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ORIGINAL ARTICLE

Lin28B/miR-92b Promote the Proliferation, Migration, and Invasion in the Pathogenesis of Preeclampsia via the DKK1/Wnt/β-Catenin Pathway

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Abstract

Preeclampsia (PE) is a disease unique to pregnancy and one of the leading causes of maternal and neonatal morbidity and mortality. Our previous study found that Lin28b, an RNA-binding protein stem cell factor, is down-expressed in the placenta of preeclampsia and significantly increases the invasion of HTR-8/SVneo cells in vitro. However, the mechanism of Lin28b's role is unclear. The purpose of this study is to investigate whether Lin28B affects the biological behavior and vascular development of trophoblast cells through miR-92b and downstream signaling pathway DKK1/Wnt/β-catenin. Our study demonstrated that Lin28B promotes trophoblast invasion through miR-92b in HTR-8 cells. Further experiments showed that microRNA-92b could negatively regulate DKK1 expression in placental trophoblasts, thereby inhibiting the activity of Wnt/beta-catenin signaling pathway, thereby inhibiting the migration and invasion of trophoblasts. Furthermore, we explored the expression of DKK1 and β-catenin in the placental tissues of preeclampsia patients and 20 healthy people. This study confirmed that Lin28 acts on DKK1 through miR-92b, which affects the expression of downstream Wnt/β-catenin, inhibits the invasion of trophoblast cells and the development of placental vasculature, and participates in the occurrence of PE.

Keywords Preeclampsia · Lin28b · miR-92b · DKK1 · Wnt/β-catenin

Introduction

Preeclampsia (PE) is a disease unique to pregnancy and one of the leading causes of maternal and neonatal morbidity and mortality [1, 2]. Preeclampsia is closely related to the degree of trophoblastic infiltration and is also associated with an abnormal recasting of uterine spiral arterioles [3, 4]. Good trophoblastic infiltration is the basis for the effective establishment of uteroplacental circulation and is the key to successful pregnancy [5]. The remodeling of the uterine spiral arterioles allows the maternal blood to be fully infused into the villus space, and the formation of the placental villous blood vessels and the construction of the vascular network are the guarantee of maternal-fetal material exchange [6].

In recent years, more and more studies have found that some microRNAs (miRNA) can be abnormally expressed when preeclampsia occurs, which is closely related to the occurrence of dysfunction of trophoblast invasion and migration [1]. However, the specific molecular mechanism still needs more exploration and research [7]. A large number of reports have reported that miR-92b is closely related to the occurrence and development of many tumors, tumor cell proliferation, metastasis, invasion and apoptosis, such as digestive system tumors, lung cancer, breast cancer, urinary system tumors, invasive pituitary adenomas, and gynecological malignant tumor [8–13]. miR-92b is a member of the miR-25 family. The microRNA family has four members. Except for miR-92b, the other three are miR-25, miR-92a-1, and miR-92a-2. The initial transcript of miR-92b contains two miRNA molecules: miR-92b-5p and miR-92b-3p [7].

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It has been previously reported that overexpression of lin28b can upregulate the expression of mir-92b, thereby promoting the infiltration of tumor cells [14]. Our previous study found that Lin28B, an RNA-binding protein stem cell factor, is under-expressed in the placenta of preeclampsia. Overexpression of Lin28B in vitro cell culture significantly increased the invasion of HTR-8/SVneo cells (the trophoblast cell line). However, the mechanism by which Lin28B increases invasion is unclear. This study explored the effects of Lin28B/miR-92b on trophoblast function and the possible mechanisms in the pathogenesis of PE.

Materials and Methods

Specimen Collection and Processing

Twenty healthy women and 40 patients with preeclampsia volunteered to join this study. They were assigned to the early-onset preeclampsia group ($n = 20$) or lately-onset preeclampsia group ($n = 20$) or the normal control group ($n = 20$). Ethical approval for this study was provided by the ethics committee of the Shengjing Hospital, Shenyang, China, on March 3, 2017 (Ethical number: 2017PS230K). Fifty grams of fresh placental tissue was removed and rinsed several times in 0.9% saline, dissected into small segments measuring 1 × 1 × 1 cm, and frozen in liquid nitrogen for subsequent experiments.

