 functions as a sponge of miR-762 in trophoblast cell.** (a) Venn diagram hybrid of miRNA from three different target prediction programs. (b) pcDNA3.1-circTNRC18 or empty vector was transfected into HTR8/SVneo cells. qRT-PCR analysis was used to determine the pulldown efficiency of circTNRC18 or NC probe. *P < 0.05, **P < 0.01, ***P < 0.001 vs. corresponding control. (c) qRT-PCR analysis was used to examine the expressions of indicated miRNAs from pull-down precipitates. Normalized to internal control U6. **P < 0.01 vs. negative probe. (d) in HTR8/SVneo cells co-transfected cells with miR-762-5p mimics or miR-1250-5p mimics and circTNRC18-luciferase reporter (WT/mut). Luciferase reporter assays were used to detect luciferase activity. *P < 0.05 vs. corresponding control. (e) qRT-PCR analysis was performed to exam miR-762 level in PE placentas compared with normal pregnancy placentas, *P < 0.05 vs. normal pregnancy placentas. (f) Relationship between miR-762 expression and circTNRC18 level in PE was analysed by Spearman’s correlation analysis (R = −0.4060, P = 0.0211).
Subsequently, miR-762 mimic and Grhl2-3′-UTR-luciferase reporter were co-transfected in HTR-8/SVneo cells. The luciferase analysis showed that miR-762 mimic significantly decreased luciferase activity mediated by wild-type 3′-UTR; mutation of the miR-762-binding site could not affect the luciferase activity in the presence of the miR-762 mimic (Fig. 5c). Furthermore, overexpression of miR-762 by transfecting miR-762 mimic significantly downregulated, whereas knockdown of miR-762 by transfecting miR-762 inhibitor upregulated Grhl2 mRNA in HTR-8/SVneo cells (Fig. 5d). Additionally, we further examined the level of Grhl2 in PE placentas and normal pregnancy placentas. The result showed Grhl2 was overexpression in PE placentas and negatively correlated with miR-762 expression (Fig. 5e, f). These findings suggest that miR-762 directly suppresses Grhl2 expression and regulate trophoblast cell EMT.

CircTNRC18/miR-762/Grhl2 axis regulates trophoblast cell migration and EMT

To clarify the function of circTNRC18/miR-762/Grhl2 in trophoblast cell migration and EMT, some rescue experiments were performed. First, HTR-8/SVneo cells were transfected with pcDNA3.1-circTNRC18, miR-762 mimic, respectively, or co-transfected with pcDNA3.1-circTNRC18 and miR-762 mimic together. Western blot analysis detected EMT-related protein expression. The result revealed that overexpression of circTNRC18 downregulated Vim protein level and upregulated Grhl2 and E-cadherin protein levels. However, the effect of circTNRC18 on the expression of E-cadherin, Vim and Grhl2 could be partly reversed in the miR-762 mimic and circTNRC18 co-transfected HTR-8/SVneo cells (Fig. 6a). Furthermore, transwell assay showed overexpression of circTNRC18 in HTR-8/SVneo cells significantly promoted cell migration compared with empty vector and reversed the inhibitory effect of miR-762 overexpression on cell migration (Fig. 6b). Consistently, HTR-8/SVneo cells were transfected with si-circTNRC18, si-Grhl2, respectively, or co-transfected with si-circTNRC18 and si-Grhl2 simultaneously. As shown in Fig. 6c, knockdown of circTNRC18 reduced Grhl2 and E-cadherin expression and increased Vim level. However, knockdown of circTNRC18 induced the effects on E-cadherin, Vim and Grhl2, which could be further enhanced after co-transfection with si-circTNRC18 and si-Grhl2. As shown in Fig. 6d, compared with negative control (Fig. 6d, upper left), overexpression of circTNRC18 inhibited rather than promoted cell migration (Fig. 6d, lower left), and suppression of Grhl2 will promoted rather than inhibited cell migration, in other words, the inhibiting effect caused by overexpression circTNRC18 could be partly reversed when Grhl2 was repressed (Fig. 6d, lower right). These findings further confirm that circTNRC18/miR-762/Grhl2 axis is a regulatory pathway in HTR-8/SVneo cells migration and EMT.

