

MiR-204-5p promoted maternally expressed gene 3 (*MEG3*) through *SP1*-mediated *DNMT1* pathway in trophoblast cells in recurrent miscarriage

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online)

Adv Clin Exp Med. 2025;34(11):1841–1849

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Funding sources

The study was financially supported by the Tianjin Key Medical Discipline (Specialty) Construction Project (grant No. TJYXZDXK-043A).

Conflict of interest

None declared

Received on July 27, 2024

Reviewed on August 21, 2024

Accepted on November 13, 2024

Published online on April 8, 2025

Cite as

Liu S, Wang Y, Guo Y, Xu X, Gao Y, Cheng L. MiR-204-5p promoted maternally expressed gene 3 (*MEG3*) through *SP1*-mediated *DNMT1* pathway in trophoblast cells in recurrent miscarriage. Adv Clin Exp Med. 2025;34(11):1841–1849. doi:10.17219/acem/195925

DOI

10.17219/acem/195925

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Abstract

Background. Recurrent miscarriage (RM) affects 1–2% of couples. Maternally expressed gene 3 (*MEG3*) is aberrantly expressed in RM patients.

Objectives. To investigate a novel regulatory mechanism, we examined the *miR-204-5p*/Specificity protein 1 (*SP1*)/DNA methyltransferase 1 (*DNMT1*)/*MEG3* axis in the trophoblast cell line HTR-8/SVneo.

Materials and methods. Human trophoblast cell line HTR-8/SVneo was used and cells were transfected with siRNA targeting *SP1*, *miR-204-5p* mimics, pcDNA3.1-*DNMT1*, or their negative controls (NCs). The methylation inhibitor, 5-azadC, was used to treat the cells transfected with pcDNA3.1-*SP1*. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) method was used to examine the relative RNA levels of *SP1*, *DNMT1* and *MEG3*. Western blot assay was performed to measure the protein levels of *SP1* and *DNMT1*. The dual-luciferase reporter gene assay was used to validate the *miR-204-5p* bindings to *SP1*. Functional assays were utilized to assess cell apoptosis, colony formation, migration, and invasion.

Results. *SP1* knockdown inhibited *DNMT1* and increased *MEG3* expression. The expression of *MEG3* was enhanced by methylation inhibition through 5-azadC, but *SP1* upregulation reversed this effect. *SP1* knockdown increased apoptosis and decreased migration and invasion, which was reversed by *DNMT1* overexpression. *SP1* was targeted and inhibited by *miR-204-5p*. *miR-204-5p* also inhibited *DNMT1*, and enhanced the expression of *MEG3*. *miR-204-5p* inhibited cell proliferation, migration and invasion, and promoted apoptosis. Overexpression of *SP1* partially reversed these effects by activating *DNMT1* and inhibiting *MEG3*.

Conclusions. *miR-204-5p* promoted *MEG3* expression in trophoblast cells via *SP1*-mediated *DNMT1* inhibition, leading to reduced cell migration, proliferation and invasion, as well as increased apoptosis. This study reveals a novel regulatory axis in trophoblast cells, providing insights into potential regulatory mechanisms in RM.

Key words: *MEG3*, *SP1*, *DNMT1*, recurrent miscarriage, trophoblast cells

Background

Recurrent miscarriage (RM) affects 1–2% of women attempting to conceive, imposing both emotional and clinical burden.¹ Despite extensive research, the underlying causes of RM remain incompletely understood, often involving a complex interplay of genetic, epigenetic and environmental factors, including endocrine disruptors such as mancozeb.^{2–7}

Recent advances in molecular biology have revealed the crucial role of extravillous trophoblast cells in maintaining a successful pregnancy by mediating implantation and placental development.⁸ Previous research has also shown that severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) affects female reproductive health by targeting trophoblasts.⁹ Dysfunction of trophoblasts is a well-established contributor to pregnancy loss.

Previously, specificity protein 1 (*SP1*), also known as a transcriptional factor *SP1*, was found to be reduced in the chorionic villus tissues in the placentas from RM patients, and the downregulation of *SP1* by upregulation of *miR-4497* could induce the apoptosis of trophoblast cells, which is associated with RM occurrences.^{10,11} *SP1* regulates genes essential for cellular growth and differentiation, while *DNMT1* is correlated with DNA methylation patterns, and is of significance in maintaining genomic stability and gene expression.^{12,13} Similar to *miR-4497*, *miR-204-5p* was also involved in studies focusing on unexplained recurrent spontaneous abortion.¹⁴ *miR-204-5p* plays a role in placental development and function. For instance, *miR-204-5p* has been revealed to modulate the trophoblast cell proliferation and invasion, which are essential for proper placental development.¹⁵ Dysregulation of *miR-204-5p* was revealed to be associated with pregnancy-related disorders, including preeclampsia (PE).^{16,17} *miR-204-5p* expression was downregulated in PE and the upregulation of *miR-204-5p* was related to the decreased migration of trophoblast cells.¹⁷

Long non-coding RNA (lncRNA), such as maternally expressed gene 3 (*MEG3*), is a known regulator in tumor suppression and regulation of cell growth and death.¹⁸ Studies have unveiled that *MEG3* expression is downregulated in blood samples, embryonic villus and chorionic villus tissues from RM patients, indicating its potential role in the pathogenesis of RM.^{19–21} Further, *MEG3* was validated to stimulate the invasion and proliferation of trophoblast cells.^{20,21} Similarly, in PE, downregulation of *MEG3* was discovered to increase apoptosis of trophoblast cells and inhibit cell invasion.²² Previously, *DNMT1* was discovered to enhance methylation of *MEG3* promoter and decrease *MEG3* expression, thereby mediating the disease progression in breast cancer,²³ anemia,²⁴ diabetic retinopathy,²⁵ etc. However, such interaction between *DNMT1* and *MEG3* has not been validated in trophoblast cells.

