Downregulated hsa_circ_005243 induced trophoblast cell dysfunction and inflammation via β-catenin and NF-κB pathways in gestational diabetes mellitus

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Research

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Abstract

Background: Gestational diabetes mellitus(GDM) is a common obstetric pregnancy complication, which poses a serious threat to the health of pregnant women and newborns. The specific etiology and pathogenesis of this disease have not been fully clarified, it is reported to be related with insulin resistance, inflammatory response and genetic factors etc. Circular RNA(circRNA) is a special kind of non-coding RNA, which have been attracted much attention in recent years. It has been reported that circRNAs may play a regulatory role in pregnancy-related diseases, including GDM.

Methods: Previously we reported a circRNA, hsa_circ_005243, which was identified by RNA-sequencing. In this study we detected its expression in 20 GDM pregnant women and 20 normal controls using quantitative reverse transcription polymerase chain reaction analysis. Further in vitro experiments were conducted after hsa_circ_005243 knockdown in HTR8-S/Vneo cells, cell proliferation and migration ability was tested, the secretion of inflammatory factors (TNF-α and IL-6) were detected by ELISA. Then we detected the expression of β-catenin and increased nuclear factor kappa-B (NF-κB) signaling pathways which was related to GDM in the mechanism study.

Results: We found the expression of hsa_circ_005243 was significantly reduced both in the placenta and plasma of GDM pregnant women. Knockdown of hsa_circ_005243 in trophoblast cells significantly suppressed cell proliferation and migration ability. In addition, increased secretion of inflammatory factors (TNF-α and IL-6) were observed after hsa_circ_005243 depletion. Further mechanism experiments showed that knockdown of hsa_circ_005243 reduced the expression of β-catenin and increased nuclear NF-κB p65 nuclear translocation.

Conclusions: Collectively, our study showed that down-regulation of hsa_circ_005243 might be associated with the pathogenesis of GDM through regulating β-catenin and NF-κB signal pathways and suggest a new potential therapeutic target for GDM.

Introduction

Gestational diabetes mellitus (GDM) is a special type of diabetes, which appeared for the first time during pregnancy or abnormal glucose metabolism(1). According to the latest statistics, the incidence of GDM in China is as high as 17.5%(2). GDM patients have a significantly increased risk of developing metabolic syndrome and type 2 diabetes after delivery(3). Therefore, timely diagnosis and therapy strategy is very important to reduce the adverse pregnancy outcome of GDM patients. The pathogenesis of GDM is very complicated and has not been fully clarified, which may be the result of multiple factors such as inflammatory factors, insulin resistance, dysfunction of islet β cells etc. The placenta is a highly specialized organ in the interface between maternal and fetal circulation with fundamental functions during pregnancy. The mechanisms of placental pathology during diabetes are still largely unclear. There was a significant inflammatory response in the placental tissues of GDM, with an increase in the number

of macrophages and the content of saturated fatty acids, which promoted the release of inflammatory factors IL-6, IL-8, and TLR2 by trophoblast cells(4).

Circular RNA (circRNA) is a special kind of RNAs with a closed loop, which has attracted much attention in recent years. With the development of high-throughput sequencing technology, more and more circRNAs have been discovered, their biological characteristics and regulatory function have been gradually revealed. CircRNAs are highly conserved, with stability, diversity and tissue specificity. They participates in regulation of gene expression at the levels of transcription and post-transcription, and thus widely involved in many physiological and pathological processes. In recent years, more and more studies have shown that circRNAs are closely related to the occurrence and development of preeclampsia, GDM and other pregnancy-related diseases(5, 6). In the previous study(7), we found a significant decrease of hsa_circ_0005243 in the placenta of GDM pregnant women by high-throughput RNA sequencing. Here, we investigated the possible regulatory function of this circRNA in trophoblast cell line HTR8-S/Vneo and its potential mechanism was primarily explored.

Materials And Methods

Patients

From April 2017 to December 2018, 20 parturient women diagnosed with GDM in the obstetrics department of Changzhou maternal and child health hospital affiliated to Nanjing medical university and 20 normal adjust control women were selected. The diagnosis of GDM was conducted by 75 g glucose tolerance test at 24-28 weeks, excluding multiple births, premature delivery, delivery age less than 20 years old or over 40 years old, pre-pregnancy patients with hypertension, diabetes, chronic liver and kidney diseases, thyroid and other endocrine diseases.

Informed consent was obtained from each participant and this study was approved by the ethics committee of the hospital.

Cell culture and transfection

Human trophoblast cell line HTR-8/SVneo were cultured in 1640 supplemented with 10% FBS(Gibco) and 1% penicillin/streptomycin at 37°C with 5% CO_2. Cells (2×10^5) were seeded in 6-well plates and transfected with small interfering RNAs (siRNAs) targeting hsa_hsa_circ_0005243 using Lipofectamine 3000 (Invitrogen, USA) according to the manufacture's protocol. The knockdown efficiency of siRNAs was determined by qRT-PCR, their sequences were listed as: siRNA-1, 5'-UGA CCA UCA UCU ACA ACA UTT-3', 5'-AUG UUG UAG AUG AUG GUC ATT-3'; siRNA-2, 5'-CCA UGA ACC CGC ACG ACA UTT-3', 5'-AUG UCG UGC GGG UUC AUG GTT-3'; siRNA-3, 5'-CCU ACA AGG UCU AUG CUG ATT-3', 5'-UCA GCA UAG ACC UUG UAG GTT-3', all the siRNAs and negative controls were obtained from RiboBio (Guangzhou, China).

CCK8 assay

Cells were digested and seeded into 96-well cell culture plate (Corning, USA) with a concentration of 3× 10^3 cells/mL. Cells viability was measured after culture for 24hr, 48hr and 72hr by adding 10 μL CCK8 reagent (DOJINDO Laborataries, Japan). After incubated at 37℃ for 3 hr, the OD value at 450 nm was detected by a Microplate Reader (BioTek, Winooski, VT, USA).

Colony formation assay

The cells in logarithmic phase were digested into suspension and inoculated in 6-well cell culture plates containing 2 mL medium, shake the culture plates gently to make the cells disperse evenly. Then the cell culture plates were transplanted into a CO_2 incubator, with 37℃ and 5% for 24 hours until it adhered to the wall. After 12 days, the medium was discarded and the culture was terminated, carefully soaked twice with PBS, fixed for 15 minutes with 5 mL absolute ethanol. After discarding the fixative solution, add Jimsa dye solution (ThermoFisher, USA) for 10-30 minutes, then wash the dye solution slowly with running water and dry the air, after photographed the clones were counted.