Cell Culture

The trophoblast cell line (HTR-8/SVneo) cells and human umbilical vein endothelial cells (HUVEC) were obtained from ATCC (MD, USA) and the cells were maintained in RPMI-1640 medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, CLARK Bioscience, Australia), 100 μg/mL streptomycin, and 100 U/mL penicillin (Solarbio, Beijing, China) in a humidified incubator containing 5% CO_2 at 37 °C.

miRNAs, Plasmids, and Transfection Reagents

The miRNAs plasmids including CRISP/Csa9 control plasmid/activation plasmid were purchased from Santa (Santa Cruz, USA). The trophoblast cell transfection was performed by using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, USA). miR-92b-5p mimics and miR-92b-5p inhibitor were purchased from Ruibo biotechnology (Ruibo bio, Shanghai, China). Reverse transcription kit was purchased from Promega (Promega, USA). Annexin V-FITC/PI kit was purchased from Keygentec (Keygentec, Shanghai, China). Matrigel was purchased from BD Biosciences (BD Biosciences, USA).

Cell Proliferation Assay

Cell viability was evaluated by Cell Counting Kit-8 (CCK-8) as previously reported [15]. In brief, 1×10^4 cells/well were cultured in a 96-well plate. After 24 or 48 h of transfection, cell viability was tested using a Cell Counting Kit-8 (Beyotime Company, Jiangsu, China) by measuring the absorbance at 450 nm using a BioTek spectrophotometer (ELX 800, BioTek, USA).

Annexin V-FITC and PI Double Staining Assay

Apoptotic and necrotic cells were assessed and quantified by the Annexin V-FITC kit according to the previously reported manufacturer's instructions [16]. After the indicated treatment, cell pellets were collected and resuspended in 195 μl Annexin V-FITC buffer. Annexin V-FITC (5 μl) and PI (5 μl) were added to 1×10^5 cells, mixed well with the cell suspension, incubated for 15 min, then placed in an ice bath in the dark, and passed through flow cytometry. FACS (Becton Dickinson, USA)..

Transwell Invasion Assay

The invasion ability of the HTR-8 cells was evaluated using Matrigel (Becton Dickinson, San Jose, CA) as the coat of filter membranes. The HTR-8 cells were seeded in the upper chamber of 24-well transwell plate. The upper chamber was filled with serum-free medium, while the lower chamber was filled with complete medium containing 10% FBS. Then the upper chamber cells were then wiped off incubated for 36 h. Methanol was immobilized on the lower chamber of the membrane and photographed after Giemsa staining. Five fields of view were taken under high magnification to count, and the mean value was repeated three times in each group.

Wound Healing Assay

After the medium in the six-well plate was changed to serum-free medium to starve the cells for 12 h, a 200 μl pipette tip was placed along the culture plate to form a "metallurgical" scratch, forming a central blank area, washed with PBS once, and under the microscope, the scratch area was recorded (0 h), and the culture was continued for 24 h, observed, and photographed (24 h).The relative migration distance of the cells was analyzed using Image J software, and the experiment was repeated 3 times and averaged.

Quantitative Real-Time PCR

The cells were transfected for 24 h and the total cellular RNA was extracted by RNeasy RNA Isolation Kit. The concentration and purity of each group were determined. Two

micrograms of RNA was reverse transcribed into cDNA and the reverse transcription system was 20 μl. Primers were as follows: miR-92b, forward: ATATCAGATCTCCAACTCCC CAGCGCG; reverse: CGCTAGAATTCGACCCACTT ACTCACGCTGGC; β-actin, forward: ATCAAGAT CATTGCTCCTCCT; reverse: CTGCTTGCTGATCC ACATCTG. Amplification procedure: 95 °C, 10 min, predenaturation; 95 °C, 15 s; 60 °C, 1 min (40 cycles total), 72 °C extension 10 min. The relative expression level of the target gene as a criterion, the relative expression level were calculated using the $2^{-\Delta\Delta Ct}$ method [17].

Tube Formation Assay

HUVEC cells were resuspended in serum-free medium and seeded at a density of 3.0×10^4/well in 96-well plate. After culturing for 1 h, the medium was aspirated and the corresponding conditioned medium containing 1% FBS was replaced. After, the reticular and tree-like structures formed by HUVEC cells were observed under a microscope, and the number of branch points was counted.

Luciferase Reporter Assay

In this experiment, a plurality of pISO-UTR plasmids (30 ng/well) were mixed with the expression plasmid of Lin28B and siRNA (20 nM/100 nM per well, respectively) and co-transduced into cells. pRL-TK (1 ng/well) was used as an internal reference. For each experiment, 4 wells were repeated for each plasmid/RNA combination. After 24 h of transfection, the medium was aspirated, washed once with PBS, and lysed with Promega's Passive Lysis Buffer (40 μl/well) for half an hour at room temperature. Pipette 30 μl of lysate and add an equal volume of substrate to obtain a firefly enzyme reading, then add StopBuffer (30 μl/sample) to obtain Renilla luciferase reading. The ratio of the two readings is taken as the final value of one well.