Discussion

Effective prediction and intervention of PE are important for early pregnancy [24]. Although some biological markers have been reported functions potential biomarkers for PE and play an important regulatory role in PE processes, such as placental growth factor (PLGF), soluble fms-like tyrosine...
kinase-1 (sFlt-1) and placental protein 13 (PP13) [25]. The underlying mechanism needs further verification. Non-coding RNAs play critical regulatory roles in the disease process, especially circRNA, which has been found to be involved in a variety of disease processes in recent years [26]. As a closed circular RNA molecule, circRNA effectively avoids the action of RNA exonuclease, enabling it to maintain stable expression [27]. Although some circRNAs have been reported as biomarkers of PE in recent years [21]. However, the role of circRNA in PE and its molecular mechanism are still unclear. In this study, we confirmed the has_circ_0006772 (circTNRC18) was significantly upregulated in PE placentas. Upregulation of circTNRC18 inhibited trophoblast cells EMT by regulating EMT-related protein levels. Our results showed that circTNRC18 sponged miR-762 in trophoblast cell, relieved miR-762 repression of transcriptional factor Grhl2 level, thus regulated EMT-related downstream gene expression and affected PE progression.

EMT is the process by which immotile epithelial cells obtain the migration and invasion abilities and transform to attain mesenchymal cell characteristics. During pregnancy, the EVT, which gain the migration and invasion abilities through EMT, could invade the uterine decidua to remodel the spiral arteries. Dysfunction migration and invasion of EVTs are contributed to the failure of placentation [28]. Thus, EMT is critical for the success of pregnancy. Previous studies have found higher level of E-cadherin and lower level of vimentin in the PE placental tissue [29–31]. Furthermore, a number of studies revealed that dysregulation of noncoding RNA may influence trophoblast cell EMT at the molecular level. For example, Zou investigated that Lnc RNA SPRY4-IT1 was overexpressed in PE placentas. Upregulation of SPRY4-IT1 inhibited EMT of HTR-8/SVneo cells by modulating WNT/β-catenin pathway [32]. Yang found that microRNA-431 induced trophoblast migration and invasion by inhibiting ZEB1 expression in PE [33]. Additionally, ZEB2 was also considered as a molecular regulator of EMT in trophoblast cells [22]. In this study, we first demonstrated that circTNRC18 is upregulated in PE placentas. Moreover, circTNRC18 as an EMT-inhibitory regulator affected the trophoblast cell migration. Overexpression of circTNRC18 markedly decreased trophoblast cell migration and EMT. On the contrary, depletion of circTNRC18 revealed the opposite result. It is consistent with upregulation of circTNRC18 expression in PE placentas, suggesting that increased circTNRC18 inhibited trophoblast cell migration and EMT, contributing to PE aggressiveness.

Grhl2 as an important wound-healing transcription factor has been wildly investigated in tumour EMT and metastasis, including breast cancer [34], non-small cell lung cancer [35], gastric cancer [36] and ovarian cancer [36]. However, the expression of Grhl2 was also regulated by miRNA in post-transcriptional level. miR-217 and miR-194 have been reported directly targeted Grhl2 3’UTR. Our study demonstrated that miR-762, as a medium of Grhl2 and circTNRC18, regulated Grhl2 protein expression by targeting its 3’UTR. Interestingly, Grhl2 mRNA was up-regulated in PE placentas and correlated with miR-762 expression. Whether miR-762 regulates Grhl2 expression directly or indirectly needs to be investigated further.

Because of the sponging function of miRNA, circRNAs serve a key role in the regulation of gene expression [40]. Numerous circRNAs are considered to have the characteristic of cell-type
specificity and disease specificity, indicating that circRNA may be an important biomarker in multiple types of diseases [41–43]. In recent years, circRNAs were reported to be an important component of the cellular signalling network that regulates the EMT program. Growing evidence shows that hundreds of circRNAs play an inhibitor or promoter role in human EMT [20]. circRNAs are mostly reported to function as miRNA sponge and form circRNA-miRNA-mRNA axis to exert its biological effect on gene regulation. Yang showed that circAMOTL1 which sponged miR-193a-5p, leading to reduced miR-193a-5p activity and increased levels of Pcdha regulates EMT in metastatic progression of prostate cancer [44]. Long reported that circPTK2, a sponge of miR-429/miR-200b-3p, inhibits non-small cell lung cancer cell EMT and metastasis induced by TGF-β [45]. Additionally, hsa_circ_0001649 [46], circRNA_0023642 [47] and circ_0067934 [48] have been identified to regulate migration and invasion in different cancers by regulating EMT. Despite recent studies in EMT-related circRNAs, circRNA-mediated EMT during PE development is still little known. In this study, we show that circTNRC18 functions as miR-762 sponge, reducing miR-762 expression. In addition, we further verified the promoting role of miR-762 in EMT-associated events. Therefore, more functions of circTNRC18 in PE pathogenesis and prognosis need to be further investigated.

