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MicroRNA-132 stimulates the growth and invasiveness of trophoblasts by targeting DAPK-1

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Abstract. – OBJECTIVE: The purpose of this study was to elucidate the regulatory effects of microRNA-132 on the growth and invasiveness of trophoblasts, thus influencing the development of preeclampsia (PE).

PATIENTS AND METHODS: Placenta tissues from 24 PE pregnancies and 24 healthy nancies were collected. Expression level croRNA-132 and DAPK-1 in collected pla ta tissues were detected. Then, the regulato fects of microRNA-132 and DAPK-1 on exp sion levels of apoptosis-associated genes, v bility, and invasiveness in trop were as sessed. Finally, through Dua cife report- er assay, the binding rela ship b een microRNA-132 and DAPK-1 determ

RESULTS: The res ed NA-132 was downr ated centa of PE pregnancies, whi APK-1 w pregulated. Overexpression roRNA-132 lated viability and in but inhibi apoptosis in troph sts. B s, it was found that DAPK-1 w the target binding microRNA-132, a negative co ion was identified b en th ir expressio levels. Notably, the expre n of DAPK-1 inhibited viability a vasi ess, bu stimulated apoptosis in troph

CONC ONS: croRNA-132 stimulates erativ i sive capacities and inhibits ap sis in blasts by targeting DAPK-1.

K 2, DAPK-1, Trophoblasts, Preeclampsia.

Introduction

Preeclampsia (PE) is a multisystemic vascular syndrome, which is one of the leading causes of ternal and fetal mo ality worldwide. The idence of PE s approximately 2-8%, and induced dea account for 14% of all mate deaths wo vide[1-3]. It is estimated that PE cau 00,00 tant deaths and 70,000 maternal dea year[4]. In addition, PE damages the liver, kidneys, brain, and coagulation system pregnancies and infants, and it also en- isks for babies, including dysplasia and premature birth. Hence, PE is a severe disease that poses treats on both pregnancies and babies[5]. PE is diagnosed based on the following criteria: (1) newly onset hypertension after 20 weeks of gestation, and (2) combination of one or more of the following symptoms (newly onset proteinuria, thrombocytopenia, impaired liver or renal function, pulmonary edema, newly onset visual impairment or central nervous system abnormalities). It is reported that the pathogenesis of PE is related to chronic inflammation, oxidative stress, placental dysplasia, inadequate immune tolerance, genetic factors, imbalance of anti-angiogenic factors and pro-angiogenic factors, and placental ischemia and hypoxia[6]. Since placental delivery is the only effective treatment for PE, it is generally believed that environmental disturbances in the placenta are responsible for PE.

MicroRNAs (miRNAs) are small, single-chain non-coding RNAs containing 19-25 nucleotides. By binding 3′-untranslated region (3′-UTR) of target mRNAs, miRNAs regulate post-transcription expressions of mRNAs by degrading them or inhibiting their translation[7]. During the pregnancy, multiple miRNAs are dynamically expressed

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in placenta tissues. Vital functions of miRNAs in placenta development and functions have been identified[8]. Previous papers have reported the involvement of microRNA-132 in many types of human diseases. In bladder cancer, microRNA-132 inhibits metastasis and epithelial-mesenchymal transition (EMT) *via* the TGF-β1/SMAD2 pathway[9]. By targeting E2F5, microRNA-132 inhibits proliferative and migratory abilities in vascular smooth muscle cells with high-glucose induction[10].

DAPK-1 is a kinase associated with cell death, which is involved in tumor suppression and cell death[11]. In this paper, it was found that microRNA-132 was able to affect viability, invasiveness, and apoptosis in trophoblasts. Moreover, DAPK-1 was proven to be the target gene binding microRNA-132 and involved in trophoblast behaviors. The results of this study provide novel ideas for prevention and treatment of PE.