In this study, we hypothesized that *miR-204-5p/SP1/DNMT1* axis plays a pivotal role in modulating trophoblast cell invasion, proliferation and apoptosis, processes essential for placental development and successful pregnancy, through *MEG3* modulation via epigenetic modifications.

Objectives

Our research aims to elucidate a novel modulatory mechanism of *miR-204-5p* in trophoblast cells via *SP1/DNMT1/MEG3* pathway and its impact on cell invasion, proliferation and apoptosis.

Materials and methods

Culture of cells

HTR-8/SVneo cells, derived from human first-trimester chorionic villi, were obtained from Procell (Wuhan, China). The cells were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, USA) at 37°C in a humidified atmosphere containing 5% CO₂.

Cell transfection and treatment

Cells were reseeded in 6-well plates at a density of 5×10^5 cells per well and incubated overnight. On the 2nd day, cells were transfected with specific siRNAs, overexpressed plasmids, miRNA mimics, or their negative controls (NC) (GenePharma, Tianjin, China), using Lipofectamine 2000 kit (Invitrogen, Waltham, USA). Following the protocols, cells were transfected with 50 nM siRNA targeting *SP1* (si-*SP1*) or negative control siRNA (si-NC) (GenePharma). For miRNA overexpression, cells were transfected with 50 nM *miR-204-5p* mimics (*miR-204-5p*), mimics negative control (NC mimics). For overexpression of *SP1* or *DNMT1*, cells were collected for transfection, with 2 µg of pcDNA3.1 empty vector (Ctrl), pcDNA3.1-*SP1* (*SP1*) or pcDNA3.1-*DNMT1* (*DNMT1*) (GenePharma). In addition, the methylation inhibitor, 5-azadC (1 µM; Aladdin, Tianjin, China) was used to treat the cells for methylation inhibition.

RNA isolation and reverse transcription quantitative polymerase chain reaction method

RNA was extracted from all cell groups using TRIzol Kit (Beyotime, Beijing, China). Complementary DNA (cDNA) was synthesized using a PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan). The reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay was performed using the SYBR Taq II Kit (TaKaRa) on Step OnePlus RT-PCR (Applied Biosystems, Waltham, USA). The specific primers used for RT-qPCR are listed below:

miR-204-5p:

Forward(F) 5'-TGCGGTTCCCTTTGTCATCCTATG-3';
Reverse (R) 5'-GTCGTATCCAGTGCAGGGTCCGAG-GTCACTGGATACGACAGGCATAG-3'.

U6:

F 5'-CTCGCTTCGGCAGCACA-3',
R 5'-AACGCTTCACGAATTTGCGT-3'.

SPI:

F 5'-CTGGTCCCATCATCATCCGG-3',
R 5'-TGTTTGGGCTTGTTGGTTCT-3'.

DNMT1:

F 5'-TGGTGAAGACGCCAGTGGA-3',
R 5'-CGTGGCTGTGGAGGGATTTTCG-3'.

MEG3:

F 5'-CGGCTGAAGAACTGCGGATGG-3',
R 5'-CGTGGCTGTGGAGGGATTTTCG-3'.

GAPDH:

F 5'-GCACCGTCAAGGCTGAGAAC-3',
R 5'-TGGTGAAGACGCCAGTGGA-3'.

The expression levels were analyzed in Microsoft Excel 2013 software (Microsoft Corp., Redmond, USA) using the $2^{-\Delta\Delta C_t}$ method, with U6 (for *miR-204-5p*) or GAPDH (for the rest) as the house keeping reference.

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer with phenylmethylsulfonyl fluoride (PMSF) (Beyotime). Proteins were separated by using the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) methods, and transferred to polyvinylidene difluoride (PVDF) (Millipore, St. Louis, USA). The membranes were then blocked in 5% skim milk for 1 h at room temperature and then incubated overnight at 4°C with the following primary antibodies: SPI (1:1000, #5931; Cell Signaling Technology (CST), Danvers, USA), DNMT1 (1:1000, #5032; CST) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10,000, AB2000; Abways, Tianjin, China). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:20,000, AB0101; Abways) for 1 h at room temperature. Immunoblots were then visualized using an enhanced chemiluminescence (ECL) kit, and images were captured using the SCG-W3000 imaging system (ServiceBio, Wuhan, China).