EdU

In order to evaluate the proliferation ability of trophoblast cells, EdU assay was performed using keyFluor555 Click-iTEdU imaging detection Kit (Keygentec, Nanjing, China) according to the manufacturer's protocol. Cells were fixed with 4% paraformaldehyde, then incubated with 2 mg/mL glycine for 5 min, 200 mul of 1×Apollo® staining solution was added to each well and incubated in a bleached shaker for 30 min at room temperature, away from light, after that washed with PBS, 100μL penetrant agent (0.5% TritonX-100in PBS) was added. After nucleus of the cells was stained with Hoechst 33342, cells are photographed by a high-content imaging system(MD Micro, USA).

Migration assay

For the in vitro Transwell migration assay, the transfected cells were digested and adjusted to a density of 1 ×10^5/mL, 100 μL cell suspension was added to the upper chamber(Corning Incorporated, USA), and 700 μL medium containing FBS was added to lower chamber. Cell culture plate was then placed in 37 C, 5% CO_2 incubating for 24 hr. Wiped off the cells in the upper chamber using a cotton swab, while the cells on lower surface of membranes were fixed with formaldehyde and stained with 0.1% crystal violet(Sigma, USA). After incubated in 37℃ for 30 min, take it out and washed with PBS, 3-5 fields were randomly selected and photographed and the migrated cells were counted under an inverted Microscope(Olympus, Japan).

For the wound healing assay, cells in logarithmic growth phase were digested and inoculated into a six-well plate. After 24hr, when the cell aggregation reached about 60%, the sterile nozzle was used to evenly draw lines in the plate. The floating cells were washed with PBS, and then fresh medium was placed in a cell culture box for further culture. After 24 hours, the cells were taken out and photographed (with a magnification of 200×), and the migration distance of cells was measured.

Elisa

Cells were seeded in a 6-well plate (Corning Incorporated), after transfection, the medium was replaced with new culture medium. The culture medium was then centrifuged for 20 minutes at 1000 ×g to remove cell debris and impurities. The concentrations of TNF-α and IL-6 in the medium was detected by Elias kit (Mlbio, Shanghai, China) according to the manufacturer's protocol. The absorbance (OD value) of each group was measured at 450 nm by a Microplate Reader (MD SpectraMax M3, USA).

Western blot

The transfected cells were harvested and lysed in the lysate containing protease inhibitors. Then protein concentrations were determined by the BCA kit (Thermo Fisher Scientific, USA). After denaturation, the proteins were separated by 12% SDS-PAGE, then transferred to the PVDF membrane (Merck Millipore, Darmstadt, Germany). After blocked by 5% defatted milk, the membranes with proteins were incubated with diluted primarily antibodis at 4℃ overnight as follows: anti-c-myc (1:2000), anti-cyclinD1 (1:3000), anti-survivin (1:3000), anti-β-catenin (1:1000), anti-p65 (1:2000), anti-laminin B (1:3000) and anti-β-actin (1:3000), all purchased from Abcam (Cambridge, UK). The membranes were then washed by TBST and incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Beyotime, China) for 1 hr. After washed by TBST the membranes were incubated with enhanced chemiluminescence reaction reagent (BeyoECL Plus, Beyotime, China) and photographed by Luminescence imaging system (Tanon, Shanghai, China)

qRT-PCR

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). The expression of circRNA and GAPDH was detected using SYBR Premix Ex Taq system (Takara, Madison, WI, USA) based on the manufacturer’s instructions. RT-PCR was performed using primer with the sequences listed as follows: hsa_hsa_circ_0005243, forward, 5'-TTATCTACATGCACCTGCGCT-3', reverse, 5'-AAGTGACAAGCTAGCCCTCAT-3', GAPDH, forward: 5'-CAAATTCCATGGCACCGTCA-3', reverse: 5'-AGCATCGCCCCACTTGATTT-3'. The PCR reactions were conducted as follows: denaturation at 95°C for 10 min, amplification 40 cycles of 95°C for 10 s and 58°C for 15 s, followed by treatment at 70°C for 30 s. The relative expression levels were determined by comparing the Ct values of the target genes to those of the GAPDH gene.

Flow cytometry

For the cell apoptosis analysis, after transfection cells were digested with 0.25% trypsin (without EDTA) and collected, washed twice with PBS and stained using Annexin V-FITC Apoptosis Detection Kit (Beyotime, China) and analyzed by flow cytometry (FACS Calibre, BD, USA) . The ratio of apoptotic cells was counted and obtained.

Immunofluorescence

Slides of cells were fixed by 4% polyformaldehyde, washed by PBS, then treated with 0.5% Triton X-100, after that the slides were blocked by 5% BSA for 30 min. Cells were then incubated with antibodies against β-catenin and p65 (1:100, abcam) at 4°C overnight .After 3 washes with PBS, FITC-conjugated secondary antibody (1:100, abcam) was added and incubated at 37°C for 1 hr away from light. Slides were stained by DAPI for 5 min, then the expression of protein in cells were observed under a laser confocal microscopy (Zeiss LSM710, Germany), 3 three photographs were randomly taken.

Results

1、Characterization of hsa_circ_0005243 in GDM

In our previous study, we found that the expression of hsa_circ_0005243 in GDM placenta was lower than that in the control group by high-throughput RNA sequencing(7). Then qRT-PCR results verificated that the expression of hsa_circ_0005243 decreased significantly in both placentas (Fig1 A) and plasmas (Fig1 B) from 20 GDM patients compared with 20 normal controls. The diagnostic value of hsa_circ_0005243 in plasma was further evaluated by ROC curve analysis(Fig1 C), results showed that the area under the curve (AUC) was 0.75 (p<0.01). Hsa_circ_0005243 originates from the *TMEM184B* (Transmembrane protein 184B) gene and consists of the head-to-tail splicing of exon 2,3,4 with a length of 517 bp(Fig1 D). In addition, we found that hsa_circ_0005243 was resistant to Rnase R treatment compared with linear mRNA(Fig1D)。

2、Downregulated hsa_circ_0005243 supressed trophoblast proliferation and induced apoptosis

To further explore the potential functions of hsa_circ_0005243 in trophoblast cells, interference siRNAs (si-circRNA and si-NC) targeting hsa_circ_0005243 were transfected into human trophoblast cell line HTR8-S/Vneo. Then interference efficiency was verified after transfection, all the 3 si-circRNAs reduced the expression of hsa_circ_0005243 significantly (Fig2 A), among which si-circNRA-2 had the best effect, so it was chosen for further experiments. CCK8 results indicated that the cell viability in the si-circRNA group was significantly lower than that of the si-NC group 48h and 72h after transfection(Fig2 B). Next, through the EdU experiment we found that the number of EdU positive cells was significantly fewer in si-circRNA group than that in the control group(Fig2 C), similar results were shown in the clone formation experiment (Fig2 D), the number of clones in the si-circRNA group was significantly fewer than that in the si-NC control group. Besides, flow cytometry results showed that the apoptosis cell rate was significantly higher after hsa_circ_0005243 knockdown(Fig2 E).