In summary, our findings reveal that circTNRC18/miR-762/Grhl2 axis serves a key role in trophoblast cell migration and EMT and correlates to PE progression. circTNRC18/miR-762/Grhl2 axis provides a new potential therapeutic target for PE.

Materials and methods

Clinical samples

Thirty pairs of PE and normal placentas were collected from the pregnant women who underwent caesarean at the Department of Obstetrics, Second Hospital of Hebei Medical University, from July 2016 to October 2018. PE diagnosis was according to ACOG 2013 diagnostic criteria. Tissues were collected from the mother surface of the placenta as well as near the root of the umbilical cord. The study protocol was approved by the Ethics Committee of Second Hospital of Hebei Medical University and verbal and written consent was obtained from each participant.

Cell culture and transfection

Human extravillous trophoblast cell line HTR-8/SVneo and human placenta cell lines 3A-suband were maintained in our laboratory. All cells were cultured in RPMI 1640 medium (Gibco, Beijing, China) supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Human normal placenta cell line Hs 795. Pl (ATCC CRL-7526®) was purchased from American Type Culture Collection. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (Gibco, Beijing, China) supplemented with 10% FBS and two antibiotics above. All cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. Cell transfection was following Lipofectamine 2000 (Invitrogen) manufacturer’s protocol. The miR-762 mimics, mimic-NC, miR-762 inhibitor, inhibitor-NC, si-Grhl2, si-circTNRC18 and si-NC were purchased from GenePharma Co., Ltd (Shanghai, China). The sequence of si-RNA as follows: si-circTNRC18-1#: GAG AUG GGA GAU AUC CGG CTT; si-circTNRC18-2#: AAG AGA UGG GAG AUA UCC CTT; si-Grhl2: GGU GGU GAA AGC UGA AGA UTT; circTNRC18 overexpression vector (#C201601-3) and luciferase reporter vector (#C201602-1) were constructed by Biocare Biotechnology Co., Ltd (Shijiazhuang, China) and confirmed by Sanger sequencing. In brief, cell seeded in 6-well plate with 70% confluent at the time of transfection. 4 µg DNA or 10 pmol RNA or 10 µl Lipofectamine 2000 diluted by 250 µl serum-free medium, respectively. After the 5-min incubation, combine the diluted DNA/RNA with diluted Lipofectamine 2000 (total volume = 500 µl). Mix gently and incubate for 20 min at room temperature. Add the 500 µl of complexes to each well containing cells and medium. After 24 ~ 48 h of transfection, the cells were harvested and lysed for Western blotting, and the total RNA was extracted for qRT-PCR.

Transwell migration assay

8-µm pore size transwell filters (Costar, Cambridge, Massachusetts) were used to detect HTR-8/SVneo cell migration ability. Briefly, 3 × 104 cells/well HTR-8/SVneo cells were seeded on the upper chambers with serum-free medium. The lower chambers were added the medium contained 10% FBS. After culturing at 37°C and 5% CO2 for 24 h, cells with higher migration ability on the upper side of the chamber were migrated into the lower chamber. The migration cells number was counted in three randomly selected areas under a light microscope.

Wound-healing migration assay

1 × 10⁵ HTR-8/SVneo cells were seeded in 6-well plates until with 95% confluent then scratched by 200-mL tip. After 24 h of growth in serum-free medium, images of the cells were pictured by microscope. The recovered area was calculated according to the formula: (mean wounded breadth × mean remained breadth)/mean wounded breadth × 100 (%). Each experiment was performed three times independently.

