

Circular RNA hsa_circ_0081343 promotes trophoblast cell migration and invasion and inhibits trophoblast apoptosis by regulating miR-210-5p/DLX3 axis

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Abstract

Background: Various circular RNAs (circRNAs) are dysregulated in the placenta of fetal growth restriction fetuses, but their role and regulatory mechanisms have not been fully elucidated. Herein, we aimed to elucidate the role of hsa_circ_0081343 in regulating the migration, invasion, and apoptosis in the human extravillous trophoblast HTR-8 cells.

Methods: circRNA and miRNA levels were examined using quantitative real-time PCR (RT-PCR). Overexpression plasmid constructs and siRNA were used to overexpress and knockdown hsa_circ_0081343, respectively. Transwell assay and flow cytometry analysis were performed to evaluate the effect of hsa_circ_0081343 or miR-210-5p on migration, invasion, and apoptosis. Protein levels were analyzed using western blot. Dual luciferase activity assay and anti-AGO2 RNA immunoprecipitation (RIP) assays were performed to identify the relationship between miR-210-5p and hsa_circ_0081343.

Results: Hsa_circ_0081343 expression was significantly downregulated in 37 FGR placental tissues as compared to healthy placental control tissues. Hsa_circ_0081343 overexpression possibly inhibits apoptosis by downregulating the expression of cleaved caspase 3 and caspase 9 and alleviates the migration and invasion of HTR-8 cells by inducing the expression of MMP2 and MMP9. The dual luciferase activity and anti-AGO2 RIP assay results showed that hsa_circ_0081343 binds to miR-210-5p. miR-210-5p overexpression eliminated the effect of hsa_circ_0081343 overexpression in HTR-8 cells. Finally, DLX3 was identified as a direct target of miR-210-5p.

Conclusions: Hsa_circ_0081343 regulates the migration, invasion, and apoptosis of HTR-8 cells via the hsa-miR-210-5p/DLX3 axis. Thus, hsa_circ_0081343 plays a key role in the etiology and pathogenesis of FGR implicating its importance as a novel candidate for targeted FGR therapy.

Background

Fetal growth restriction (FGR) refers to a condition in which the fetus does not maintain its intrauterine potential for growth and development. This condition further results in the fetal birth weight being less than 2500 g after 37 weeks of gestation, which is below the 10th percentile of the normal weight or less than two standard deviations below the mean weight for the same gestational age [1, 2]. It is a relatively common obstetric condition with intercurrent of 5-10 % in all pregnancies and a contribution to 30 % of stillbirths [3-5]. FGR is a multifactorial disorder and the associated placental dysfunction has been linked to the deceleration of fetal growth. This dysfunction can result in a reduction in oxygen and nutrient supply from the mother to the fetus [5]. However, the precise molecular mechanisms of placental development and function remain unclear.

In our previous study, we found that the expression profiles of numerous circular RNAs (circRNAs), a class of single-stranded molecules[6], were dysregulated in the placenta of FGR fetuses [7]. Moreover, we found

that hsa_circ_0000848 is involved in regulating migration, invasion, and apoptosis of trophoblast cells, suggesting that it may play a role in the etiology and pathogenesis of FGR [7]. CircRNAs exist widely in mammalian cells and are involved in the regulation of gene expression at the transcriptional or post-transcriptional levels [8, 9]. Recent studies have found that circRNAs can bind to microRNAs and function as competing endogenous RNA (ceRNA), bind to RNA-binding proteins to regulate gene transcription, or undergo translation as a template [10, 11]. Although circRNAs are regarded as important biological molecules and are associated with disease pathogenesis, the role of circRNAs in FGR is not well documented.

In this study, we further confirmed the expression of differentially expressed circRNAs in FGR placenta identified by circRNA microarray in our previous study; and evaluated the functional role and molecular mechanism of hsa_circ_0081343 (6). The placenta is a multifunctional organ that is essential for fetal development and survival. Trophoblast cells are specialized cells in the placenta that mediate interactions between the fetus and mother at the fetomaternal interface. In the human placenta, there are three major trophoblast subpopulations: the cytotrophoblast, extravillous cytotrophoblast (EVT), and syncytiotrophoblast (ST). HTR-8/SVneo is a mature trophoblast cell model which maintains the basic characteristics of the original cells. It is currently used as a representative of cell models, such as FGR or preeclampsia. Hence, we investigated the effect of hsa_circ_0081343 on the migration, invasion, and apoptosis in the human extravillous trophoblast HTR-8 cells. In addition, we explored the molecular mechanism of hsa_circ_0081343 from the perspective of ceRNA.

Materials And Methods

Tissue samples

Thirty-seven pregnant women with FGR and thirty seven healthy pregnant women enrolled in this study were the same as the participants in our previous study [7]. Their detailed characteristics have been reported in our previous study. Placental tissues were collected in RNA later (Sangon Biotech, Shanghai, China) after delivery and frozen in liquid nitrogen. This study was approved by the Ethics Committee of the Shenzhen Maternity and Child Health Hospital. All patients provided the written informed consent.

RNA isolation and quantitative real-time PCR (RT-PCR) assay

Total RNA from tissue samples or cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quantified using the NanoDrop ND-1000. Reverse transcription (RT) was performed to obtain cDNA using ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA). Random primers were used as the RT primer for detecting circRNA. The RT primer for detecting miRNAs was a special stem-loop primer based on the principle of the stem loop method. Quantitative RT-PCR analysis was

performed using SYBR GREEN qPCR Super Mix (Promega,USA). GAPDH was used as an internal control for circRNAs and mRNA. U6 was used as an internal control for miRNA. All assays were performed in three independent experiments. The data were calculated using the $2^{-\Delta\Delta Ct}$ method to represent the relative expression level of RNAs. The primers used in qRT-PCR are shown below. The forward and reverse primer sequences for hsa_circ_0081343 were AACGAGAACAAGTTTGCTGTG and AGTCGATGCCAGTCATTCTC respectively. The forward and reverse primer sequences for GAPDH were GGGAAACTGTGGCGTGAT and GAGTGGGTGTCGCTGTTGA respectively. The forward primers for miR-210-5p, miR-545-3p, and miR-597-3p were ACACTCCAGCTGGGAGCCCCTGCCACCGCACAC, ACACTCCAGCTGGGTCAGCAAACATTTATTGTG, and ACACTCCAGCTGGGTGGTTCTCTTGTGGCTCA respectively. The universal reverse primer for all the miRNAs was CTCAACTGGTGTCTGTTGA. The forward and reverse primer sequences for U6 (RNA, U6 small nuclear 1) CTCGCTTCGGCAGCACA and AACGCTTCACGAATTTGCGT respectively.