Patients and Methods

Sample Collection

A total of 24 PE pregnancies and 24 pregnancies undergoing regular pre-natal examination in Zibo Maternal and Child Health Hospital from January 2016 to December 2 were enrolled, and their placental tissues were collected. This study was approved by the Ethics Committee of Zibo Maternal and Child Health Hospital. Signed written informed consent was obtained from all participants before the study. This study was conducted in accordance with the Declaration of Helsinki.

Cell Culture

HTR-8/SVneo cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) containing 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, HyClone Laboratories, South Logan, UT, USA) in a 5% CO_2 incubator at 37°C.

Transfection

Transfection plasmids, including microRNA-132 mimic, microRNA-132 inhibitor, overexpression plasmid of DAPK-1 and NC, were provided by GenePharma (Shanghai, China). Transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RA, followed by determination of RNA concentration using Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA, USA). Complementary deoxyribonucleic acids (cDNAs) were obtained using the reverse transcript II RT and their mRNA levels were determined using the miScript SYBR Green PCR Kit (Qiagen, Hilden, German). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference. The mRNA levels were calculated by $2^{-\Delta\Delta CT}$. The primer sequences are as follows: microRNA-132: 5′-GTAACA TA GCCA-3′ (forward), CAGGGTC GGTATT-3′ (reverse), 5′-GCTTCGC CAGCACA-3′ (forward), 5′-AACG CACGAATTTGCGT-3′ (reverse), Bax: 5′-CC GAGGTCTTTTTC-C -3′ (forward), 5′- CAGCCCATGATG-C CTGAT-3′ (reverse), Bcl-2: 5′-GGTGGGGT-GGTGTGTG (forward), 5′-CGGTTCAG-C TCAGTC C-3′ (reverse), GAPDH: 5′-C GGA TGAGAGCAAGAG-3′ (forward), GGAATTGTGAGGGAGATG-3′ (reverse), DAPK-1: 5′-ACGTGGATGATTAC-ACACC-3′ (forward), 5′-TGCTTTTCT-CATTTCT-3′ (reverse).

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA) for extracting proteins (Beyotime, Shanghai, China). After concentration determination, the protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. The membranes were then incubated with primary and secondary antibodies. Finally, band exposure and grey value analysis were finally conducted.

Cell Counting Kit-8 (CCK-8)

Cells were inoculated into a 96-well plate. At the appointed time points, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added in each well. Then, the absorbance at 450 nm of each sample was recorded.

Dual-Luciferase Reporter Assay

Cells were inoculated in a 24-well plate with 5×10^3 cells per well. After co-transfection with DAPK-1 WT/DAPK-1 MUT and miRNA-132 mimic/NC for 48 h, relative Luciferase activity was finally measured (Promega, Madison, WI, USA).

Transwell

A total of 2.5×10^4 cells were applied on the upper of a transwell insert pre-coated with Matrigel (Corning, Corning, NY, USA), and 750 μL of complete medium was added in the bottom. After 36-h cell culture, the transwell insert was taken out and fixed in 95% methanol for 20 min. Through 10-min violet crystal staining and phosphate-buffered saline (PBS) washing, the cells retained on the upper chamber were wiped off, while those invading to the bottom were captured and counted in 6 randomly selected fields (200×) (Nikon, Tokyo, Japan).

Statistical Analysis

GraphPad software Version 6.0 (La Jolla, CA, USA) was used for data analysis. All data were expressed as mean ± SD (standard deviation). The paired two-tailed *t*-test was used for comparing differences between two groups. $p<0.05$ considered as statistically significant.

Results

MicroRNA-132 was Downregulated in Placenta of PE Pregnancies

Compared with placenta tissues of healthy pregnancies, microRNA-132 was downregulated in those of PE pregnancies (Figure 1A). To uncover the potential influence of microRNA-132 on the development of PE, the transfection efficacy of microRNA-132 mimic and inhibitor was tested (Figure 1B, 1C). In addition, the overexpression of microRNA-132 markedly stimulated the invasiveness in HTR-8/SVneo cells (Figure 1D).