Western Blot

Western blotting analysis was performed as described previously [16]. Placental tissue homogenates and cell lysis solutions collected and washed three times with prechilled PBS, followed by the addition of 50 μl of protein lysate containing a 1:100 protease inhibitor and phosphatase to obtain a protein solution. Protein was quantified by BCA method. In brief, cell lysates were separated on 12% SDS polyacrylamide gel and transferred onto PVDF (Millipore Corporation) membranes, and stained with primary antibodies. The Lin28B, DKK1, β-catenin antibody (Cell Signaling Technology, USA) were encapsulated with 5% skimmed milk: Lin28B (1:1000 dilution), DKK1, and β-catenin (1:1000 dilution). β-actin (1:12000) protein served as an internal control. The results were visualized by chemiluminescence using ECL advance reagent.

Statistical Analysis

All the data were analyzed by GraphPad Prism 5.0 and were expressed by mean ± SD. Multivariate analysis of variance were performed using one-way ANOVA followed by a Tukey's multiple comparison test. $P < 0.05$ was considered to be statistically significant.

Results

Effects of Lin28b and miR-92b on the Proliferation Induce Cellular Apoptosis in of HTR-8 Cells

We examined the cell proliferation after 24 and 48 h. As shown in Fig. 1a, at 24 h, the comparison between the siLin28b group and the mimics miR-92b group showed that the relative activity of the cells was significantly increased after transfection of mimics miR92b. This indicates that mimics miR-92b can promote the proliferation of HTR-8 cells. At 48 h, the overexpressed lin28B+inhibitor miR92b group and the siLin28b group showed a significant decrease in cell viability compared with the corresponding control group. Besides, compared with the overexpressed lin28B+inhibitor miR-92b group, the cell viability of the latter was also significantly decreased, indicating that inhibition of miR-92b harms the proliferation of HTR-8 cells (Fig. 1b).

Next, the effects of Lin28b and miR-92b on apoptosis of HTR-8 cells were further evaluated by AV-PI double staining. As shown in Fig. 1c and d, the apoptosis rate increases after overexpression of lin28B. However, if the inhibitor miR-92b is simultaneously transfected, the apoptotic rate is significantly reduced. Similarly, when silencing lin28B was simultaneously added to miR-92b mimics, the apoptotic rate was significantly reduced.

Effects of Lin28b and miR-92b on the Migration and Invasion on HTR-8/SVneo Cells and Tube Formation on HUVEC Cells

Next, we investigated the effects of Lin28b and miR-92b on HTR-8 cell migration and angiogenic ability of HUVEC cells. As shown in Fig. 2a, the migration rate of HTR-8 cells was significantly increased compared with the control group after overexpression of lin28B, either after 24 h or after 48 h. After transfection with miR-92b inhibitor, cell migration was significantly decreased. However, after transfection of miR-92b mimics, cell migration was significantly increased.

As shown in Fig. 2b, the invasive ability of the cells was enhanced after overexpression of lin28B, while the transfected silencing lin28B decreased the invasive ability of the cells. After transfected with miR-92b inhibitor, the invasive ability