Cell culture and transfection

HTR-8/SVneo cells, purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI1640 (Gibco, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (Gibco), 1 % penicillin/ streptomycin, and 1 % L-glutamine at 37 °C in a humidified incubator with 5 % CO₂. The full length of hsa_circ_0081343 (position: chr7:98985662-98985884; spliced length: 223 nt) was cloned into the pLCD5H-ciR plasmid (Guangzhou Genesee Biotech Co.,Ltd, China) by DNA synthesis *in vitro* to construct the hsa_circ_0081343 overexpression vector (ov-circ_0081343). Empty pLCD5H-ciR plasmid was used as a negative control (NC). Two small interfering RNAs (siRNA) targeting hsa_circ_0081343 and named siRNA-1 (sense sequence: GGAGAAUGACUGGCAUCGATT) and siRNA-2 (sense sequence: GACUGGCAUCGACUGGGCCTT) were designed to include splice junctions to avoid degrading linear mRNA which is later processed into circRNA. The sense sequence of the negative control siRNA (si-NC) was UUCUCCGAACGUGUCACGUTT. The negative control miRNA (miR-NC, UCACAACCUCCUAGAAAGAGUAGA), miR-210-5p mimics (AGCCCCUGCCCACCGCACACUG), miR-210-5p inhibitor (CAGUGUGCGGUGGGCAGGGGCU), and miR-NC inhibitor (UCUACUCUUUCUAGGAGGUUGUGA) were synthesized by GenePharma Co (Shanghai, China).

HTR-8 cells were seeded in six-well plates and transfected using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. To investigate the effect of hsa_circ_0081343, HTR-8 cells were divided into four groups: si-NC group transfected with si-NC; si-circ_0081343 group transfected with siRNA-2; NC group transfected with empty pLCD5H-ciR plasmid; and ov-circ_0081343 group transfected with ov-circ_0081343. To investigate whether miR-210-5p overexpression alleviates the effect of hsa_circ_0081343 overexpression, HTR-8 was divided into three groups and transfected with the following scheme: ov-circ_0081343+ miR-NC, ov-circ_0081343+ miR-210-5p, and NC+ miR-NC.

Transwell assay and flow cytometry analysis

After 24 h of transfection, the Transwell assays were performed to examine the migration and invasion capabilities of the indicated groups using the same method as described in our previous study [7]. The migrated or invaded cells at the bottom of the filter membrane were photographed using a light microscope (Nikon, Tokyo, Japan) at 200X magnification. The cell number was counted in each photograph of five randomly selected fields and the average was used as the number of cells migrated or invaded per field. Each experiment was repeated three times.

After 24 h of transfection, cells were analyzed for apoptosis using flow cytometry. The cell staining was performed using an Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China) and apoptosis levels were analyzed by flow cytometry using the same method as described previously [7].

Western blot analysis

The concentrations of total proteins extracted using RIPA strong buffer (Beyotime, Shanghai, China) were quantified with Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Western blot analysis was carried out using the same method as described in our previous study [7]. The primary antibodies of MMP2, MMP9, caspase 3, and caspase 9 were the same as those used in our previous study [7]. In addition, anti-DLX3 antibody (1:800, ab64953) was also used.

Dual luciferase activity assay

The fragments of wild type linear hsa_circ_0081343 and DLX3 3' untranslated regions (UTR) were amplified and cloned into the dual-luciferase miRNA target expression vector GP-mirGLO (Promega, Madison, WI, USA) and named as wild type circ_0081343 and wild type 3' UTR respectively. The binding sequence of miR-210-5p on wild type circ_0081343 and wild type 3' UTR plasmids were mutated by site-directed mutagenesis using one-step overlap extension PCR, and named as mutant circ_0081343 and mutant 3'UTR respectively. HTR-8 cells were plated on 24-well plates and co-transfected with 100 ng of indicated recombinant plasmids, and 50 nM of miR-210-5p mimic or miR-NC. After 48 h of transfection, firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Three independent experiments were performed.

Anti-AGO2 RNA immunoprecipitation (RIP) assay

RIP were carried out according to the instructions of Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA). Briefly, approximately 1×10^7 cells were harvested post transfection with miR-210-5p mimic or miR-NC. Cell pellets were lysed in polysome lysis buffer supplemented with protease inhibitor cocktail and RNase inhibitor. Partial cell lysate (20 μ L), termed as input, was collected for use as a positive control. Subsequently, 100 μ L cell lysates were incubated with magnetic bead IgG or Ago2 antibody complex at 4 °C overnight. Next day, the complex was washed according to the manufacturer's instructions. RNA was extracted and purified. The level of hsa_circ_0081343 in purified RNA was detected by qRT-PCR.

Statistical analysis

Statistical analyses were performed using the SPSS 18.0 software and GraphPad Prism version 7.0. Data are expressed as mean \pm standard deviation based on three independent experiments. Differences between two groups were analyzed using unpaired *t*-tests when the data were normally distributed or non-parametric *t*-tests when the data were not normally distributed. Differences between more than two groups were analyzed using one-way ANOVA. *P* values < 0.05 were considered statistically significant.

Results

Hsa_circ_0081343 expression is significantly lower in FGR placenta as compared to healthy placenta.

In our previous study [7], the results of circRNA microarray had shown that hsa_circ_0081343 expression levels were lower in FGR placental tissues than in healthy placental tissues. Its sequence reported in the circBase database is shown in Fig. 1A. In this study, we further investigated its expression profile in 37 pairs clinical samples. The results of qRT-PCR showed that hsa_circ_0081343 expression levels are significantly downregulated in the 37 FGR placental tissues as compared to the 37 healthy placental tissues (Fig. 1B). The sequencing analysis of the DNA fragment of qRT-PCR verified the presence of the splice junction of hsa_circ_0081343 as shown in circBase (Fig. 1C).