Dual-luciferase reporter assays

The binding sites were predicted using StarBase database (<https://rnasysu.com/encori>). The pmirGLO-*SPI*-3'UTR-wild type (*SPI*-UTR-wt) and mutant type (*SPI*-UTR-mt) were constructed using the pmirGLO vector (Promega, Madison, USA). Cells were co-transfected with 50 ng of *SPI*-UTR-wt or *SPI*-UTR-mt and 50 nM *miR-204-5p* mimics/inhibitors, or respective controls (GenePharma). After 48 h, luciferase activity was measured on a multi-functional microplate after using the Dual-Luciferase Reporter Assay kit (Beyotime).

Flow cytometry for apoptosis analysis

Apoptosis was assessed using Cell Apoptosis Kit (Bioss, Beijing, China). Transfected cells were collected, washed with precooled phosphate-buffered saline (PBS) and suspended using binding buffer. As per the manufacturer's protocol, cells were stained in Annexin V-FITC and propidium iodide (PI) for 15 min at room temperature in dark. Cell apoptosis was analyzed with flow cytometry (BD FAC-SCanto II; BD Biosciences, Franklin Lakes, USA).

Colony formation assay

Cells after transfection were collected and reseeded in 6-well plates with a density of 600 cells per well. Cells were observed every day till the formation of colonies. Cells were fixed using 4% paraformaldehyde for 20 min and then stained with 0.1% crystal violet for 30 min in dark. Colonies with more than 50 cells were counted in each group.

Transwell assays

The transwell chambers with 8-μm pores were obtained from Jet Biotech (Guangzhou, China). For migration assays, cells were washed in PBS twice and seeded in the upper chamber with serum-free medium at a density of 5×10^4 per well, while culture medium with 15% FBS was added in the lower chamber. For invasion assays, the upper chambers were pre-coated with Matrigel (Corning Company, Corning, USA). Cells were observed under an optical microscope (model Ts2; Nikon Corp., Tokyo, Japan) every 6 h. After 36 h, cells that had migrated or invaded to the lower surface were washed using PBS twice gently and fixed using 4% paraformaldehyde for 20 min, and then stained in crystal violet for 30 min. Cells were then observed and counted under an optical microscope (model Ts2; Nikon Corp.) from 5 different fields.

Statistical analyses

Due to the small sample size, non-normal distribution was assumed. The statistical significance of difference was determined with the nonparametric Kruskal–Wallis test with Dunn's post hoc test, and Mann–Whitney U test for comparison within 2 groups using GraphPad v. 9.0 (GraphPad Software, San Diego, USA). Bonferroni correction was performed for multiple testing. The results from statistical analysis in this study were included in Supplementary Tables 1 and 2. The figures were generated based on statistical data and cell images from experiments mentioned above using GraphPad. Data in replicates are presented in figures as scatter dots with the median and range lines. The p-values less than 0.05 were considered statistically significant.