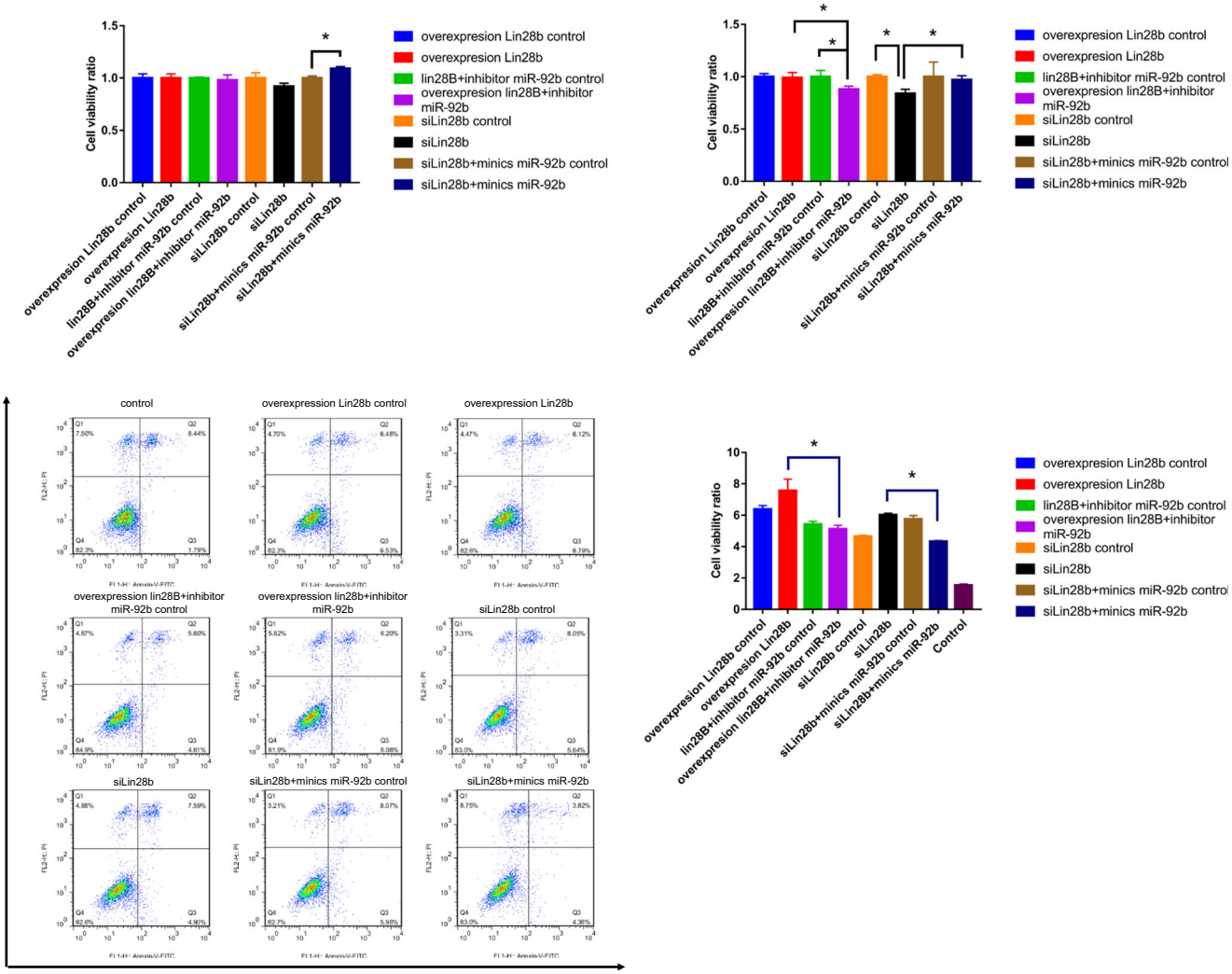

Fig. 1 Lin28b and miR-92b on the proliferation induces cellular apoptosis in of HTR-8/SVneo cells. **a** Cell viability of HTR-8 cells transfected with overexpression Lin28b, overexpression Lin28b plus miR-92b inhibitor, siRNA Lin28B and transfecting miR-92b mimics after 24 h and 48 h (**b**). **c** Quadrant analysis, the fluorescence characteristics of Annexin V/PI staining. **d** The statistical histogram of **c**. Data are showed as mean ± SD ($n = 3$); $^*P < 0.05$

was weakened, but the invasive ability of the cells was significantly enhanced after transfection of miR-92b mimics.

Next, we examined the tube formation ability of HUVEC cells. As shown in Fig. 2c, the relative value of nodes decreased significantly after silencing Lin28B, and the relative value of tube length was decreased significantly. This indicates that Lin28b plays an important role in angiogenesis of HTR-8 cells.

Effects of Lin28b and miR-92b on the Expression of Lin28B, DKK1, and β-Catenin and mRNA Level on HTR-8 Cell

As shown in Fig. 3a, lin28B increased significantly compared with the control group after overexpression of lin28B and addition of inhibitor miR-92. However, after silencing lin28B and adding mimics miR-92b, the expression of lin28B was significantly decreased. After overexpression of lin28B, the expression of DDK1 was significantly reduced compared with the control group. After the addition of inhibitor miR-92, the expression of DDK1(Dickkopf-1) was significantly increased, indicating that the expression of DKK1 can be upregulated after transfection of inhibitor miR-92. After silencing lin28B, the expression of DKK1 was significantly increased. After silencing lin28b+mimicscs miR-92b, the expression of DKK1 was significantly decreased, indicating that the expression of DKK1 was downregulated after transfection with mimics miR-92b. After overexpression of lin28B, the expression of β-catenin was significantly higher than that of the control group, but its expression was significantly decreased after the addition of inhibitor miR-92, indicating that the transfection of inhibitor miR-92 can downregulate the expression of β-catenin. After silencing lin28B and then adding mimics miR-92b, the expression of β-catenin was