Hsa_circ_0081343 promotes migration and invasion; and inhibits apoptosis in HTR-8 cells.

Considering the dysregulated expression of hsa_circ_0081343 in FGR placental tissues, we further explored its function by overexpressing or silencing hsa_circ_0081343 in HTR-8 cells. Our results show that hsa_circ_0081343 expression levels were increased in HTR-8 cells upon transfection with ov-circ_0081343 as compared to those transfected with the empty vector or NC group (Fig. 2A). Further, siRNA-2 demonstrated the highest efficacy in silencing the expression of hsa_circ_0081343 at 100 nM as compared to the si-NC group (Fig. 2B). In subsequent assays, the transfection of the siRNA-2 at 100 nM

was termed as the si-circ_0081343 group. Overall, these results demonstrate that hsa_circ_0081343 was successfully overexpressed or silenced in HTR-8 cells.

Subsequently, we examined the effect of hsa_circ_0081343 overexpression or knockdown on the migration, invasion, and apoptosis of HTR-8 cells. Our immunoblotting studies revealed that both MMP2 and MMP9 levels are increased upon overexpressing circ_0081343 in HTR-8 cells (ov-circ_0081343). However, their levels are down-regulated when circ_0081343 expression is silenced (si-circ_0081343) as compared to the negative control siRNA (si-NC) (Fig. 2C). Furthermore, cleaved caspase 3 and cleaved caspase 9 expression levels were found to be decreased in the ov-circ_0081343 group as compared to the NC group, but were increased in the si-circ_0081343 group as compared to the si-NC group (Fig. 2C). The results of flow cytometry analysis revealed that the percentage of early apoptotic cells was lower in the ov-circ_0081343 group than in the NC group, but was higher in the si-circ_0081343 group than in the si-NC group (Fig. 2D). The results of transwell assay further revealed that the number of migrated or invaded cells per field was more in ov-circ_0081343 group as compared to the NC group, but less in si-circ_0081343 group as compared to si-NC group (Fig. 2E). Overall, these results indicate that hsa_circ_0081343 overexpression promotes cell migration and invasion and inhibits apoptosis in HTR-8 cells. However, silencing hsa_circ_0081343 shows opposite effects.

Hsa_circ_0081343 interacts with miR-210-5p.

It has been reported that circRNAs function as ceRNA. Thus, we analyzed the presence of miRNA response elements in hsa_circ_0081343 sequence. Based on the sequence complementary matching score of hsa_circ_0081343 and miRNAs, top three miRNA candidates; namely, miR-545-3p, miR-210-5p, and miR-597-3p were chosen for the further studies. The results of qRT-PCR show that miR-210-5p and miR-597-3p expression levels are significantly upregulated in 37 FGR placental tissues than in 37 healthy placental tissues (Fig.3A). A review of literature revealed that miR-210-5p is a hypoxia-regulated miRNA [12]. Moreover, hypoxia is frequently associated with FGR [13]. Consequently, we focused on the relationship between miR-210-5p and hsa_circ_0081343. The binding site of miR-210-5p on hsa_circ_0081343 is shown in Fig. 3B. Additionally, we identified their relationship using a dual luciferase activity assay and an anti-AGO2 RIP assay. The results of the dual luciferase activity assay disclosed that the relative luciferase activity of miR-210-5p mimic plus wild type circ_0081343 co-transfection group is evidently decreased as compared to the miR-NC plus wild type circ_0081343 co-transfection group. However the relative luciferase activity is unaltered between miR-210-5p mimic plus mutant circ_0081343 co-transfection group and miR-NC plus mutant circ_0081343 co-transfection group (Fig. 3C). These results suggest that miR-210-5p can bind to the linear sequence of hsa_circ_0081343. The results of the anti-AGO2 RIP assay indicate that miR-210-5p mimic transfection increases the hsa_circ_0081343 level in RNAs enriched by AGO2 (Fig. 3D), indicating that miR-210-5p evidently interacts with hsa_circ_0081343 in HTR-8 cells.

DLX3 is a direct target of miR-210-5p.

To explore the mechanism underlying the hsa_circ_0081343/miR-210-5p axis, we predicted the targets of miR-210-5p. Among all the predicted targets, DLX3 is known to be dysregulated in idiopathic FGR placentae [14]. Thus, we explored whether DLX3 is a target of miR-210-5p and whether the hsa_circ_0081343/miR-210-5p axis regulates DLX3. The binding site of miR-210-5p on the 3' UTR of DLX3 is shown in Fig. 3E. The result of the dual luciferase activity assay shows that the relative luciferase activity of miR-210-5p mimic + wild type 3' UTR co-transfection group is evidently decreased as compared to the miR-NC + wild type 3' UTR co-transfection group. However, the relative luciferase activity remains unchanged between miR-210-5p mimic + mutant 3' UTR co-transfection group and miR-NC + mutant 3' UTR co-transfection group (Fig. 3F). These results suggest that miR-210-5p can directly interact with the 3' UTR of DLX3. The results of western blotting studies show that DLX3 protein levels are down-regulated in HTR-8 cells upon overexpression of miR-210-5p as compared to the miR-NC group (Fig. 3G). Additionally, the DLX3 levels were found to be increased in HTR-8 cells upon transfection with the miR-210-5p inhibitor as compared to the miR-NC inhibitor group (Fig. 3G). Furthermore, the DLX3 protein levels were found to be augmented in the ov-circ_0081343 group as compared to the NC group, but reduced in the si-circ_0081343 group as compared to the si-NC group (Fig. 3H).

miR-210-5p overexpression rescues the effect of hsa_circ_0081343 overexpression in HTR-8 cells.