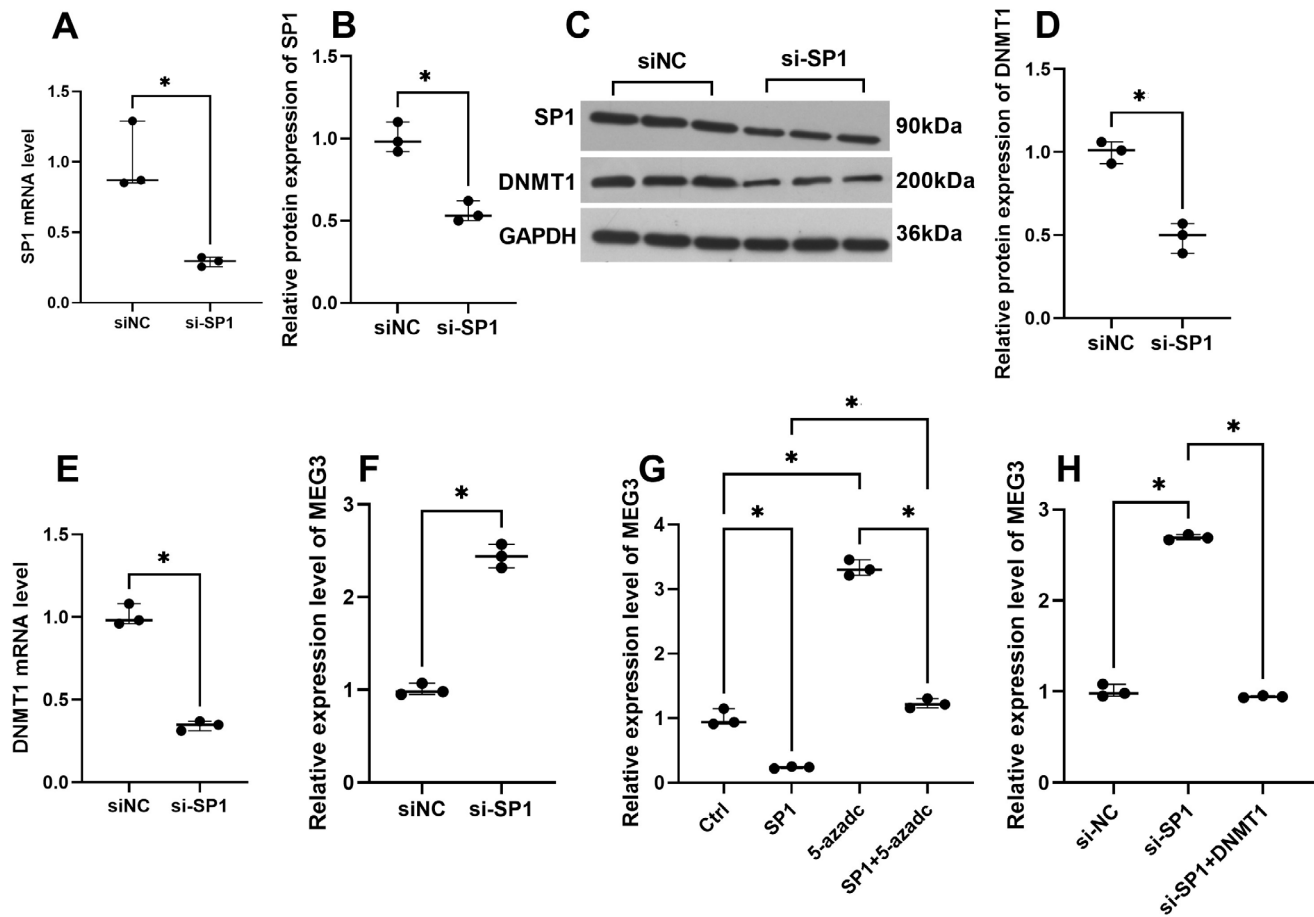


Fig. 1. Downregulation of *SP1* promoted *MEG3* through methylation inhibition in trophoblast cells. A. HTR-8/SVneo cells were transfected with siRNA that targeted *SP1* (si-*SP1*) and its control (si-NC). The reverse transcription quantitative polymerase chain reaction (RT-qPCR) method was used to determine the mRNA levels of *SP1* with reference to GAPDH; B–D. Western blot method was used to analyze the protein expression levels of *SP1* and *DNMT1*; E, F. The RNA levels of *DNMT1* and *MEG3* were analyzed by applying the RT-qPCR method; G. Cells were transfected with pcDNA3.1 empty vector (Ctrl) and plasmid overexpressing *SP1* (*SP1*), and then the transfected cells were further divided by treatment of methylation inhibitor, 5-azadC. *MEG3* expression was measured; H. Cells were transfected with si-*SP1* alone, or co-transfected with pcDNA3.1 plasmid overexpressing *DNMT1* (*DNMT1*) and si-*SP1*. *MEG3* expression was measured. Data in replicates are presented in figures as scatter dots with the median and range lines. Kruskal–Wallis test with Dunn's post hoc and Bonferroni correction was performed for multiple comparisons, and Mann–Whitney U test for comparison within 2 groups

* $p < 0.050$. *SP1* – specificity protein 1; *DNMT1* – DNA methyltransferase 1; si-*SP1* – siRNA targeting *SP1*; si-NC – siRNA negative control; *MEG3* – maternally expressed gene 3.

Results

Downregulation of *SP1* promoted *MEG3* through methylation inhibition in trophoblast cells

SP1 mRNA and protein levels were inhibited in HTR-SV/neo cells transfected with si-*SP1* (Fig. 1A–C). Furthermore, *SP1* knockdown in cells inhibited both *DNMT1* mRNA and protein levels (Fig. 1C–E). Additionally, *SP1* knockdown upregulated *MEG3* expression, an effect that was reversed by *DNMT1* overexpression (Fig. 1F,H). A methylation inhibitor, 5-azadC, promoted the *MEG3* expression and overexpression of *SP1* could partly reverse the effect of 5-azadC (Fig. 1G). The findings suggest that *SP1* knockdown enhanced *MEG3* by *DNMT1*-mediated methylation in trophoblast cells.

SP1 knockdown decreased trophoblast cell invasion, proliferation and migration and increased apoptosis through *DNMT1* modulation

Downregulation of *SP1* induced cell apoptosis in HTR-SV/neo cells but the overexpression of *DNMT1* could counteract this (Fig. 2A). Colony formation results showed that the number of colonies was decreased by *SP1* knockdown but was restored by the overexpression of *DNMT1* (Fig. 2B). Cell migration and invasion were also inhibited by *SP1* knockdown and restored by *DNMT1* overexpression (Fig. 2C,D).

miR-204-5p inhibited *SP1/DNMT1* by targeting *SP1* in HTR-8/SVneo cells

Based on predictions from the Starbase database, miR-204-5p is predicted to bind to *SP1*, and the putative binding