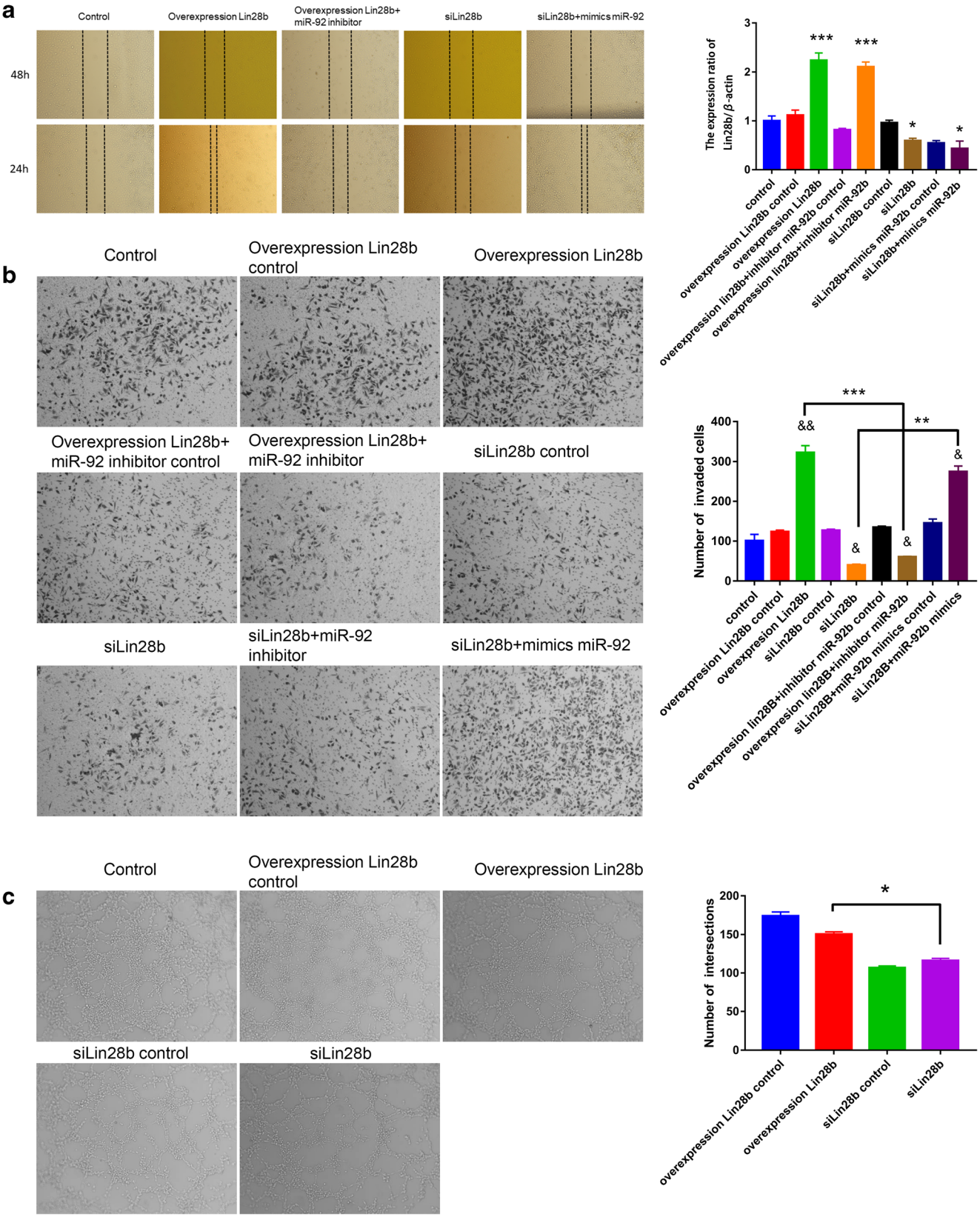

Fig. 2 Lin28b and miR-92b on the migration and invasion on HTR-8/SVneo cells and tube formation on HUVEC cells. **a** The cell migration rate HTR-8 cells transfected with overexpression Lin28b, overexpression Lin28b plus miR-92b inhibitor, siRNA Lin28B and transfecting miR-92b mimics after 24 h and 48 h. **b** The invasive ability of HTR-8 cells after treatment as described above. **c** The number of tubes and after treated with overexpression or silence Lin28b