To further confirm whether hsa_circ_0081343 plays a role of a competing endogenous RNA for miR-210-5p, we evaluated whether miR-210-5p overexpression can rescue the effect of hsa_circ_0081343 overexpression in HTR-8 cells. Our qRT-PCR results revealed that the expression of hsa_circ_0081343 is higher in the ov-circ_0081343 + miR-NC and ov-circ_0081343 + miR-210-5p groups as compared to the NC + miR-NC group (Fig. 4A). In addition, the expression of miR-210-5p is higher in the ov-circ_0081343 + miR-210-5p groups as compared to both the ov-circ_0081343 + miR-NC or NC + miR-NC groups (Fig. 4A). These results suggest that miR-210-5p and/ or hsa_circ_0081343 were successfully overexpressed in HTR-8 cells. The results of western blotting studies showed that the expression of MMP2 and MMP9 were decreased in ov-circ_0081343 + miR-210-5p groups as compared to the ov-circ_0081343 + miR-NC group (Fig. 4B). Cleaved caspase 3 and cleaved caspase 9 expression levels were on the other hand, found to be increased in ov-circ_0081343 + miR-210-5p groups as compared to that in the ov-circ_0081343 + miR-NC group (Fig. 4B). Our flow cytometry data reveals that the percentage of early apoptotic cells was higher in the ov-circ_0081343 + miR-210-5p groups than in the ov-circ_0081343 + miR-NC group (Fig. 4C). The results of transwell assay reveal that the number of migrated or invaded cells number per field were less in the ov-circ_0081343 + miR-210-5p group as compared to ov-circ_0081343 + miR-NC group (Fig. 4D). These results indicate that miR-210-5p overexpression alleviates the effect of hsa_circ_0081343 overexpression on the migration and invasion potential as well as the apoptosis of HTR-8 cells.

Inhibition of miR-210-5p suppresses apoptosis and promotes migration and invasion of HTR-8 cells.

To evaluate the potential role of miR-210-5p in FGR, we analyzed the effect of miR-NC inhibitor and miR-210-5p inhibitor on apoptosis, migration, and invasion of HTR-8 cells. The results of western blotting studies showed that both MMP2 and MMP9 expression levels are upregulated in the miR-210-5p inhibitor groups as compared to the miR-NC inhibitor group (Fig. 5A). In contrast, the cleaved caspase 3 and cleaved caspase 9 expression levels are down-regulated in the miR-210-5p inhibitor groups than in the miR-NC inhibitor group (Fig. 5A). Our results of flow cytometry analysis indicate that the percentage of cells undergoing early apoptosis is lower in the miR-210-5p inhibitor group as compared to that in the miR-NC inhibitor group (Fig. 5B). The results of the transwell assay reveal that migration or invasion of cells per field is evidently higher in the miR-210-5p inhibitor group than in the miR-NC inhibitor group (Fig. 5C). Overall, these results suggest that the inhibition of miR-210-5p suppresses the apoptosis and promotes the migration and invasion of HTR-8 cells.

Discussion

Emerging reports indicate that circRNAs regulate important functions and are closely correlated with diverse human diseases [15, 16]. In our previous study, we had presented the differential circRNA expression profiles in the placenta of FGR fetuses. We also demonstrated that hsa_circ_0000848 promotes migration and invasion and inhibits apoptosis by sponging hsa-miR-6768-5p in HTR-8 cells. As a number of dysregulated circRNAs have been identified, it is imperative to further explore the role of other dysregulated circRNAs in the disease context. In the present study, we found that the expression of hsa_circ_0081343 is significantly downregulated in FGR placental tissues as compared to healthy controls. Sequence analysis of the DNA fragment of qRT-PCR proved that hsa_circ_0081343 is a genuine circRNA. These results suggest that hsa_circ_0081343 may play a vital role in the pathogenesis of FGR. Hence, we further analyzed its role in HTR-8 cells, which is a cell line derived from human extravillous trophoblasts.

Previous studies have shown that the apoptosis level in placental trophoblasts is lower in healthy pregnancy than in FGR, and that it may contribute to the placental pathology characteristic of FGR [17, 18]. Accordingly, in this study, we initially investigated the effect of hsa_circ_0081343 on the apoptosis of HTR-8 cells. We found that hsa_circ_0081343 overexpression decreases while its knockdown increases the apoptosis level of HTR-8 cells. Moreover, we found that hsa_circ_0081343 overexpression could enhance and hsa_circ_0081343 knockdown could weaken the migration and invasion capabilities of HTR-8 cells. Extravillous trophoblast cells migrate to and invade the uterine wall, leading to remodeling of the maternal vasculature [19-21]. The maintenance of migration and invasion capabilities of extravillous trophoblast cells is important for the normal development of the fetus. Our results suggest that hsa_circ_0081343 may play a protective role in the pathogenesis of FGR, and it may serve as a potential

therapeutic target for FGR. To the best of our knowledge, we believe that ours is the first report that has studied the functional role of hsa_circ_0081343.

CircRNAs bind to microRNAs and function as “miRNA sponges”. As a result, the suppression effect of miRNAs on the target genes is relieved that result in increased target gene expression. This regulatory mechanism is termed as the competitive endogenous RNA (ceRNA) mechanism [22, 23]. To explore the molecular mechanism of hsa_circ_0081343 from the perspective of ceRNA, we investigated the relationship between hsa_circ_0081343 and miR-210-5p. All our results demonstrate that hsa_circ_0081343 can directly bind to miR-210-5p in HTR-8 cells. Our results show that miR-210-5p overexpression relieves the effect of hsa_circ_0081343 overexpression in HTR-8 cells. These results suggest that hsa_circ_0081343 may function as a competing endogenous RNA by sponging miR-210-5p. This hypothesis is further supported by the promotion of apoptosis and suppression of the migration and invasion capabilities of HTR-8 cells upon transfection with miR-210-5p inhibitor. We believe that this study is the first to report the potential role of miR-210-5p in the pathogenesis of FGR. Dysregulated expression of miR-210-5p under hypoxic conditions further supports our study, as hypoxia is frequently associated with FGR [12, 13].

According to the ceRNA mechanism, circRNA/miRNA axis plays a crucial role in biological phenomena by affecting the translation of target mRNA. Therefore, it is vital to identify the target of hsa_circ_0081343/miR-210-5p to fully elucidate the molecular mechanisms. DLX3 is a member of the homeodomain transcription factor and vertebrate-free distant homeobox gene family. Its function and regulatory mechanism in the placenta have been reported [24, 25]. DLX3 is required for the generation of a functional chorioallantoic placenta and targeted deletion of the mouse DLX3 gene results in embryonic death between days 9.5 and 10 due to placental defects [26]. Chui et al. have reported that DLX3 is expressed in proliferating and differentiating cells of the human placenta; thus, DLX3 may play an important role in normal placental development [24]. Our present study demonstrates that miR-210-5p can bind to the 3' UTR of DLX3 and that it can decrease the protein level of DLX3. The results indicate that DLX3 is a direct target of miR-210-5p. Moreover, hsa_circ_0081343 overexpression increases the protein level of DLX3. Therefore, we hypothesize that DLX3 is the target of hsa_circ_0081343/miR-210-5p axis, and that hsa_circ_0081343 promotes trophoblast cell migration and invasion and inhibits trophoblast apoptosis by regulating the miR-210-5p/DLX3 axis.