3、Kncokdown of hsa_circ_0005243 inhibited migration ability of trophoblast cells

The normal migration of trophoblast cells is important for the maintenance of placental function(8), therefore, we investigated the effect of hsa_circ_0005243 on the migration ability of trophoblast cells. *In vitro* Transwell results suggested that the migration ability of trophoblast cells decreased significantly after transfected with si-circRNA(Fig3 A), the number of migratory cells in the knockdown group was significantly fewer than that in the control group(Fig3 B). The wound healing assay results also indicated

that the migrated distance in the si-circRNA group was significantly shorter than that in the control group(Fig3 CD).

4、Knockdown of hsa_circ_0005243 elevated the levels of inflammatory cytokines TNF-α and IL-6

In the GDM pathogenesis, inflammation plays a significant role and various inflammatory mediators are considered to be risk factors leading to GDM development(9) .Therefore, ELISA was used to detect the levels of relevant inflammatory factors in the culture medium. After hsa_circ_0005243 knockdown, the levels of TNF-***α*** (Fig4 A) and IL-6 (Fig4 B) in the medium increased significantly compared with si-NC group.

5、The potential mechanism of regulation of hsa_circ_0005243 on trophoblast cell function and inflammation

To further investigated the potential molecular mechanisms of hsa_circ_0005243 in regulating trophoblast cell functions, after hsa_circ_0005243 knockdown, relevant signaling pathway protein expressions were detected by western blot. We found that the protein expression of β-catenin was significantly decreased. Also, the expression of its related downstream molecules (c-myc, cyclinD1, survivin) were also significantly decreased (Fig5 AB). In addition, we found that nuclear NF-κB expression was increased after hsa_circ_0005243 depletion(Fig5 CD), and also immunofluorescence assay indicated an increase in nuclear translocation of its p65 subunit(Fig5 E).

Discussion

Gestational diabetes refers to varying degrees of abnormal glucose metabolism during pregnancy, which affect maternal and infant health seriously. About 2–5% of all pregnant women could develop GDM and the prevalence has increased considerably during the last decade(10). GDM could cause dystocia, macrosomia, neonatal hypoglycemia and other dangerous complications. Therefore, timely diagnosis and active treatment are very important to reduce adverse pregnancy outcomes in GDM patients. Although the pathogenesis of GDM is not completely understood, current studies have found it is similar to the pathogenesis of T2DM(type 2 diabetes mellitus), it is the outcome of the synergistic effect of internal genetic and external environmental factors. Pregnant women with a family history of T2DM had a significantly increased risk of GDM(11).

CircRNAs are a special class of non-coding RNA molecules with a closed circular structure, in recent years with the development of high-throughput sequencing technology, circRNAs have been reported to have important regulatory functions in a variety of diseases(12, 13), thus they were considered to have potential values as diagnostic and therapeutic targets for some diseases. CircRNAs could participate in gene regulation by miRNA sponges that block the inhibitory effect of miRNAs on their target genes(14), or bind to RNA binding proteins (RBP) to regulate gene expression(15), some circRNAs even have a protein-coding function of their own(16). Placenta is an important organ to maintain mater-fetus material transport, it affects the supply and transformation of fetal nutrition, and is of vital importance to mater-

fetus gas and material exchange. The placental changes were associated with GDM(10, 17).In recent years, differential expression of circRNA in placenta has been reported(5, 7), some circRNAs were abnormally expressed in GDM patients and may play a potential role in the development of GDM(18). These research indicated that circRNAs may be involved in the pathogenesis and pregnancy outcome. However, the research on circRNAs and GDM is is few and not in-depth.

In this study we found that the expression of hsa_circ_0005243 decreased significantly in the placentas of patients with GDM, in vitro experiment showed that knockdown of hsa_circ_0005243 suppressed cell proliferation and migration in trophoblast cell line HTR8-S/Vneo. Placental trophoblast is one of the most active cells in pregnancy, its dysfunction will lead to abnormal exchange of mother and fetus, imbalance of immune inflammation and so on, which will lead to adverse pregnancy. Although the pathogenesis of GDM is still unclear, it is reported that inflammatory reaction has played an important role(19). We found the levels of inflammatory factors TNF-α and IL-6 were increased after hsa_circ_0005243 knockdown. TNF-α is a cytokine secreted mainly by monocyte macrophages. During pregnancy, the placenta also secretes TNF-α, which has a wide range of biological functions. It can promote the aggregation and adhesion of inflammatory cells and damage vascular endothelial cells. TNF-α levels were reported to be positively correlated with body mass index, involved in glucose and lipid metabolism, and closely related to insulin resistance and gestational diabetes mellitus(20–22). IL-6 not only plays an important role in the regulation of immune and inflammatory response, but also plays an important role in the balance of energy metabolism. It is involved in the development of GDM,, its expression is significantly increased in the placenta and plasma of GDM pregnant women(23, 24).