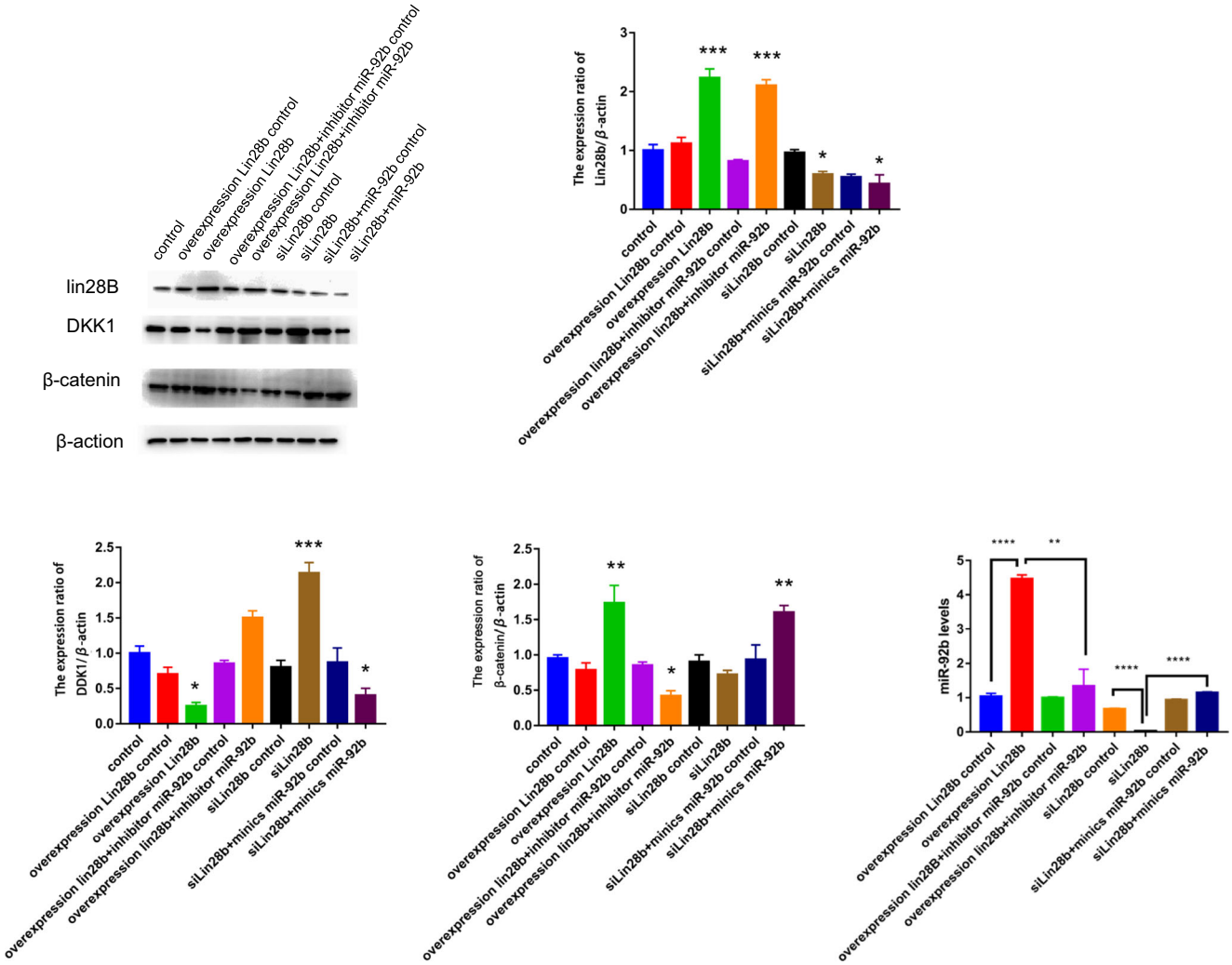

Fig. 3 Lin28b and miR-92b on the expression of Lin28B, DKK1 and β-catenin and mRNA level on HTR-8/SVneo cell. **a** The expression of Lin28b, DKK1, and β-catenin. **b**–**d** Statistical analysis of the gray value of the above proteins. **e** The mRNA level of miR-92b. Data are showed as mean ± SD ($n = 3$); $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$

significantly increased, indicating that the expression of β-catenin was upregulated after transfection with mimics miR-92b. Figure 3b, c, and d were the statistical analysis f Fig. 3a.