While our study conclusively proves that hsa_circ_0081343 regulates the pathogenesis of FGR, it also has certain limitations. First, numerous miRNA response elements were found to be present on the sequence of hsa_circ_0081343. Hence, the existence of other miRNAs as potential downstream target cannot be

ruled out. In addition, it is necessary to construct an FGR animal model to study the effects of hsa_circ_0081343 *in vivo*.

In conclusion, our study demonstrates that hsa_circ_0081343 expression level is significantly down-regulated in FGR placental tissues. The *in vitro* assays have shown that hsa_circ_0081343 promotes migration and invasion; and inhibits apoptosis via the hsa-miR-210-5p/DLX3 axis in HTR-8 cells. Based on the importance of the role of EVT in FGR [21, 27], we hypothesize that hsa_circ_0081343 plays a vital role in the etiology and pathogenesis of FGR. Furthermore, our results implicate the potential of hsa_circ_0081343 as novel candidate for targeted FGR therapy.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

HW, JX, and JL contributed to the conception and design of the study. HW wrote the first version of the manuscript. HW, CL, and XW performed the experiments. JZ and ZX constructed the figures. YL and BL were involved in the clinical data analysis. All authors contributed to revising the manuscript, and read and approved the submitted

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Availability of data and materials

The data from this study are available in this published article.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Shenzhen Maternity and Child Health Hospital (No. SFYLS[2020]047) at 6th Aug. 2020 as per the guidelines established in the Declaration of Helsinki. All patients provided the written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

A
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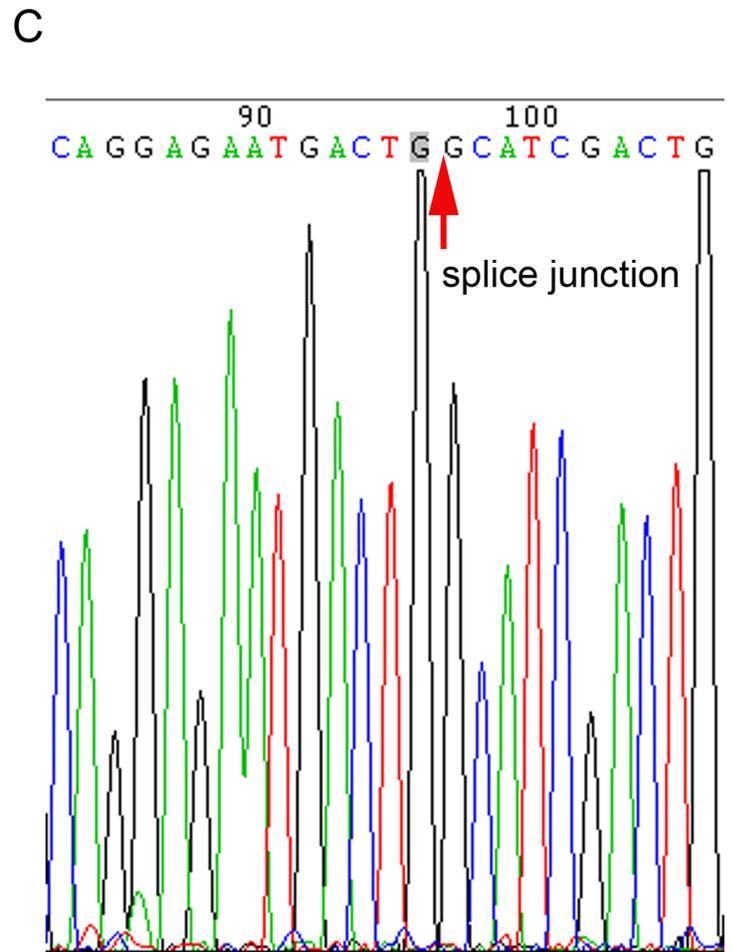
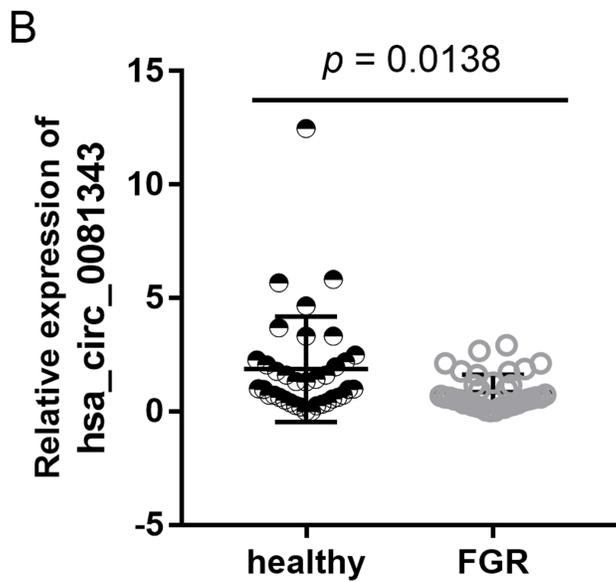


Figure 1

Hsa_circ_0081343 expression is down-regulated in FGR placenta as compared to healthy controls. A: hsa_circ_0081343 sequence reported in circBase database. B: hsa_circ_0081343 expression level in FGR and healthy placental tissues (n=37). C: the splice junction of hsa_circ_0081343 shown in circBase verified by sequencing the DNA fragment of qRT-PCR.