In our study Elisa results showed that the levels of TNF-α and IL-6 were significantly higher in the hsa_circ_0005243 knockdown group, which indicated that it may play a role in inflammation. In order to explore the potential mechanism, we detected the signal pathway molecules related to GDM, and found the expression of β-catenin decreased significantly, along with its downstream genes. β-catenin is a downstream component of Wnt signaling pathway, which plays an important role in cell proliferation, apoptosis, migration and invasion etc(25). The WNT/β-catenin-catenin signaling pathway was reported to play a key role in the differentiation of the trophoblastic stem cells, fusion of chorionic allantoic cells and placental morphological development in pregnant rats(26). The expression level of β-catenin was downregulated in the placental tissues of the preeclampsia group and the hypoxia/reoxygenation HTR8/SVneo cells(27). Changes in related genes of the Wnt canonical pathway were observed, including downregulation of Wnts, fzds, β-catenin, apc, and gsk3β, which suggesting regulation of Wnt expression by hyperglycemia in different embryonic tissues(28). These investigations suggest that maternal diabetes may suppresses Wnt signaling(29). Therefore, it is suggested that the trophoblast dysfunction after hsa_circ_0005243 depletion may be involved in the pathogenesis of placental pathological state. In addition, a decreased nuclear NF-κB p65 expression was observed. NF-κB pathway is the central link of regulating immune response, stress response, apoptosis and inflammation, and it is also the common pathway of many inflammatory processes. NF-κB belongs to the nuclear transcription factor family, it is a dimer composed of p50 and p65 protein subunits. NF-κB p65 could interact with cytokines (TNF-α, IL-6 etc.) and forms positive feedback regulation, which then amplifies the inflammatory response in

GDM(30–32). After being activated by inflammatory factors such as TNF-α and IL-6, the p65 subunit enters into the nucleus, regulates a series of inflammatory factors and related gene expression, and further feedback increases the expression of the above-mentioned inflammatory factors that cause insulin resistance, leading to the decrease of insulin sensitivity. Through immunofluorescence we also found that p65 protein in the nucleus increased after the hsa_circ_0005243 knockdown.

Conclusions

In this study we found that hsa_circ_0005243 was down-regulated in GDM placenta, in vitro cell experiments demonstrated that downregulated hsa_circ_0005243 suppressed trophoblast proliferation and migration, the levels of inflammatory cytokines Il-6 and TNF-α were increased significantly. In the follow-up mechanism study, depletion of hsa_circ_0005243 significantly reduced the expression of β-catenin and its downstream related gene expressions. Also, the expression of NF-κB, which mediates the inflammatory response was increased, its subunit p65 nuclear translocation levels was increased. Although our research provides new potential new molecular targets for the pathogenesis of GDM, considering the complexity of the regulatory mechanism, additional in vivo and in vitro studies are recommended to further verify its potential function.

Abbreviations

GDM: Gestational diabetes mellitus; qRT-PCR: quantitative reverse transcription polymerase chain reaction; circRNA: Circular RNA; NF-κB: nuclear factor kappa-B; TMEM184B:transmembrane protein 184B

Declarations

Acknowledgements

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Authors' contributions

HYW, WBZ and LZS carried out the assays and participated in designing the study. GTS carried out clinical consultation. WBZ, BY carried out laboratory tests and performed the statistical analysis. HYW and LZS conceived the study, participated in its coordination and draft the manuscript.

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Availability of data and materials

All data generated or analysed in this study are included in this published article

Ethics approval and consent to participate

Informed consent was obtained from each participant. The study design and protocol were reviewed and approved by the ethics committee of Changzhou Maternity and Child Health Care Hospital affiliated to Nanjing Medical University (Approval No:CZFY20160103).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

References

1. 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018. Diabetes care. 2018;41(Suppl 1):S13-s27.
2. Xu T, Dainelli L, Yu K, Ma L, Silva Zolezzi I, Detzel P, et al. The short-term health and economic burden of gestational diabetes mellitus in China: a modelling study. BMJ open. 2017;7(12):e018893.
3. ACOG Practice Bulletin No. 190 Summary: Gestational Diabetes Mellitus. Obstetrics and gynecology. 2018;131(2):406-8.
4. Stirm L, Kovarova M, Perschbacher S, Michlmaier R, Fritsche L, Siegel-Axel D, et al. BMI-Independent Effects of Gestational Diabetes on Human Placenta. The Journal of clinical endocrinology and metabolism. 2018;103(9):3299-309.
5. Yan L, Feng J, Cheng F, Cui X, Gao L, Chen Y, et al. Circular RNA expression profiles in placental villi from women with gestational diabetes mellitus. Biochemical and biophysical research communications. 2018;498(4):743-50.
6. Jia N, Li J. Role of Circular RNAs in Preeclampsia. Disease markers. 2019;2019:7237495.
7. Wang H, She G, Zhou W, Liu K, Miao J, Yu B. Expression profile of circular RNAs in placentas of women with gestational diabetes mellitus. Endocrine journal. 2019;66(5):431-41.
8. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. Thrombosis research. 2004;114(5-6):397-407.
9. Majcher S, Ustianowski P, Tarnowski M, Dziedziejko V, Safranow K, Pawlik A. IL-1beta and IL-10 gene polymorphisms in women with gestational diabetes. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet. 2019:1-6.

10. Monteiro LJ, Norman JE, Rice GE, Illanes SE. Fetal programming and gestational diabetes mellitus. Placenta. 2016;48 Suppl 1:S54-s60.
11. Retnakaran R, Connelly PW, Sermer M, Zinman B, Hanley AJ. The impact of family history of diabetes on risk factors for gestational diabetes. Clinical endocrinology. 2007;67(5):754-60.
12. Holdt LM, Kohlmaier A, Teupser D. Circular RNAs as Therapeutic Agents and Targets. Frontiers in physiology. 2018;9:1262.
13. Yu T, Wang Y, Fan Y, Fang N, Wang T, Xu T, et al. CircRNAs in cancer metabolism: a review. Journal of hematology & oncology. 2019;12(1):90.
14. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature. 2013;495(7441):333-8.
15. Wang KW, Dong M. Role of circular RNAs in gastric cancer: Recent advances and prospects. World journal of gastrointestinal oncology. 2019;11(6):459-69.
16. Legnini I, Di Timoteo G, Rossi F, Morlando M, Briganti F, Sthandier O, et al. Circ-ZNF609 Is a Circular RNA that Can Be Translated and Functions in Myogenesis. Molecular cell. 2017;66(1):22-37.e9.
17. Edu A, Teodorescu C, Dobjanschi CG, Socol ZZ, Teodorescu V, Matei A, et al. Placenta changes in pregnancy with gestational diabetes. Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie. 2016;57(2):507-12.
18. Wu H, Wu S, Zhu Y, Ye M, Shen J, Liu Y, et al. Hsa_circRNA_0054633 is highly expressed in gestational diabetes mellitus and closely related to glycosylation index. Clinical epigenetics. 2019;11(1):22.
19. Plows JF, Stanley JL, Baker PN, Reynolds CM, Vickers MH. The Pathophysiology of Gestational Diabetes Mellitus. International journal of molecular sciences. 2018;19(11).
20. Wang SL, Liu PQ, Ding Y, Peng W, Qu X. [Maternal serum tumor necrosis factor-alpha concentration and correlation with insulin resistance in gestational diabetes]. Zhonghua fu chan ke za zhi. 2004;39(11):737-40.
21. Cseh K, Baranyi E, Melczer Z, Csakany GM, Speer G, Kovacs M, et al. The pathophysiological influence of leptin and the tumor necrosis factor system on maternal insulin resistance: negative correlation with anthropometric parameters of neonates in gestational diabetes. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology. 2002;16(6):453-60.
22. Nicholson W, Wang NY, Baptiste-Roberts K, Chang YT, Powe NR. Association between adiponectin and tumor necrosis factor-alpha levels at eight to fourteen weeks gestation and maternal glucose tolerance: the Parity, Inflammation, and Diabetes Study. Journal of women's health (2002). 2013;22(3):259-66.
23. Nergiz S, Altinkaya OS, Kucuk M, Yuksel H, Sezer SD, Kurt Omurlu I, et al. Circulating galanin and IL-6 concentrations in gestational diabetes mellitus. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology. 2014;30(3):236-40.