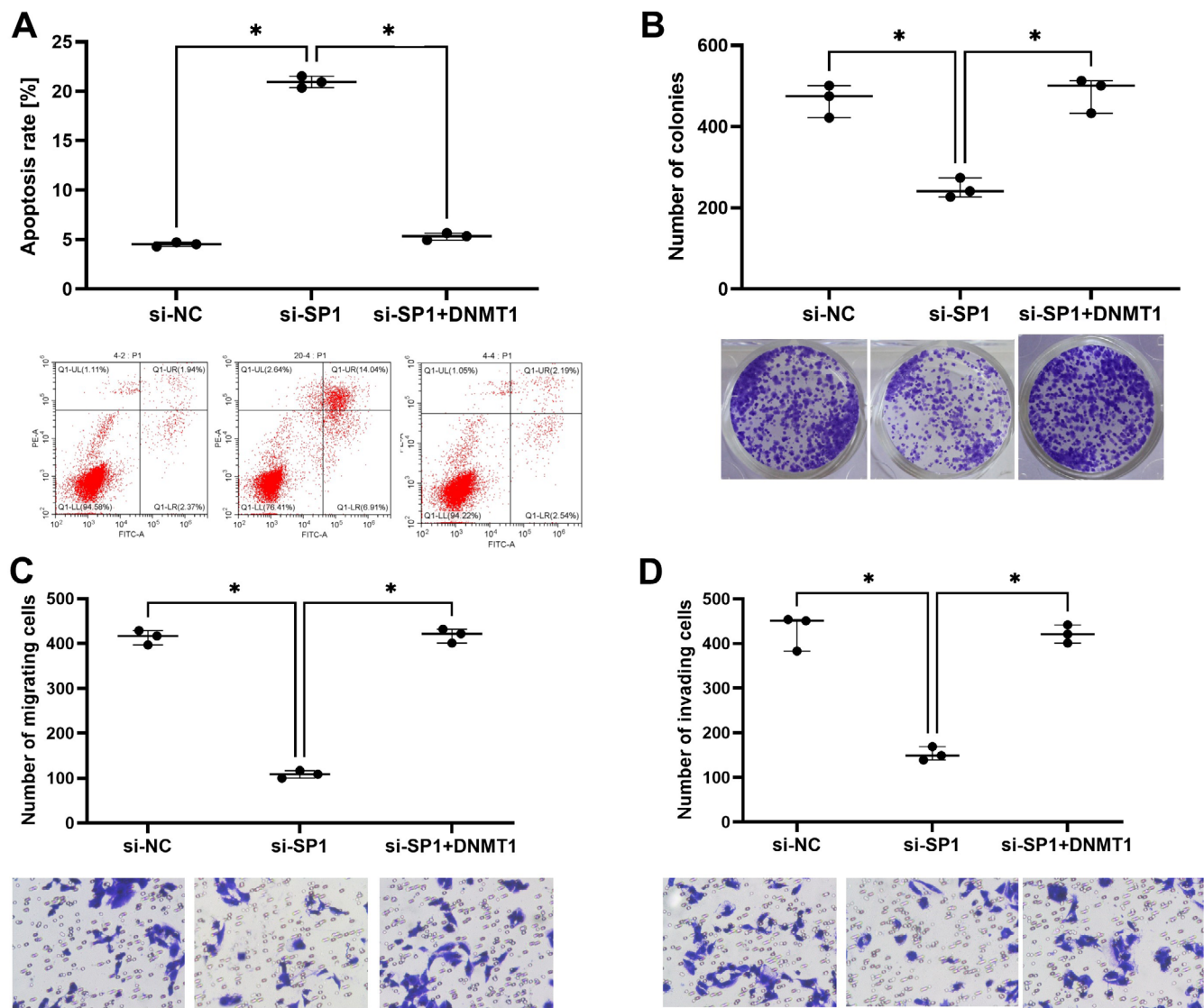


Fig. 2. *SP1* knockdown decreased cell invasion and migration and increased apoptosis through *DNMT1* modulation in trophoblast cells. HTR-8/SVneo cells were transfected with si-*SP1* alone, or together with pcDNA3.1-*DNMT1*. **A.** Flow cytometry method was used to analyze cellular apoptosis; **B.** Colony formation assays were performed to analyze cell proliferation; **C,D.** Transwell assays were performed to analyze the cell capability of invasion and migration in each group. Data in replicates are presented in figures as scatter dots with the median line and range lines. Kruskal–Wallis test with Dunn's post hoc and Bonferroni correction was performed for multiple comparisons, and Mann–Whitney U test for comparison within 2 groups

* $p < 0.050$. *SP1* – specificity protein 1; *DNMT1* – DNA methyltransferase 1; si-*SP1* – siRNA targeting *SP1*; si-NC – siRNA negative control; *MEG3* – maternally expressed gene 3.

sites are illustrated in Fig. 3A. The cells were transfected with *miR-204-5p* mimics (*miR-204-5p*) or its negative control (NC mimics), *miR-204-5p* inhibitor or its negative control (inhibitor NC), and the pmirGLO plasmids containing the *SP1*-UTR-wt/mt. Dual luciferase reporter gene assays were performed to analyze the relative luciferase activity, and results showed that the relative luciferase activity was significantly reduced in cells co-transfected with *miR-204-5p* and *SP1*-UTR-wt and increased in cells transfected with *miR-204-5p* inhibitor and *SP1*-UTR-wt (Fig. 3B). In addition, *miR-204-5p* upregulation inhibited *SP1* and *DNMT1* in mRNA and protein levels (Fig. 3C–G). Conversely, overexpression of *miR-204-5p* upregulated *MEG3* expression, an effect that was reversed

by overexpression of *SP1* (Fig. 3H). The inhibitory effect of *miR-204-5p* on *SP1*/*DNMT1* could be reversed by overexpression of *SP1* (Fig. 3I–K).

miR-204-5p regulated the invasion and migration of trophoblast cells by targeting *SP1*

Overexpression of *miR-204-5p* inhibited cell proliferation, invasion and migration but the inhibitory effect was recovered by *SP1* overexpression in HTR-8/SVneo cells (Fig. 4A,C,D). On the contrary, *miR-204-5p* overexpression enhanced apoptosis rates and *SP1* overexpression reversed this (Fig. 4B).