MicroRNA-132 Stimulated Proliferative Ability and Inhibited Apoptosis in Trophoblasts

CCK-8 assay revealed that overexpression of microRNA-132 mimic markedly elevated viability, while the knockdown of microRNA-132 yielded the opposite trend (Figure 2A). Subsequently, the expression levels of apoptosis-associated genes were determined. The results found that microRNA-132 negatively regulated Bax level, and positively regulated Bcl-2 level, suggesting the inhibitory effect of microRNA-132 on trophoblast apoptosis (Figure 2B-2D).

DAPK-1 was the Target Gene Binding MicroRNA-132

Through bioinformatics analysis, the binding sequences of the 3'UTR of microRNA-132 and DAPK-1 were identified (Figure 3A). Based on the binding sequences, wild-type and mutant-type DAPK-1 vectors were constructed. Luciferase activity in wild-type DAPK-1 was negatively regulated by microRNA-132, verifying the binding

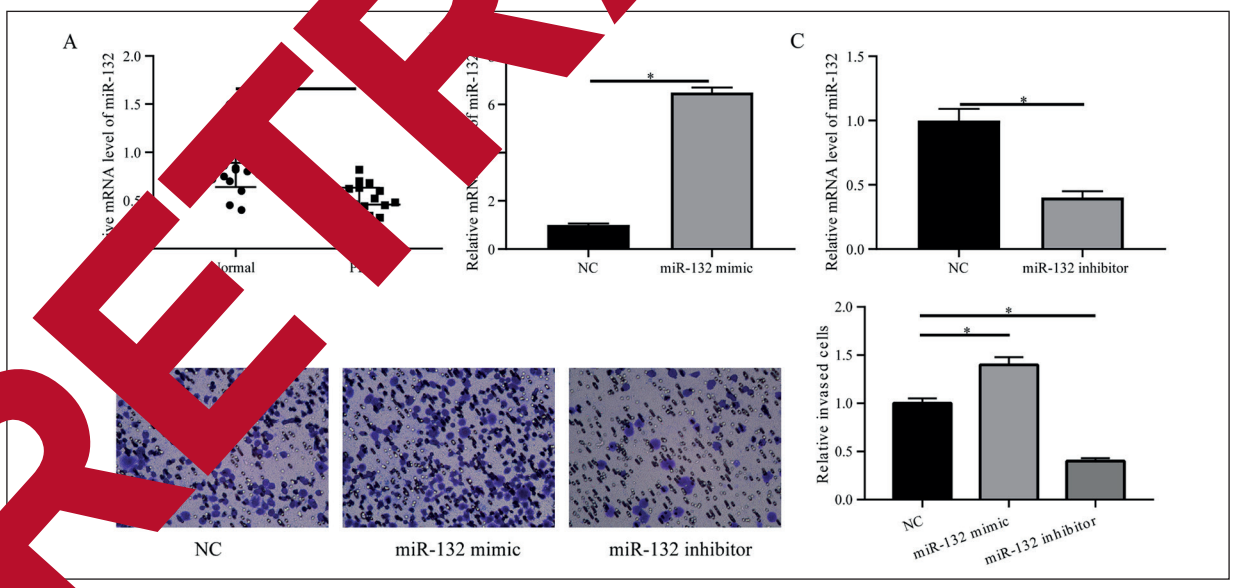

Figure 1. MicroRNA-132 is downregulated in placenta of PE pregnancies. **A,** MicroRNA-132 levels in placenta tissues of healthy pregnancies (n=24) and PE pregnancies (n=24). **B,** Transfection efficacy of microRNA-132 mimic in HTR-8/SVneo cells. **C,** Transfection efficacy of microRNA-132 inhibitor in HTR-8/SVneo cells. **D,** Invasiveness in HTR-8/SVneo cells transfected with NC, microRNA-132 mimic or microRNA-132 inhibitor (magnification: 200×).

