Research Article

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Asiaticoside reverses the inhibition effect of miR-184 on proliferation, migration and AKT phosphorylation of HTR-8/Svneo cells

https://doi.org/10.1515/tjb-2024-0014
Received January 21, 2024; accepted November 22, 2024; published online December 31, 2024

Abstract

Objectives: Physiological changes of trophoblast cells are associated with gestational diabetes mellitus (GDM), and miR-184 involved in the development of GDM can be a potential therapeutic target. Asiaticoside (AS) has potential therapeutic effects on GDM, but its effect on miR-184 in HTR-8/Svneo cells is a relatively unworked area. The present study aimed to explore the effect of AS and miR-184 on physiological changes of HTR-8/Svneo cells.

Methods: After the cytotoxicity assay of AS, HTR-8/Svneo cells were transfected with miR-184 mimics and/or treated with AS. The mRNA level of miR-184 in different groups was determined by real-time reverse transcription polymerase chain reaction, the cell viability was detected by cell counting kit-8 assay, the cell migration was measured by scratch test, the protein expressions of matrix metalloproteinase-2 (MMP2), MMP9, protein kinase B (AKT) and p-AKT were determined by western blot.

Results: Eighty μM AS with a treatment time of 48 h had no cytotoxicity and even promoted cell viability of HTR-8/Svneo cells. AS could significantly inhibit the mRNA level of miR-184 in HTR-8/Svneo cells ($p<0.05$). The overexpression of miR-184 significantly suppressed the cell viability, migration, and protein expressions of MMP2, MMP9 and p-AKT/AKT; however, AS was able to reverse the inhibition effect of miR-184 to increase the cell viability, migration and protein expressions of MMP2, MMP9 and p-AKT/AKT in HTR-8/Svneo cells ($p<0.05$).

Conclusions: AS can reverse the inhibition effect of miR-184 on HTR-8/Svneo cells to facilitate cell proliferation, migration and AKT phosphorylation.

Keywords: asiaticoside; gestational diabetes mellitus; miR-184; HTR-8/Svneo cell; AKT

Zhilan Jia and Ya Long contributed equally to this work and share first authorship.

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Introduction

Gestational diabetes mellitus (GDM) can lead to severe short-term and life-long complications in the mothers or their offsprings. The incidence of GDM is increasing globally with reported rates ranging from 8.1 to 24.2 % in China [1]. Research suggests that GDM may be related to physiological changes of trophoblast cells in the placenta [2]. High glucose (HG) is one of the key features of GDM, which can impair the normal development of the placenta, facilitating abortion and malformation of the fetus [3]. Trophoblast cells, as a special type of placental cells, are important in embryo implantation, as well as establishment and maintenance of pregnancy [4].

The suppression of proliferation and migration of trophoblast cells can lead to the maldevelopment of the placenta tissues, further resulting in the development of GDM [2, 3].

MicroRNAs (miRNAs) are short, noncoding RNA molecules and can play a post-transcriptional regulatory function [5]. Over 600 miRNAs are expressed in the placenta, and their expression levels and types change significantly with the development of the placenta [6]. MiRNAs can influence the abilities of migration and invasion in trophoblast cells [7]. It is reported that some miRNAs have relevance to the development of diabetes, which may serve as potential predictors of gestational diabetes and possess vital functions in the epigenetic regulation of GDM [8]. MiR-184, enriched in pancreatic islets and β-cells, plays a role in modulating the compensatory β-cell expansion during insulin resistance, which is an important factor in the pathophysiology of GDM [9, 10]. The study of Martinez-Sanchez et al. has indicated that miR-184 is the most down-regulated miRNA in diabetes and is regulated by the glucose sensor adenosine monophosphate activated protein kinase [11]. Moreover, it has been suggested that miR-184 is highly expressed in recurrent spontaneous abortion and can induce the apoptosis of trophoblast cells by targeting WIG1 [12]. Therefore, it can be speculated that miR-184 may be concerned with the development of GDM and also is a potential therapeutic target.

Centella asiatica has been widely used for thousands of years due to its broad spectrum of biological and pharmacological properties, including antioxidant, anti-microbial, anti-inflammatory, wound healing, cytoprotective, neuroprotective, and memory improvement effects [13]. Asiaticoside (AS, $C_{48}H_{78}O_{19}$, CAS: 16,830-15-2) is a main chemical component responsible for the pharmacological activity of *C. asiatica* [14]. Recently, AS has been shown to improve cellular changes induced by HG. For example, AS can ameliorate the inflammation and apoptosis induced by HG through the cyclic adenosine monophosphate/protein kinase A signaling pathway in retinal pigment epithelial cells [15]. In addition, AS is able to increase anti-oxidative activity and suppresses the advanced glycation end products (AGEs)/receptor for AGEs/nuclear factor-κB pathway to reduce injury induced by HG in cochlear hair cells [16]. As for *in vivo* study, AS has been reported to significantly reduce fasting blood glucose concentration in obese diabetic rats and greatly promote insulin secretion under HG stimulation [17, 18]. Hence, AS may have potential therapeutic effects on GDM. Moreover, AS is suggested to show a regulatory effect on miRNA, such as miR-635 [19] and miR-142-5p [20]. But the effect of AS on miR-184 is a relatively unworked area.