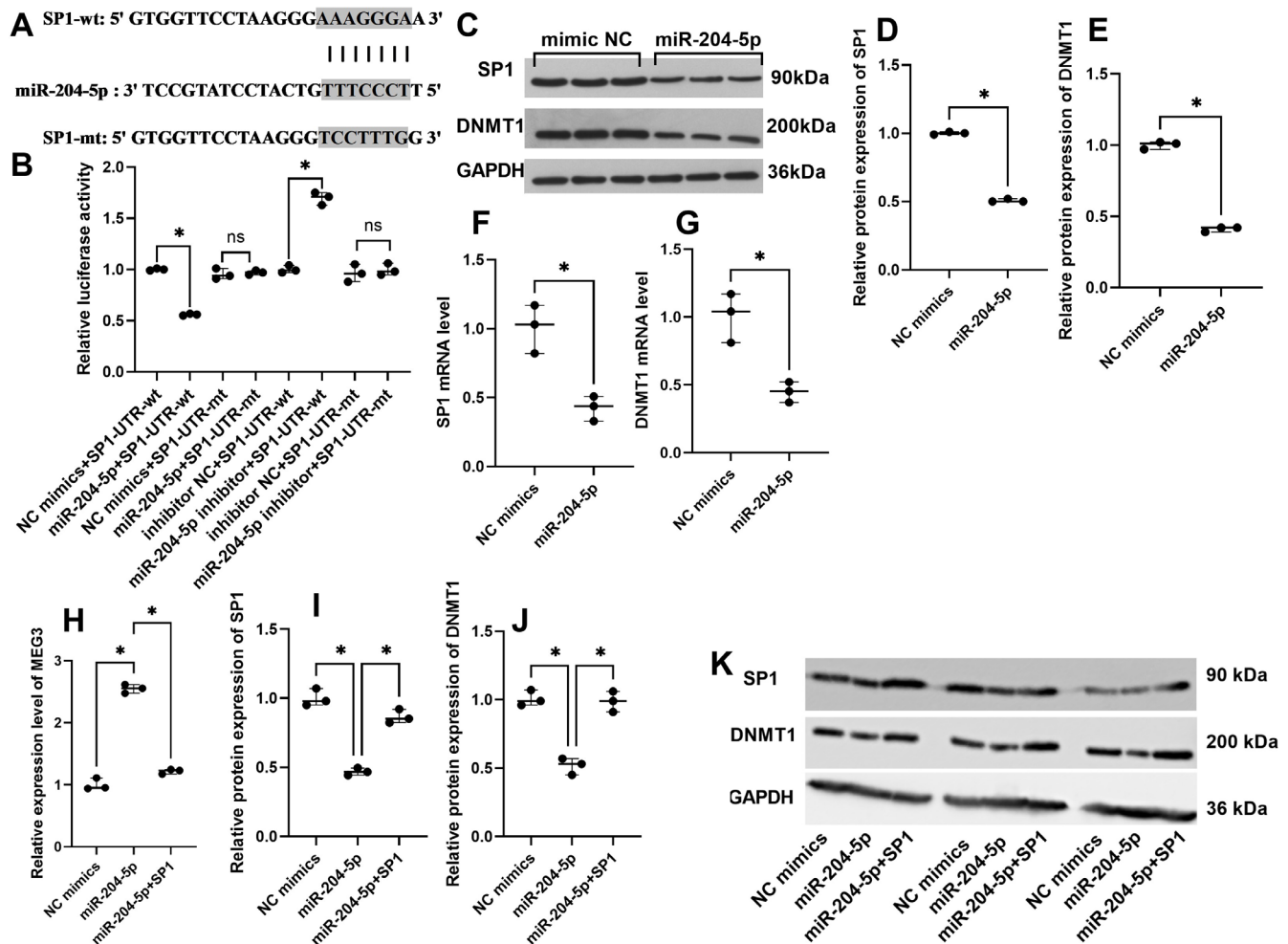


Fig. 3. *miR-204-5p* inhibited *SP1/DNMT1* by targeting *SP1* in HTR-8/SVneo cells. **A.** The binding sites between *SP1* and *miR-204-5p* were predicted using StarBase database; **B.** pmirGLO-*SP1*-3'UTR-wild type (*SP1*-UTR-wt) and mutant type (*SP1*-UTR-mt) were constructed using pmirGLO vector. Then, cells were transfected with *miR-204-5p* mimics (*miR-204-5p*) or its negative control (NC mimics), or *miR-204-5p* inhibitor or its negative control (inhibitor NC). Dual luciferase reporter gene assays were performed to analyze the relative luciferase activity; **C–E.** The protein expression levels of *SP1* and *DNMT1* were measured in cells with *miR-204-5p* upregulation; **F–G.** The mRNA levels of *SP1* and *DNMT1*; **H.** Cells were transfected with *miR-204-5p* mimic, or co-transfected with *miR-204-5p* mimic and pcDNA3.1-*SP1*. *MEG3* expression was measured; **I–K.** Western blot analysis for protein levels of *SP1* and *DNMT1*. Data in replicates are presented in figures as scatter dots with the median and range lines. Kruskal–Wallis test with Dunn's post hoc and Bonferroni correction was performed for multiple comparisons, and Mann–Whitney U test for comparison within 2 groups

* $p < 0.050$, ns – not significant. *SP1* – specificity protein 1; *DNMT1* – DNA methyltransferase 1; si-*SP1* – siRNA targeting *SP1*; si-NC – siRNA negative control; *MEG3* – maternally expressed gene 3.