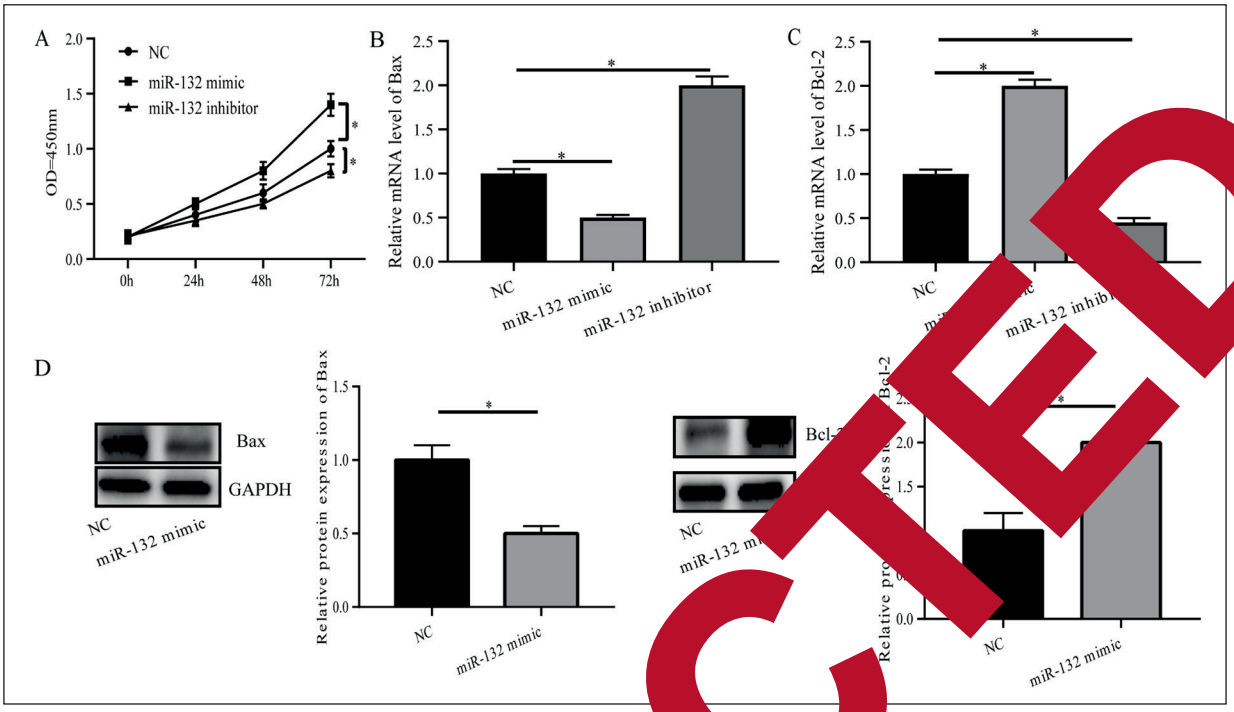

Figure 2. MicroRNA-132 stimulates proliferative ability and inhi[illegible]apoptosis [illegible]ophoblasts. **A,** Viability in HTR-8/SVneo cells transfected with NC, microRNA-132 mimic or microRNA[illegible], The mRNA level of Bax in HTR-8/SVneo cells transfected with NC, microRNA-132 mi[illegible]microRNA-132[illegible]C, The mRNA level of Bcl-2 in HTR-8/SVneo cells transfected with NC, microRNA-132[illegible]RNA-132 inhibitor. **D,** Protein levels of Bax and Bcl-2 in HTR-8/SVneo cells transfected with NC, microRNA[illegible]mi[illegible]RNA-132 inhibitor.