As shown in Fig. 3e, the mRNA level of miR-92b was significantly increased after overexpression of lin28B. After transfected with miR-92b inhibitor, the mRNA level of miR-92b was significantly decreased, which was about 1.5 folds that of the control group. When lin28B was silenced and transfected into miR-92b mimics, the expression of miR-92b was significantly increased.

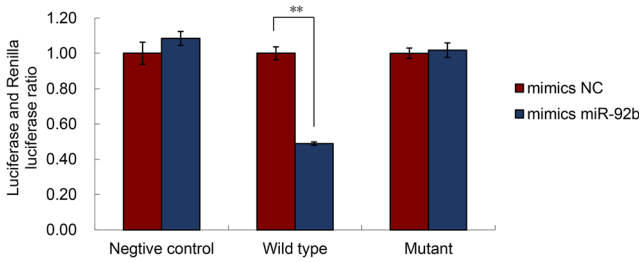

Fig. 4 Lin28B and miR-92b interaction. Data are showed as mean ± SD ($n = 3$), compared with mimics negative control; $^{**}P < 0.01$

Lin28B and miR-92b Interaction

Next, we designed a luciferase reporter gene assay to identify the site of action of miR-92b and Lin28B. As shown in Fig. 4, there was no significant difference between the negative control group and the mutant group. After infection with wild-type and miR-92b, the ratio was lower than that of wild-type and mimics negative control, and the results were significantly different. This result demonstrated that Lin28B in trophoblasts further promotes trophoblast invasion by acting on miR-92b.

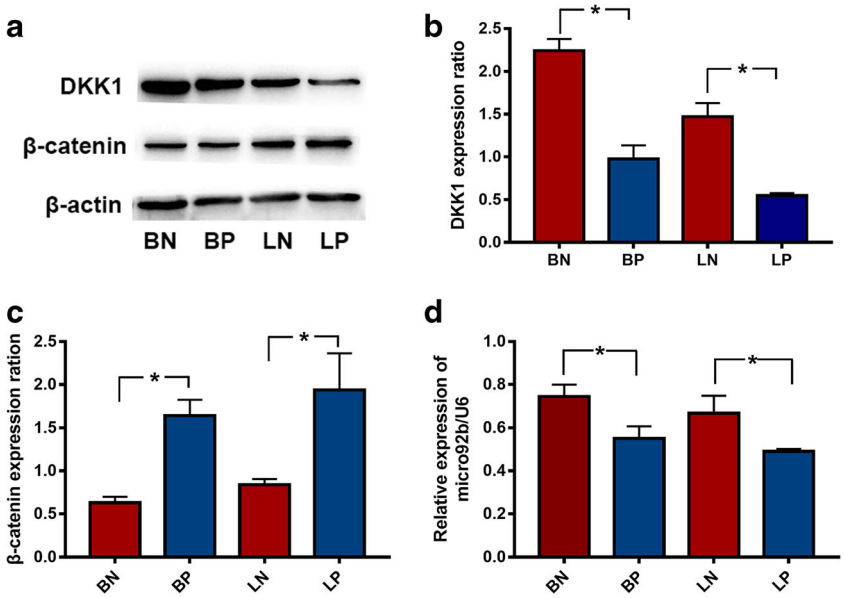

Fig. 5 Expression of DKK1, β-catenin, and miR-92b from preeclampsia and normal maternal placental tissues. **a** Protein bands of DKK1 and β-catenin. **b**, c The statistical histogram of DKK1 and β-catenin, respectively. **d** The level of miR-92b. BN early-onset preeclampsia control group, BP early-onset preeclampsia group, LN late-onset preeclampsia control group, LP late-onset preeclampsia group. Data are showed as mean ± SD ($n = 20$), *$P < 0.05$

Expression of DDK1 and β-Catenin and miR-92b Level in the Placental Tissues from Pregnant Women with PE

The expression level of DDK1 and β-catenin in the placental tissues of the preeclampsia and normal control groups was detected using western blot. For early-onset preeclampsia, the expression of DDK1 and β-catenin protein the preeclampsia group was significantly decreased compared with the control group. And, these two proteins was significantly lower than the late-onset preeclampsia group ($P < 0.05$; Fig. 5a–c). Furthermore, we further investigated the level of miR-92b. Similarly, miR-92b levels in preeclampsia group were also significantly lower than that of control group (Fig. 5d).