24. Kleiblova P, Dostalova I, Bartlova M, Lacinova Z, Ticha I, Krejci V, et al. Expression of adipokines and estrogen receptors in adipose tissue and placenta of patients with gestational diabetes mellitus. Molecular and cellular endocrinology. 2010;314(1):150-6.
25. van Amerongen R, Mikels A, Nusse R. Alternative wnt signaling is initiated by distinct receptors. Science signaling. 2008;1(35):re9.
26. Peng S, Li J, Miao C, Jia L, Hu Z, Zhao P, et al. Dickkopf-1 secreted by decidual cells promotes trophoblast cell invasion during murine placentation. Reproduction (Cambridge, England). 2008;135(3):367-75.
27. Zhuang B, Luo X, Rao H, Li Q, Liu X, Qi H. [Expression and significance of SATB1 and wnt/beta-catenin signaling molecule in the placenta of preeclampsia]. Zhonghua fu chan ke za zhi. 2015;50(4):283-90.
28. Pavlinkova G, Salbaum JM, Kappen C. Wnt signaling in caudal dysgenesis and diabetic embryopathy. Birth defects research Part A, Clinical and molecular teratology. 2008;82(10):710-9.
29. Zhao Z. TGFbeta and Wnt in cardiac outflow tract defects in offspring of diabetic pregnancies. Birth defects research Part B, Developmental and reproductive toxicology. 2014;101(5):364-70.
30. Priyanka A, Sindhu G, Shyni GL, Preetha Rani MR, Nisha VM, Raghu KG. Bilobalide abates inflammation, insulin resistance and secretion of angiogenic factors induced by hypoxia in 3T3-L1 adipocytes by controlling NF-kappaB and JNK activation. International immunopharmacology. 2017;42:209-17.
31. Lappas M, Yee K, Permezel M, Rice GE. Sulfasalazine and BAY 11-7082 interfere with the nuclear factor-kappa B and I kappa B kinase pathway to regulate the release of proinflammatory cytokines from human adipose tissue and skeletal muscle in vitro. Endocrinology. 2005;146(3):1491-7.
32. Coughlan MT, Permezel M, Georgiou HM, Rice GE. Repression of oxidant-induced nuclear factor-kappaB activity mediates placental cytokine responses in gestational diabetes. The Journal of clinical endocrinology and metabolism. 2004;89(7):3585-94.

Table

Table 1, Clinical parameters of GDM and normal controls

Characteristic	GDM(n=20)	Control(n=20)	*p* value
Age(year)	32.26±4.06	30.4±4.35	0.176
Gestational age(day)	38.63±0.59	38.81±0.71	0.403
BMI index(kg/m2)	22.17±2.1	21.04±1.95	0.082
OGTT 0h	5.32±1.58	4.4±0.36	0.021
OGTT 1h	10.35±2.95	7.37±1.33	0.001
OGTT 2h	9.24±3.73	6.24±1.05	0.003
HbA1c(%)	5.37±0.9	4.9±0.37	0.051
Birth weight(g)	3529±527.2	3306±379.64	0.143