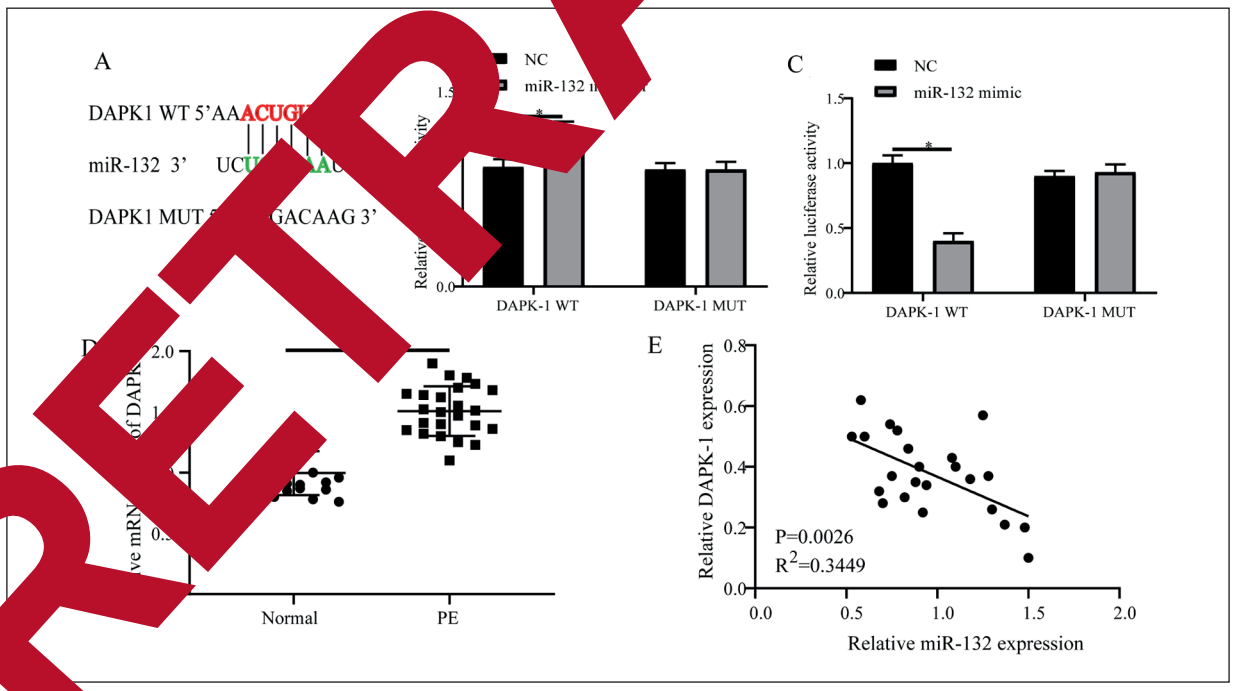

Figure 3. DAPK-1 is the target gene binding to microRNA-132. **A,** Binding sequences in 3'UTR of microRNA-132 and DAPK-1. **B, C,** Luciferase activity in HTR-8/SVneo cells co-transfected with DAPK-1 WT/DAPK-1 MUT and NC/microRNA-132 inhibitor (**B**)/microRNA-132 mimic (**C**). **D,** MicroRNA-132 levels in placenta tissues of healthy pregnancies (n=24) and PE pregnancies (n=24). **E,** A negative correlation between expression levels of microRNA-132 and DAPK-1.

between microRNA-132 and DAPK-1 (Figure 3B, 3C). Notably, the expression of microRNA-132 in the placenta of PE pregnancies was negatively correlated with systolic blood pressure, diastolic blood pressure, and 24-hour urine protein, and positively correlated with onset gestational week and neonatal weight. However, DAPK-1 had the opposite clinical characteristics and pregnancy outcomes (Table I). Subsequently, DAPK-1 was found to be highly expressed in placenta tissues of PE pregnancies (Figure 3D). MicroRNA-132 level was negatively correlated to that of DAPK-1 in the placenta (Figure 3E).

Regulatory Effects of DAPK-1 on Trophoblasts

To elucidate the involvement of DAPK-1 in the development of PE, the transfection efficacy of overexpression plasmid of DAPK-1 was first tested (Figure 4A). It was shown that the overexpression of DAPK-1 inhibited cell viability (Figure 4B) and invasiveness (Figure 4C). On the contrary, the overexpression of DAPK-1 stimulated the apoptosis in trophoblasts (Figure 4D).