Discussion

This study advances the understanding of epigenetic regulation in RM. By elucidating the *miR-204-5p/SP1/DNMT1/MEG3* axis, it provides insights into the molecular pathways contributing to trophoblast cell function and pregnancy maintenance. Specifically, it identifies *miR-204-5p* as a critical regulator of *SP1* and *DNMT1*, influencing *MEG3* expression and thereby affecting trophoblast cell behavior. These findings suggest potential therapeutic targets for managing RM, focusing on the modulation of *miR-204-5p* and its downstream effectors. In this study, we demonstrated that *SP1* knockdown leads to a significant reduction in *DNMT1* levels, which in turn upregulates *MEG3* expression. This finding is consistent with existing literature that

indicates *SP1* enhances *DNMT1* transcription.²⁶ It was unveiled previously that protein expression levels of *SP1* and *DNMT1* were significantly low in human villous specimen from RM patients, in comparison with those from the patients with induced abortion or sporadic abortion; in vitro, knockdown of *SP1* or *DNMT1* could decrease the invasion and migration in trophoblast cells.²⁷ In our study, the increase in apoptosis and decrease in migration and invasion following *SP1* knockdown in trophoblast cells also reveal critical roles of *SP1* in maintaining cell function. Further, these effects were reversed by *DNMT1* overexpression, highlighting *DNMT1*'s role in cell proliferation and motility, and indicating that *SP1* modulated the cell phenotypes via *DNMT1*. Such interactions have been well documented in cancer cells, where *SP1* increases the activity of *DNMT1*,