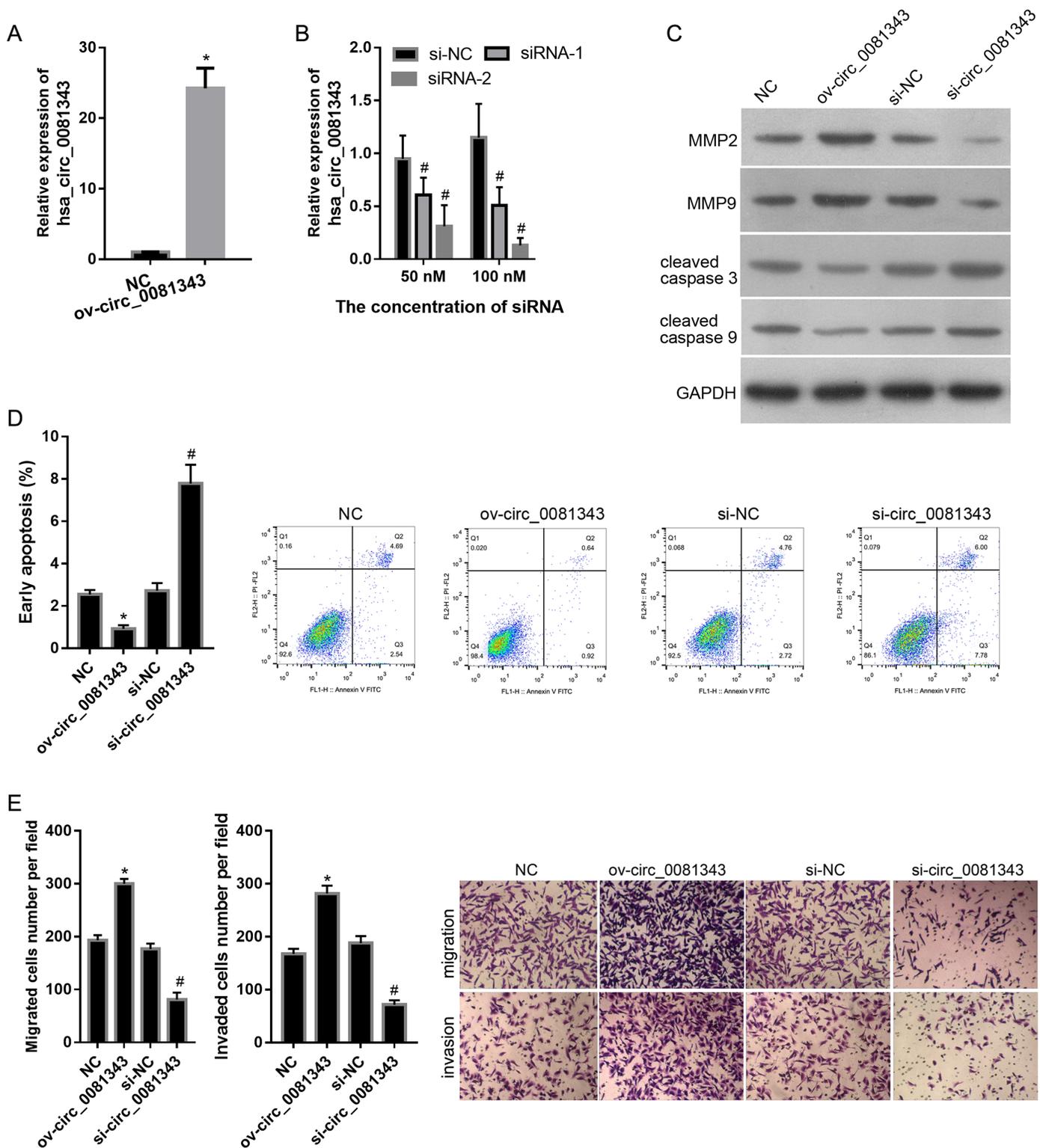


Figure 2

Effect of hsa_circ_0081343 overexpression or knockdown on the migration, invasion, and apoptosis in HTR-8 cells. A: hsa_circ_0081343 was overexpressed in HTR-8 cells by transfecting with hsa_circ_0081343 overexpression plasmid (ov-circ_0081343). Transfection with empty pLCD5H-ciR plasmid was used as a negative control (NC). B: hsa_circ_0081343 was silenced in HTR-8 cells by transfecting with two small interfering RNA (siRNA-1 and -2) targeting hsa_circ_0081343. Transfection

with negative control siRNA (si-NC) was used as control. C-E: The effect of hsa_circ_0081343 overexpression or silencing on migration and apoptosis related proteins (C), apoptosis (D), and the migration and invasion potential of HTR-8 cells (E). * $p < 0.05$, when compared to NC; # $p < 0.05$, when compared to si-NC.