Discussion

PE is featured by proteinuria and hypertensi… which seriously affects the he… of pregna… women and infants[12,13]. MiR… are …ensively involved in the developme… PE by …ediating trophoblast homeostasis… MiRN… ported to regulate ov… an… ogress… and induce caspas… pendent … apoptosis in glioma[17]. Besides… downregula… of microRNA-132 is cl… d to a poo… rognosis of colorectal …ncer[18]; … microRNA-132 can stimulate …iferative ab… and inhibit apoptosis in …creatic cancer by …tivating the Hh signa… [19].

…-1, a… antly enriched in the placenta, is ver… the ta… gene of microRNA-132. Bioinformatics analysis proposes that the expression level of DAPK-1 in the placenta is 3.5 times than that of other tissues[20]. DAPK-1 is considered to be a vital regulator for cell death and au… gy[21]. A relevant trail uncovered that DA… increases in the blood circulation of … pregnan… cies. In addition, it is also highly e… ssed in placenta tissues of PE pregnancies, s… ting that the expression of DAPK-1 in … blood … ation may be derived from the … nta[22].

DAPK-1 is an imp… t enz… e that trols cell growth thr… the ca… n ion/serine/threonine kinase p… s also i… lved in IFN-γ-induce… popto… Abn… ly silenced DAPK… observed … li… nors and hematologi… nancies[26]. …ptosis is a physiolog… act… being strictly regulated, which is characteriz… membrane blistering, dec… otential di… e of mitochondrial d… rization membrane, cytochrome C release, a… activation of …aspase-3. As a positive regu… for apoptos… DAPK-1 can be activated by n… le factors… cluding TGF-β, Fas, INF-γ, Cer… … and p-53[27]. Relevant studies[28-30] … monstrated that insufficient proliferation and metastasis of trophoblasts, as well … apoptosis, are the fundamental reasons … he trophoderm is the core organ during embryo implantation and placenta formation[31]. Trophoblast differentiation is of significance to maintain the healthy pregnancy[32]. The proliferate progression occurs in the cytotrophoblasts, which is the major mechanism responsible for the formation of villus structure in the first trimester. Moreover, miRNAs have been identified to be involved in this progression. For example, miR-376c stimulates trophoblasts to proliferate and invade through the Nodal and TGF-β pathways[33].

In this work, microRNA-132 was remarkably downregulated, while DAPK-1 was upregulated in the placenta tissues of PE pregnancies compared to those of healthy pregnancies. MicroRNA-132 was able to stimulate viability and in-

Tab… Correla… …ween miR-132, DAPK-1 and clinical features and pregnancy outcomes.

…iable	Systolic blood pressure		Diastolic blood pressure		24-hour urine protein		Onset of gestational week		Neonatal weight	
	t	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*
DAPK-1	0.359	0.012	0.710	0.001	0.685	< 0.001	-0.256	0.007	-0.413	< 0.001
miR-132	-0.525	0.003	-0.432	0.002	-0.428	0.001	0.615	0.034	0.498	0.017