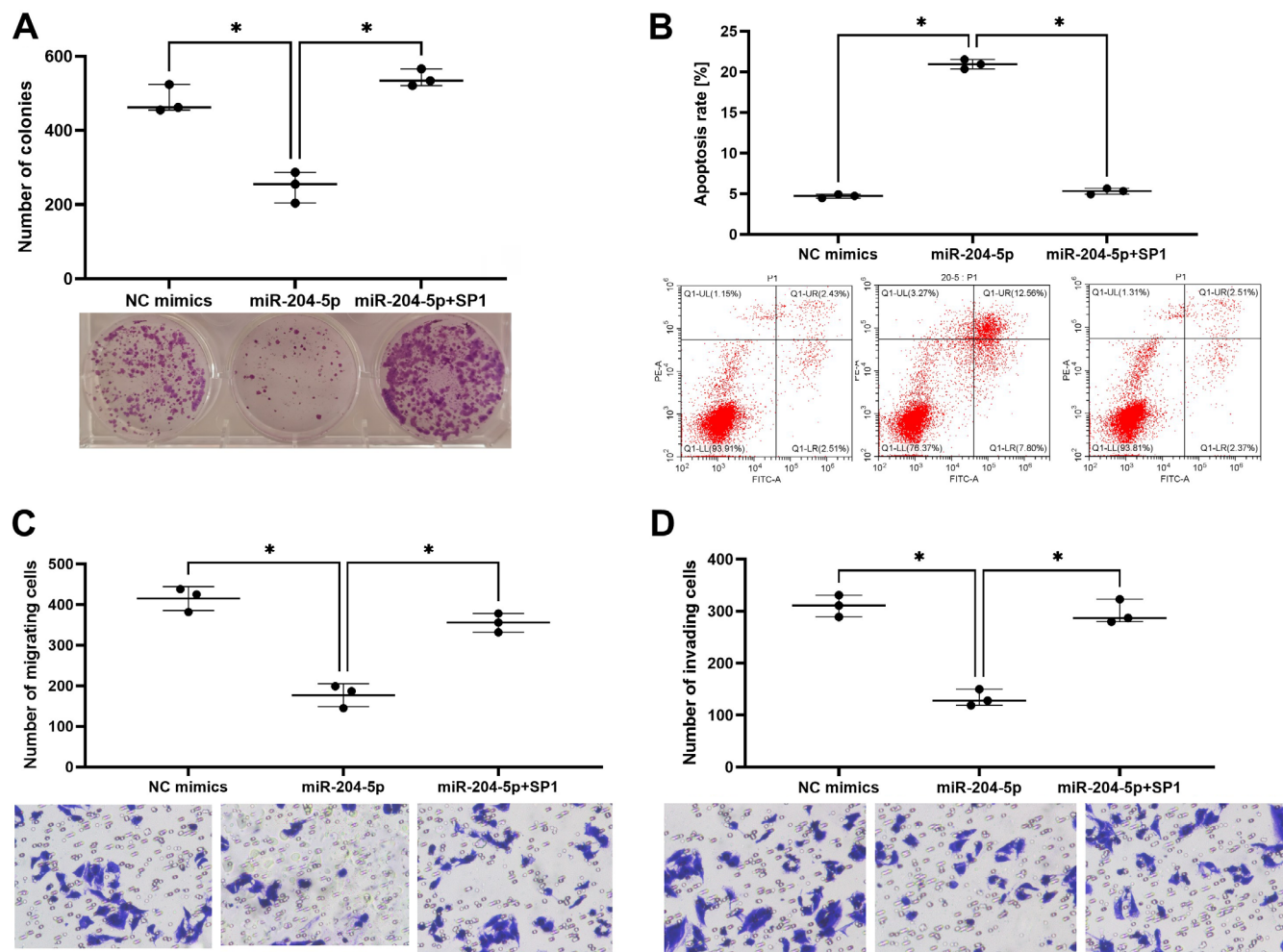


Fig. 4. *miR-204-5p* decreased HTR-8/SVneo cell invasion and migration and increased apoptosis through targeting *SP1*. Cells were transfected with *miR-204-5p* mimic, or co-transfected with *miR-204-5p* mimic and pcDNA3.1-*SP1*. A. Colony formation; B. Apoptosis rates; C,D. Migration and invasion. Data in replicates are presented in figures as scatter dots with the median and range lines. Kruskal–Wallis test with Dunn’s post hoc and Bonferroni correction was performed for multiple comparisons, and Mann–Whitney U test for comparison within 2 groups

* $p < 0.050$. *SP1* – specificity protein 1; *DNMT1* – DNA methyltransferase 1; si-*SP1* – siRNA targeting *SP1*; si-NC – siRNA negative control; *MEG3* – maternally expressed gene 3.

DNMT3A and *DNMT3B*, leading to hypermethylation of tumor suppressor gene.^{28–30} In our study, the decreased *DNMT1* levels following *SP1* knockdown suggest a similar regulatory mechanism in trophoblast cells, underscoring the broader implications of *SP1*-*DNMT1* interactions beyond oncogenesis. In addition, the methylation inhibitor, 5-azadC, was used to induce the cells and we found that *MEG3* expression was increased by 5-azadC, suggesting that *MEG3* was regulated in trophoblast cells likely due to methylation. Furthermore, upregulation of *SP1*/*DNMT1* in cells was found to partly reverse the change in *MEG3* expression induced by 5-azadC. These results suggest that *SP1*/*DNMT1* inhibited *MEG3* expression via methylation regulation.

Previously, *DNMT1* was identified to be essential for maintaining DNA methylation patterns during DNA replication, crucial for gene silencing and genomic stability.^{31,32} For instance, in breast cancer, *MEG3* expression was enhanced by knockdown of *DNMT1* through demethylation

of *MEG3* promoter.^{23,33} Our findings extend the interaction of *DNMT1*/*MEG3* to trophoblast cells, suggesting that *DNMT1*-mediated methylation is vital for placental development and function.

A significant finding of this study is that *miR-204-5p* directly targets *SP1*, leading to decreased levels of both *SP1* and *DNMT1*, which in turn upregulates *MEG3* expression in trophoblast cells. This regulatory pathway was confirmed through dual-luciferase activity, RT-qPCR and western blot methods; *miR-204-5p* has been previously identified as a tumor suppressor in various cancers, regulating genes involved in proliferation and apoptosis.^{34,35} Our study shows that *miR-204-5p* similarly impacts the apoptosis, invasion and migration of trophoblast cells, highlighting its role in reproductive health. *MEG3* expression was increased by *miR-204-5p* overexpression, which was reversed by *SP1* overexpression, indicates a complex regulatory mechanism involving *miR-204-5p*, *SP1* and *DNMT1* in trophoblast cells.

Limitations

Despite the significant findings, this study has limitations. First, the research was conducted in vitro using HTR-8/SVneo trophoblast cells, which may not fully replicate in vivo environment of placental tissue. Second, although the study identifies *miR-204-5p* as a regulator of *SP1* and *DNMT1*, the broader regulatory network involving other miRNAs and transcription factors was not explored. Additionally, the clinical relevance of these findings needs further validation through studies involving human study participants and clinical samples.

Conclusions

The elucidation of the *miR-204-5p/SP1/DNMT1/MEG3* pathway offers new perspectives on the molecular mechanisms in regulating trophoblast cells. Future research should explore therapeutic interventions targeting this pathway to improve pregnancy outcomes in preclinical animal studies. Additionally, investigating other miRNAs and lncRNAs involved in trophoblast function could further enhance the understanding of the complex regulatory networks in placental development.

Supplementary data

The supplementary materials are available at <https://doi.org/10.5281/zenodo.14043134>. The package includes the following files:

Supplementary Table 1. The results of homogeneity of variance test.

Supplementary Table 2. Statistical methods (Mann–Whitney U test) and test results used.

Supplementary Table 3. Kruskal–Wallis test with Dunn's post hoc and Bonferroni correction and test results used.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Use of AI and AI-assisted technologies

Not applicable.

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