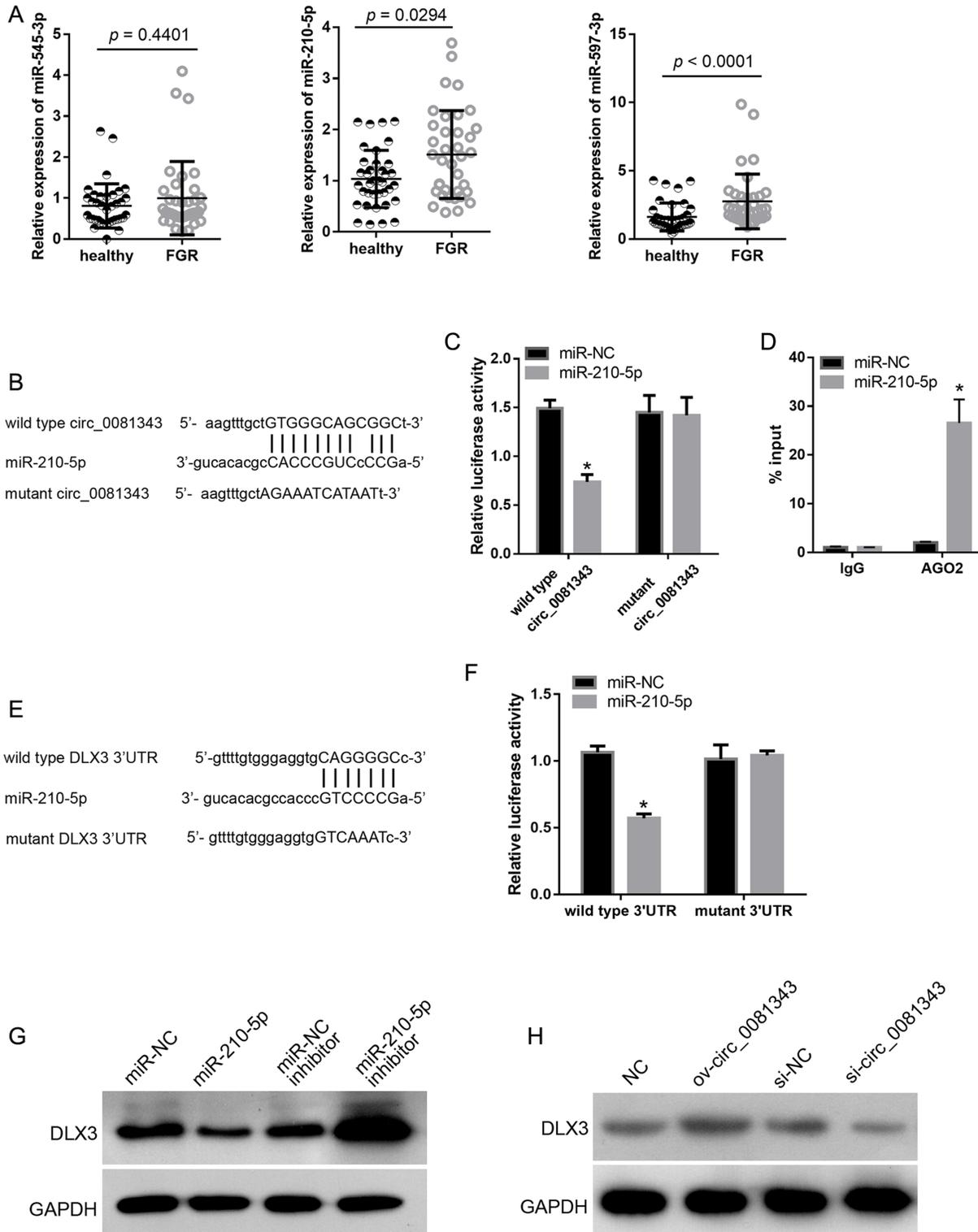


Figure 3

Hsa_circ_0081343 physically interacts with miR-210-5p and miR-210-5p directly targets DLX3 transcript. A: Expression level of miR-545-3p, miR-210-5p, and miR-597-3p in FGR and healthy placental tissues (n=37). B: The binding site of miR-210-5p on hsa_circ_0081343 sequence, and the mutant scheme of luciferase reporter vector containing linear hsa_circ_0081343 with mutated miR-210-5p binding site. C: miR-210-5p mimic co-transfection decreases the relative luciferase activity of luciferase reporter vector containing wild type linear hsa_circ_0081343 (wild type circ_0081343). D: miR-210-5p mimic transfection increases hsa_circ_0081343 level in RNAs enriched by AGO2. E: The binding site of miR-210-5p on 3' UTR of DLX3, and the mutant scheme of luciferase reporter vector containing 3' UTR of DLX3 with mutated miR-210-5p binding site. F: miR-210-5p mimic co-transfection decreases the relative luciferase activity of luciferase reporter vector containing wild type 3' UTR of DLX3 (wild type 3' UTR). G-H: Effect of the overexpression or knockdown of miR-210-5p or hsa_circ_0081343 on DLX3 protein level. *p< 0.05, when compared to miR-NC.