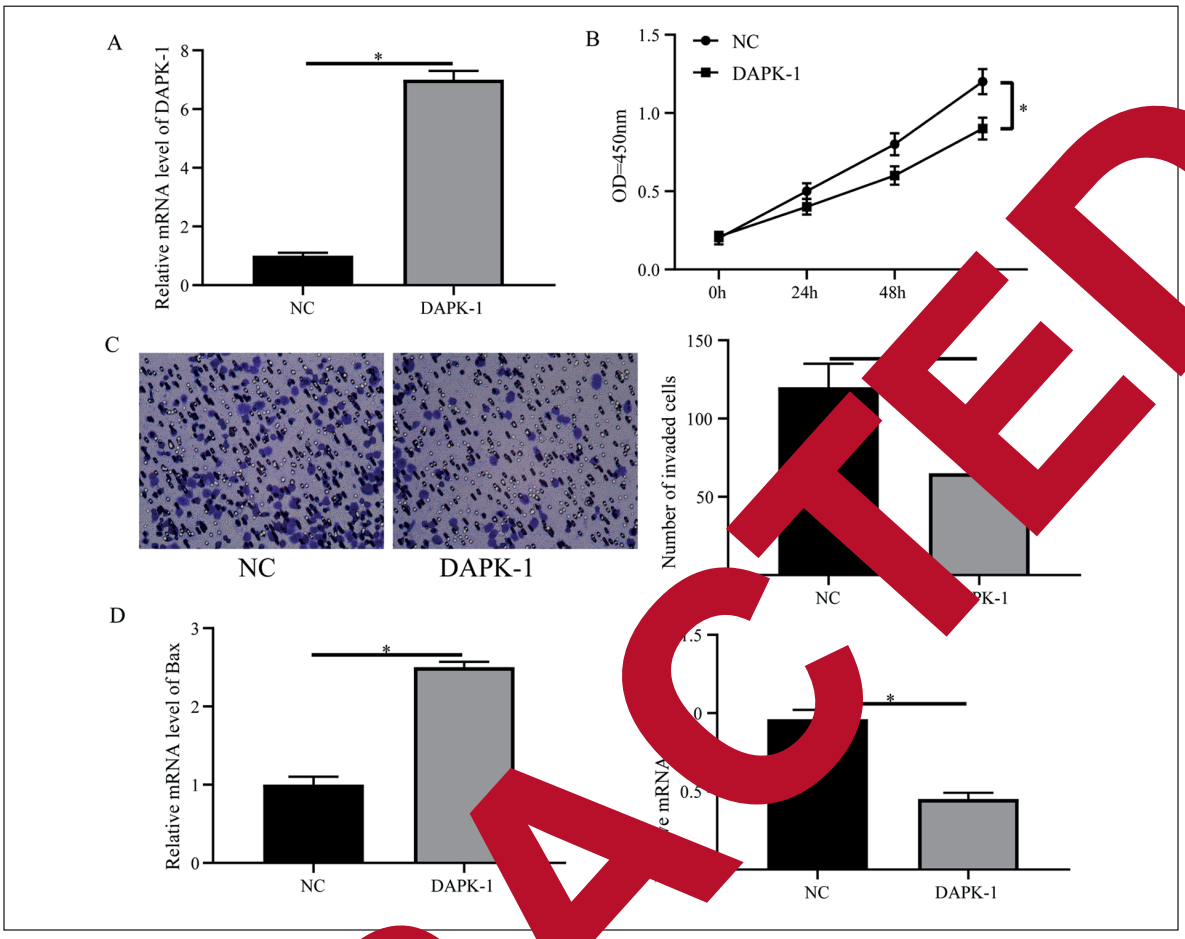

Figure 4. Regulatory effects of DA hoblasts. ansfection efficacy of overexpression plasmid of DAPK-1. **B,** Viability in HTR-8/SVneo cells tr cted w C or over ssion plasmid of DAPK-1. **C,** Invasiveness in HTR-8/SVneo cells transfected with NC or ov ression p id of DAPK (magnification: 200×). **D,** Relative levels of Bax and Bcl-2 in HTR-8/SVneo cells transfecte lC or asmid of DAPK-1.

vasiveness and i apoptosis hoblasts. In addition, D the targe e to be negatively re ated croRNA-132, and it was resp ble for the latory effects of microR 132 on viability, vasiveness, and apop in tr oblasts. The findings of this stu ovid thological basis for elucidating the ro roRNA in the trophoblast de- lopme wev nly microRNA-132 level in place etected, and its expressions in ma, dec , and maternal-fetal interface re be further detected.

Conclusions

Shortly, microRNA-132 stimulates proliferative and invasive capacities and inhibits apoptosis in trophoblasts by targeting DAPK-1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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