Discussion

Our previous study found that Lin28B, an RNA-binding protein stem cell factor, is down-expressed in the placenta of preeclampsia. Invasion of HTR-8/SVneo cells was significantly increased in in vitro cell culture. In other tumor studies, overexpression of Lin28B can upregulate the expression of miR-92b, thereby promoting tumor cell infiltration [18]. In this study, we found that overexpression of Lin28B can upregulate the expression of miR-92b, thereby promoting invasion and migration of HTR-8 cells, which may be through the wnt/β-catenin signaling pathway.

There is evidence that miR-92b is involved in metastasis, invasion, proliferation, and apoptosis of various tumors. In recent years, studies have found that it is closely related to the formation and development of a variety of tumors, and plays an important role in the proliferation, metastasis, and invasion of related tumors [19, 20]. The expression of miR-92b in our preeclampsia was also decreased in our previous study, and the relationship between the two was consistent with the results reported in the literature. Therefore, we intend to study that trophoblasts in Lin28B further promote trophoblast invasion through miR-92b. In this study, overexpression of Lin28B significantly increased the migration and invasion of HTR-8/SVneo cells and increased the number of nodes in HUVEC cells. Moreover, after silencing Lin28B, the addition of mimics miR-92b also significantly increased the infiltration ability of the cells (Fig. 2). These findings were in agreement with previous studies showing that Lin28B promoted cell invasion and migration in cancer cell lines [18]. At the same time, lin28B may work by interacting with miR-92B.

It was found that miR-92b is complementary to partial sequences in the DKK1 gene [15]. DKK1 (Dickkopf-1) is a secreted glycoprotein that inhibits wnt signaling, inhibits cell proliferation and invasion, and induces apoptosis. Recently, wnt signaling pathway has been found to play an important role in trophoblast differentiation and invasion [16, 17]. It has been studied that the DKK1 is a target gene of miR-92b through the dual luciferase reporter gene system, and miR-92b can negatively regulate DKK1 (the reverse regulator of wnt signaling pathway) to activate the wnt/β-catenin signaling pathway,which could change in the biological behavior of tumor cells [15]. In preeclampsia, our preexperiments have demonstrated that miR-92b is reduced in expression compared with the normal control group, and it is speculated that miR-92b may negatively regulate DKK1 and its DKK1 expression in placental trophoblasts. Increased, thereby inhibiting the activity of the wnt/β-catenin signaling pathway could inhibit the migration and invasion of trophoblasts. In our study, our results are consistent with the predictions (Fig. 3), so we speculated that miR-92b may negatively regulate DKK1 to increase its DKK1 expression, while β-catenin

expression is reversed, inhibiting the activity of wnt/β-catenin signaling pathway, thereby inhibiting the migration and invasion of trophoblast cells.

Thanks to its superior light signal and ultra-high signal-to-noise ratio, the dual luciferase reporter detection system is widely used for miRNA target gene validation. The miRNA acts mainly by acting on the 3'UTR of the target gene, and the 3'UTR region of the target gene can be constructed behind the reporter gene luciferase [21], and the expression of the gene can be detected by comparing or overexpressing the miRNA (monitoring fluorescence) [22]. Our study found that Lin28B was found to be a target of miR-92b which regulated the expression of Lin28B via targeting its 3'UTR (Fig. 4).

Furthermore, we examined the expression of DKK1 and β-catenin proteins and the level of microRNA-92b in tissue samples of healthy volunteers and patients. Similar to HTR-8 cells, in patients, the expression of DKK was decreased and the expression of β-catenin was upregulated, corresponding to a decrease in miR-92b levels in patients (Fig. 5).

Conclusion

In the present study, we found that Lin28B promotes trophoblast invasion through miR-92b in trophoblastic HTR-8 cells which was further demonstrated by tissue samples from volunteer patients. Overexpression of Lin28B can upregulate the expression of miR-92b, thereby promoting the invasion and migration of HTR-8 cells. The data from present study suggested that Lin28 acts on DKK1 through miR-92b, which affects the expression of downstream wnt/β-catenin, inhibits the invasion of trophoblast cells and the development of placental vasculature, and participates in the occurrence of preeclampsia.

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Data Availability Detailed data are available in supplementary materials.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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