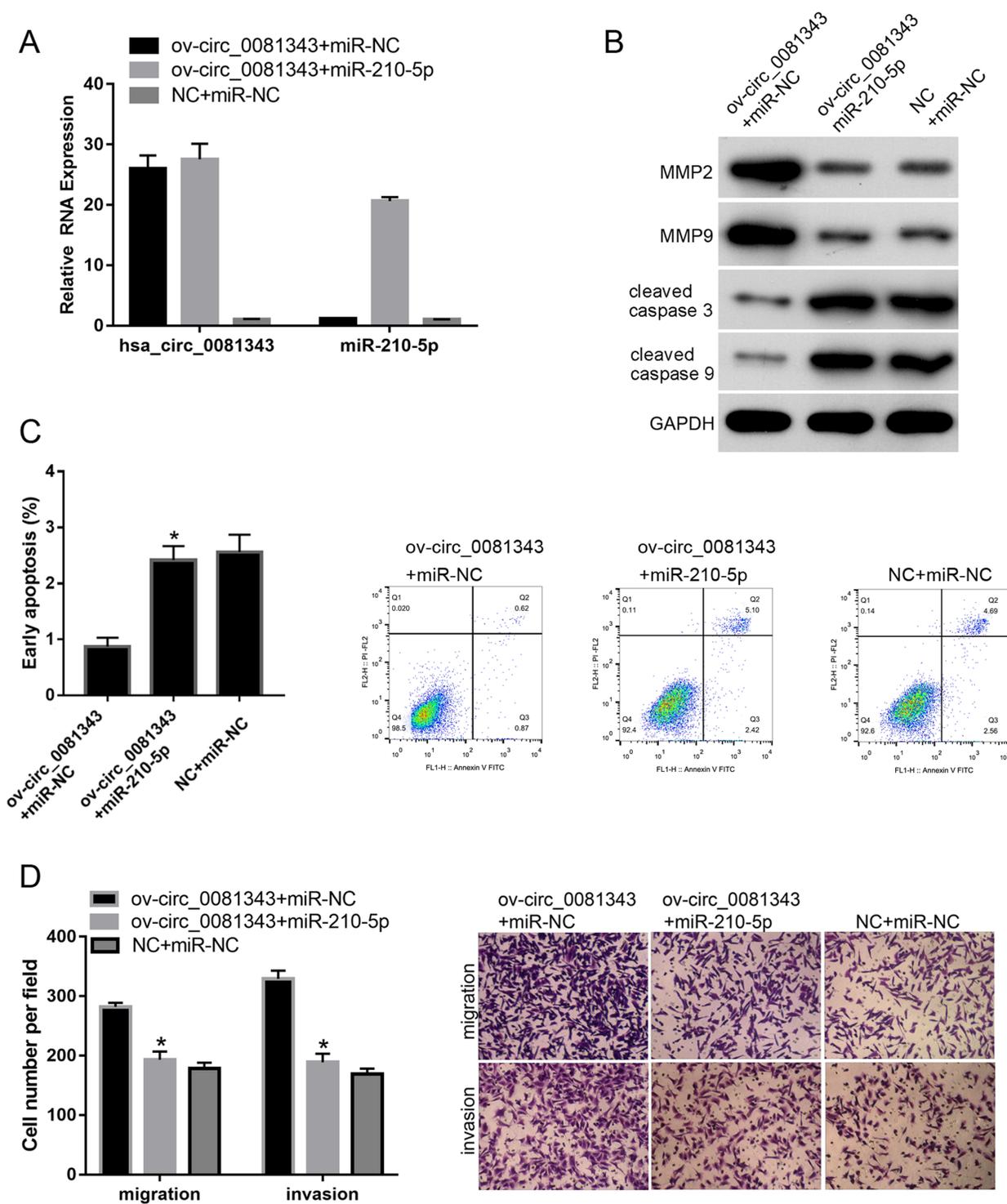


Figure 4

miR-210-5p overexpression alleviates the effect of hsa_circ_0081343 overexpression in HTR-8 cells. HTR-8 cells were transfected with ov-circ_0081343 + miR-NC, ov-circ_0081343 + miR-210-5p, or NC + miR-NC. The overexpression of miR-210-5p and hsa_circ_0081343 in the three groups was verified by qRT-PCR. The protein expression of MMP2, MMP9, cleaved caspase 3 and cleaved caspase 9 (B), early apoptosis level (C), and the migration and invasion potential (D) of HTR-8 cells under indicated co-transfection

conditions were evaluated by western blotting, flow cytometry analysis and transwell assays, respectively. * $p < 0.05$, when compared to ov-circ_0081343 + miR-NC group.

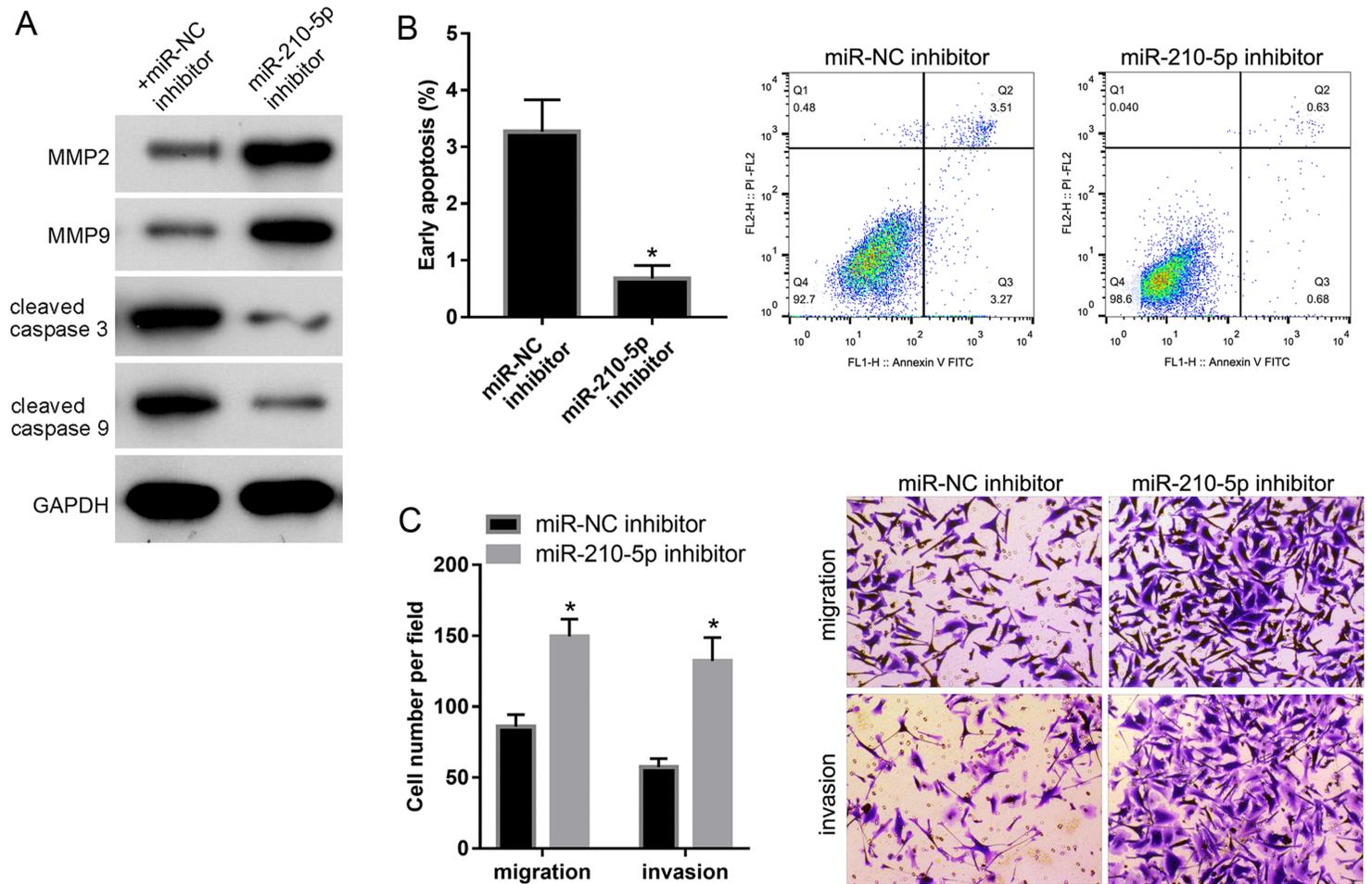


Figure 5

miR-210-5p inhibitor suppresses apoptosis and stimulates migration and invasion of HTR-8 cells. HTR-8 cells were transfected with miR-NC, ov-circ_0081343 + miR-210-5p, and NC + miR-NC. The overexpression of miR-210-5p and hsa_circ_0081343 in three groups was verified by qRT-PCR. The protein level expression of MMP2, MMP9, cleaved caspase 3 and cleaved caspase 9 (B), early apoptosis level (C), and the capabilities of migration and invasion (D) were evaluated by western blot, flow cytometry analysis and transwell assays respectively. * $p < 0.05$, when compared to ov-circ_0081343 + miR-NC group; * $p < 0.05$, when compared to miR-NC inhibitor.