Figures

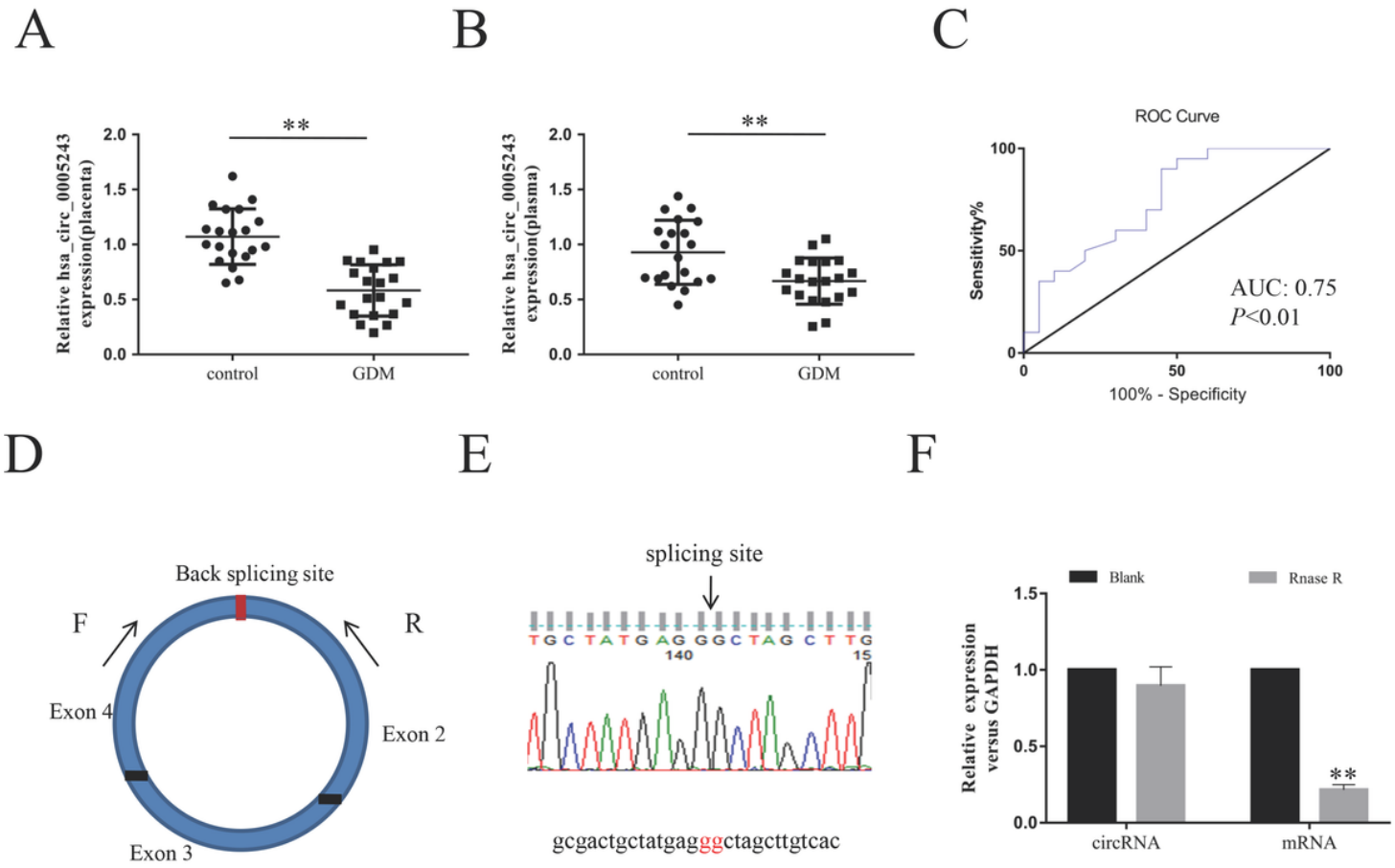

Figure 1

Characterization of hsa_circ_0005243 in GDM A-B, The relative hsa_circ_0005243 expression level was determined in placenta tissues (A) and plasma (B) samples from PE patients compared with normal controls using qRT-PCR. C, ROC curve analysis to evaluate the diagnostic value of hsa_circ_0005824. D, hsa_circ_0005243 was generated from the back-spliced exon 2, 3, 4 of the TMEM184B gene. E, The Sanger sequence was conducted to confirm the splicing site. F, qRT-PCR analysis of hsa_circ_0005243 and the linear TMEM184B mRNA after treatment with or without RNase R in HTR-8/SVneo cells.

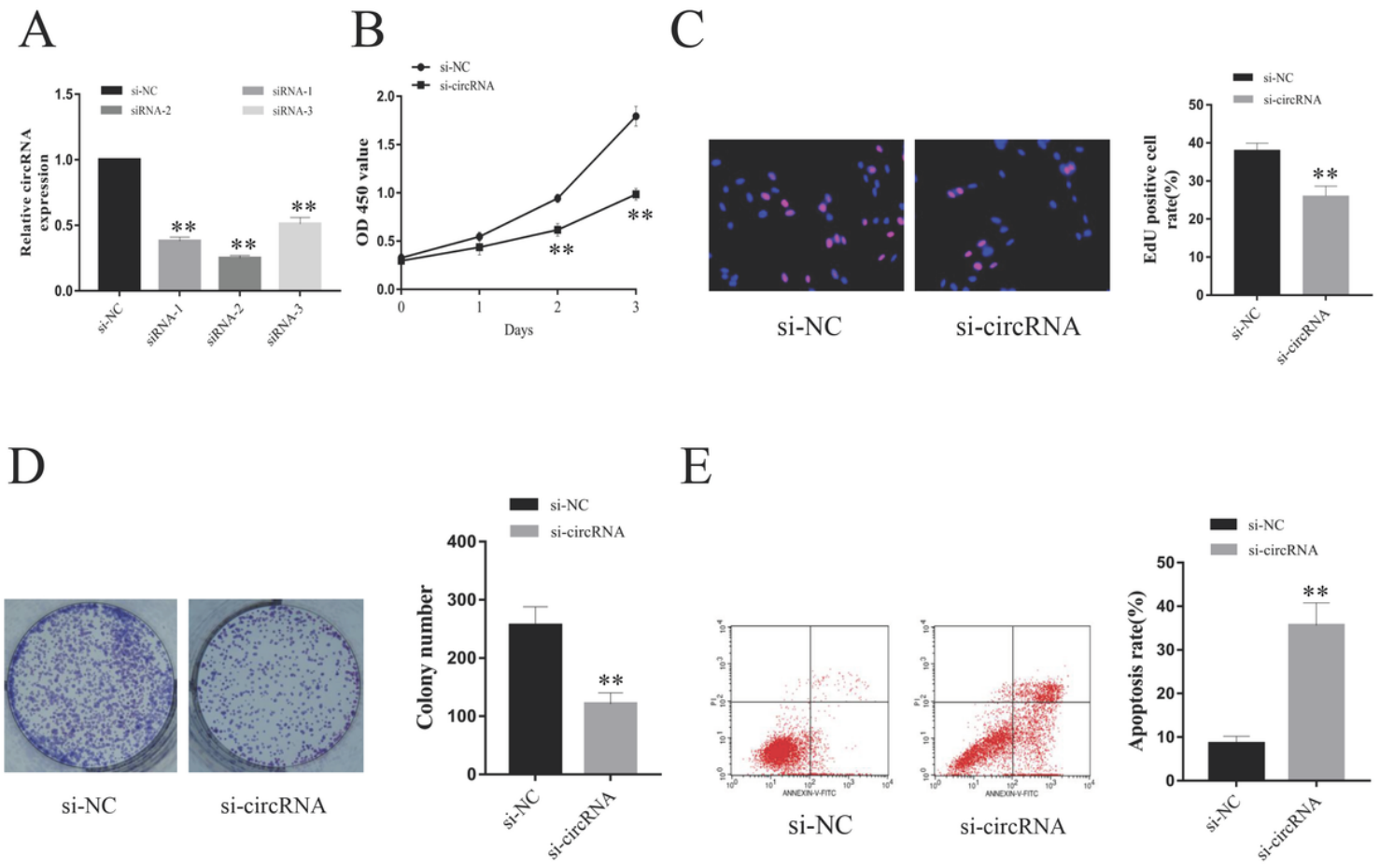

Figure 2

Downregulated hsa_circ_0005243 suppressed trophoblast cell proliferation and promoted apoptosis A, The expression of hsa_circ_0005243 was determined after transfected with siRNAs. B, The CCK8 assay showed that knockdown of hsa_circ_0005243 suppressed cell vitality. C-D, The EdU(C) and colony formation(D) assay showed that depletion of hsa_circ_0005243 inhibited cell proliferative activity. E, Flow cytometry assay showed that downregulaed hsa_circ_0005243 induced cell apoptosis. Data are mean ± SEM, **P□0.01.