RNA pull-down analysis

HTR-8/SVneo cells were incubated with biotin (Bio)-labelled oligonucleotide probes against circTNRC18 (Bio-5’- GAG CCA AGA GAT GGG AGA TAT CCC GCC CAG, GenePharma Co., Shanghai) at 37°C for 4 h. M-280 Streptavidin Dynabeads (Life Technologies) were added per 100 pmol of biotin-DNA oligos, and the mixture was then rotated for 30 min at 37°C. Beads were captured by magnets (Life Technologies) and washed five times. Each experiment was carried out in triplicate.

Immunofluorescence staining

Cultured HTR-8/SVneo cells were fixed in 4% paraformaldehyde. 10% normal goat (710,027, KPL, USA) was used for pre-
incubated. Primary antibodies: anti-E-cadherin (20,874–1-AP), anti-vimentin (10,366–1-AP) incubated the smears for 1 h in 37°C. After treating with fluoresce-labelled secondary antibodies, cells were incubated by DAPI for nuclear counterstaining. Microscopy was used to acquire the image.

Western blot analysis

RIPA buffer was used for lysing cultured cells. Then, the proteins were running on 8%-10% SDS-PAGE, and electro-transferred to a PVDF membrane (Millipore). After blocking in 5% milk in TTBS, the membranes were incubated with primary antibodies at 37°C. Then, the blots were detected by ECL (enhanced chemiluminescence) Fuazon Fx (Vilber Lourmat) Antibodies that were used as are follow: anti-E-cadherin (1:2000, ab1416, Abcam), anti-vimentin (1:2000, sc-6260, Santa Cruz), anti-Grhl2 (1:1000, ab86611, Abcam), anti-ELF5 (1:1000, ab136119, Abcam) and anti-β-actin (1:1000, sc-47,778, Santa Cruz). Images were captured and processed by FusionCapt Advance Fx5 software (Vilber Lourmat). All experiments were replicated three times independently.

Luciferase assay

HTR-8/SVneo cell were seeded in 24 well plates. For circTNRC18-miRNA luciferase assays, HTR-8/SVneo cells were co-transfected with a miRNA mimic or NC mimic (200 pmol) and circTNRC18-luciferase reporter or an empty vector together; For miR-762-Grhl2 luciferase assay, HTR-8/SVneo cells were transfected with a miRNA-762 mimic or NC mimic combined with Grhl2-3’UTR-luciferase reporter (WT or mut). A Dual-Glo Luciferase Assay System (Promega, Madison, WI) with a Flash and Glow (LB955, Berthold Technologies) was used to measure Luciferase activity.

RNA extraction and quantitative real-time PCR

QIAzol Lysis Reagent (79,306) was used for lysing tissues and cultured cells. NanoDrop 2000 (Thomer Fisher) was used for examining the purity and quality of RNA. microRNA reverse transcription was used the miScriptIRT kit (QIAGEN GmbH, D-40,724 Hilden, GERMANY) following its protocol. miScript SYBR® Green PCR kit was used for qRT-PCR of miRNA with primers: miR-762: GGG GCT GGG GCC GGG GC; miR-1250: ACG GTG CTG GAT GTG GCC; miR-1250: AGC GTG CTG GAT GTG GCC; miR-339-5p: GCT GCT CCC TGT CCT CCA GGA GCTC; miR-449a: GGC TGAG TGT ATT GTT AGC TGG; miR-432-3p: GCC TGG CAG TGT ATT GTT AGC TGG; miR-432-5p: GCT GCT CCC TGT CCT CCA GGA GCTC; miR-615-5p: GGG GGT CCC CGG TGG ATG GCT CCT CCA TGA. All data were analysed by adopting 2−ΔΔCt method.

Target prediction

For finding the potential miRNA of circTRNC18, we used the miRanda (www.microrna.org), RNAhybrid (http://bibiserv.tech fak.uni-bielefeld.de/mnahybrid/submission.html) and RNA22 (https://cm.jefferson.edu/rna22/Interactive/) ; For predicting the target gene of miRNA, we used Targetscan (http://www.targetscan.org).

Statistical analysis

Data are presented as mean ± SEM. The differences between two groups were analysed by Student’s t-test. Spearman’s correlation analysis was used for evaluating the correlation between circRNA and miRNA. P < 0.05 were considered as statistically significant. Statistical analysis was performed using Graphpad Prism 7.0 software (GraphPad Software, San Diego, CA, USA).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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