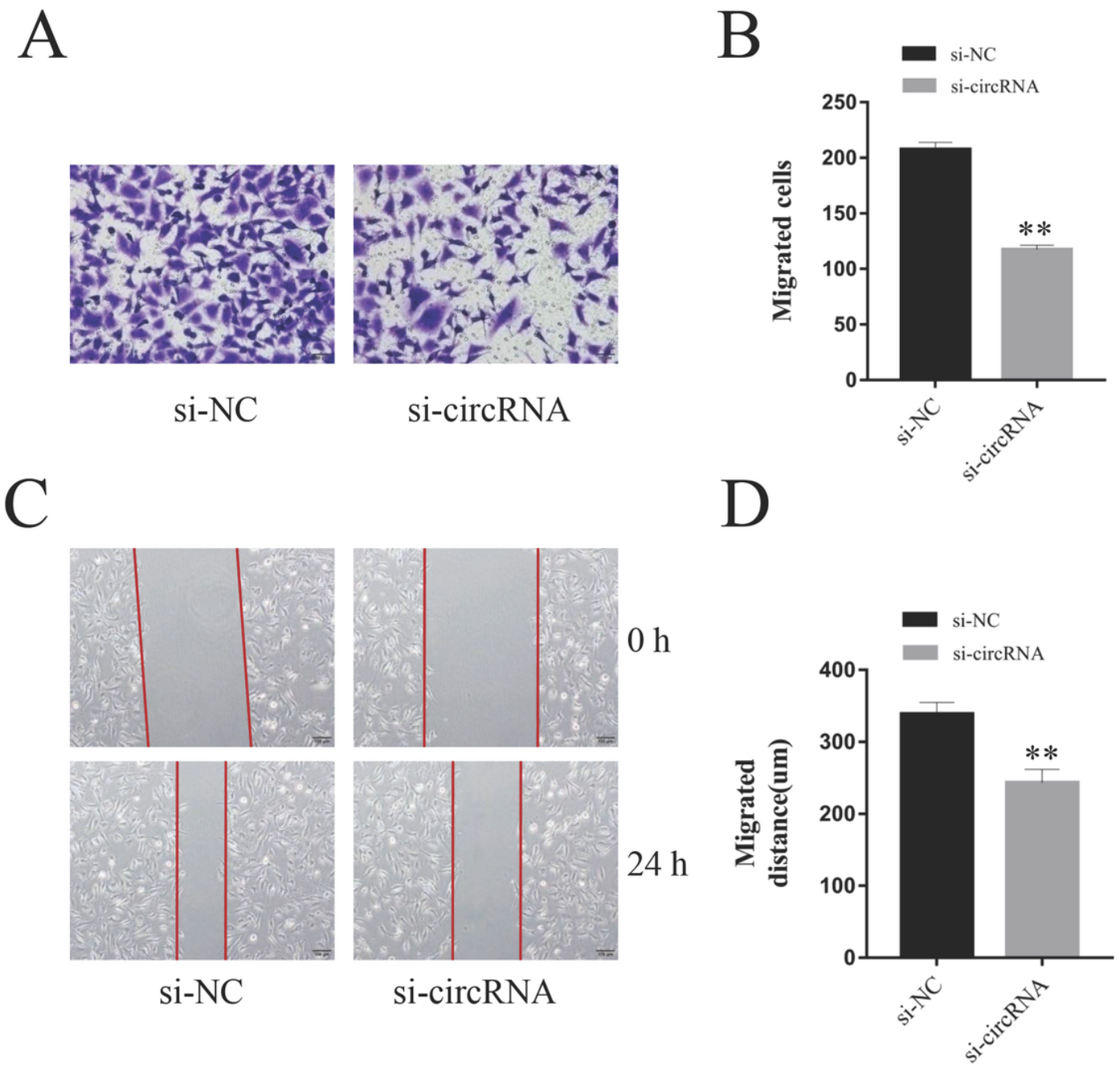

Figure 3

Knockdown of hsa_circ_0005243 inhibited cell migratory ability A, In vitro Transwell migration assay demonstrated that knockdown hsa_circ_0005243 decreased the migratory cell numbers. B, The migrated cells were counted in each group. C, Wound healing assay indicated that the migrated distance were significantly decreased after hsa_circ_0005243 knockdown. D, The migratory distance was calculated 24h after transfection. Data are mean ± SEM, **P□0.01.

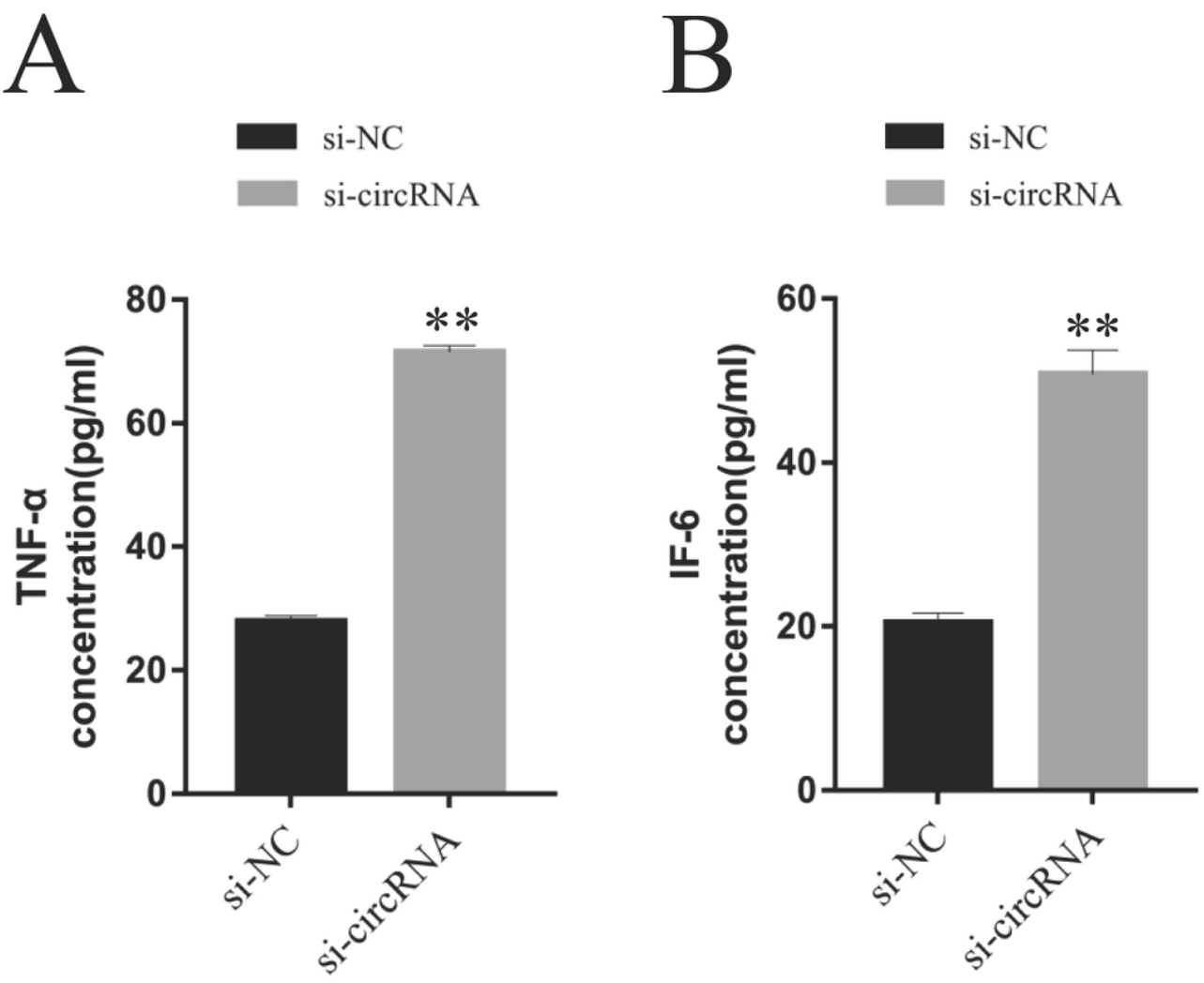

Figure 4

Knockdown of hsa_circ_0005243 promoted the levels of TNF-α and IL-6 A, Knockdown of hsa_circ_0005243 promoted the secretion of TNF-α and IL-6, the concentrations of TNF-α(A) and IL-6(B) were determined by Elisa Kit. Data are mean ± SEM, **P□0.01.

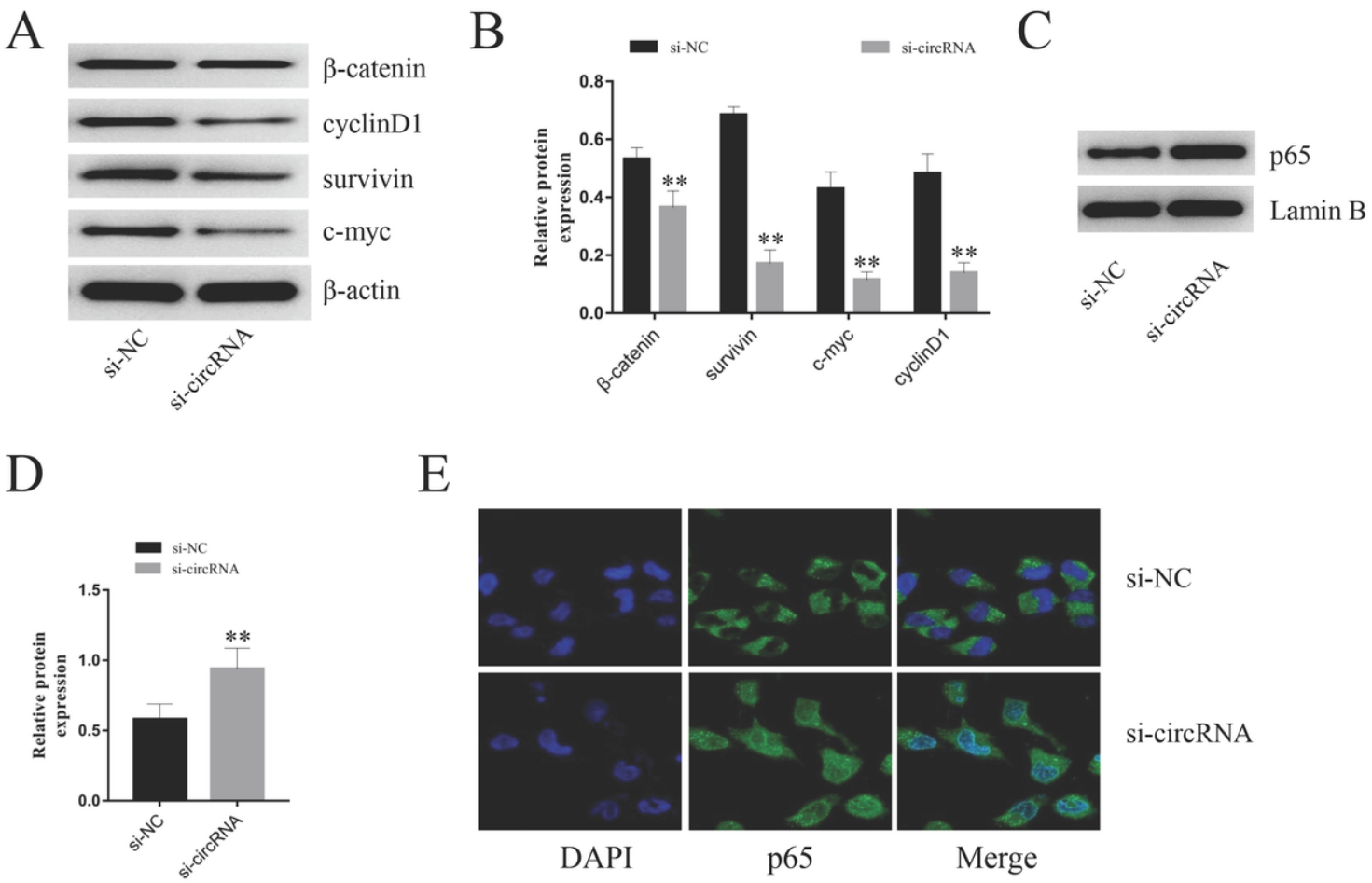

Figure 5

Potential mechanisms of hsa_circ_0005243 regulating trophoblast cell function and inflammatory factor levels A, After knockdown the expression of hsa_circ_0005243 in HTR-8/SVneo cells, the expression of β-catenin, c-myc, cyclinD1 and survivin were measured by western blot. B, Quantitative analysis of protein expression. C-D, The expression of p65 protein in the nuclear was elevated after hsa_circ_0005243 knockdown. E, Confocal immunofluorescence assay showed that hsa_circ_0005243 deletion increased NF-kB p65 subunit nuclear translocation. Data are mean ± SEM, **p□0.01.