As previously described, miR-184 can be a potential therapeutic target of GDM, and AS has a regulating effect on miRNA, which is speculated to contribute to the prevention and treatment of GDM via the effect on miR-184. However, the relevant area has been relatively unexplored. Therefore, this is the first study focused on miR-184 to investigate the effect of AS on HTR-8/Svneo cells, with the goal of providing promising drugs to prevent and treat gestational diabetes.

Materials and methods

Cell culture

HTR-8/Svneo cells, a common cellular model of trophoblasts, were acquired from the American Type Culture Collection (no. CRL3271, Rockville, MD, USA). HTR-8/Svneo cells were placed in RPMI 1640 medium (Invitrogen, Shanghai, China) containing 10 % fetal bovine serum (Invitrogen, MA, USA) and 100 U/mL penicillin/streptomycin (Thermo Fisher, MA, USA), and cultured at 37 °C with 5 % CO_2.

Cell transfection

MiR-184 mimics and mimic negative control (NC) were designed and purchased from Ribobi (Guangzhou, China). After reaching 60 % cell confluence, HTR-8/Svneo cells were transfected with 10 μM miR-184 mimics or mimic NC at 37 °C with 5 % CO_2 for 48 h. The transfection was performed using Lipofectamine® 2000 (Invitrogen, Shanghai, China), and real-time reverse transcription polymerase chain reaction (RT-PCR) was used to measure the expression of miR-184 after transfection [21].

Cytotoxicity assay and grouping

After being seeded in 96-well plates and reached 80 % cell confluence, cytotoxicity assay of HTR-8/Svneo cells was conducted with different concentrations (0, 20, 40, 60, 80, 100, 120, 140, 160, and 180 μM) of AS and different treatment times (12, 24 and 48 h). Then, cell viability was measured by cell counting kit-8 (CCK-8) assay to obtain the optical density (OD) value at 450 nm [22]. Based on the cytotoxicity assay, the optimum concentration and treatment time of AS were determined and further used in the follow-up experiments.

Whereafter HTR-8/Svneo cells were divided into six groups. The control group was normal HTR-8/Svneo cells, the mimic group was cells transfected with miR-184 mimics for 48 h, the mimic NC group was cells transfected with mimics NC for 48 h, the AS group was cells treated with optimum concentration of AS for optimum time, the mimic + AS was

cells which were transfected with miR-184 mimics for 48 h and then treated with AS similar to the AS group, the mimic NC + AS was cells transfected with mimics NC and then treated with AS. The cells in different groups were prepared for the subsequent experiments, which were performed in triplicate and repeated at least three times.

RT-PCR

The total RNAs of the six groups were extracted using the Trizol reagent kit (Invitrogen, MA, USA) and reversely transcribed into cDNA by PrimeScript II 1st strand cDNA synthesis kit (Takara, Shiga, Japan). RT-PCR was conducted in StepOnePlus real-time PCR (AB International, CA, USA) according to the instrument of SYBR Premix Ex *Taq*II (Takara, Shiga, Japan). Taking glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* as an internal reference, the relative mRNA levels of miR-184 were detected by the $2^{-\Delta\Delta Ct}$ method [23]. The primer sequences were as follows: miR-184 forward 5′-TACGACTATGTGACCTG-CCTG-3′ and reverse 5′-TGGTTCAACTCTCCTTTCCA-3′, GADPH forward 5′-CAATGACCCCTTCATTGACC-3′ and reverse 5′-GACT-CTTGCCCTTCGAACAG-3’.

CCK-8 assay

HTR-8/Svneo cells were seeded in 96-well plates and received the intervention of different groups, then 10 μL CCK-8 (Dojindo Laboratories, Kyushu, Japan) was added in each well. After incubation for 2 h and washing, the OD value at 450 nm was detected in a microtiter plate reader (Thermo Fisher Scientific, Waltham, USA). The cell viability was calculated as follows: $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\ \%$ [22].

Scratch test

After being seeded in 6-well plates, HTR-8/Svneo cells were transfected for 48 h to 100 % confluency. The cell layers were scratched by a 20 μL sterile pipette tip and then cultured with a serum-free medium or medium containing AS. After the incubation for 24 h, images of the cell layers were captured by microscopy. The widths of the scratch were analyzed by the Olympus CellSens Dimension software (version 1.7, Olympus, Tokyo, Japan), where the cell migration rate was expressed as a percentage of the healing wound area relative to the initial wound area [24].

Western blot

Western blot was used to measure the protein levels of matrix metalloproteinase-2 (MMP2), MMP9, and protein kinase B (AKT). Total proteins of cells in different groups were extracted according to the instrument of the tissue or cell total protein extraction kit (Chundu Bio, Wuhan, China). After the determination of protein concentration, 50 μg extracted proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Cosmo Bio, Tokyo, Japan) and transferred to polyvinylidene fluoride membranes (Millipore, MA, USA). After being sealed with 5 % fat-free milk, the membranes were incubated overnight at 4 °C with primary antibody, anti-MMP2, MMP9, AKT, p-AKT (1:500, catalog no: bs-4605R, bs-4593R, bs-0115R, bs-2720R, Bioss, Beijing, China), which were diluted by 0.01 M tris buffered saline (pH=7.4). Then, the membranes were washed and incubated at room temperature for 1 h with a secondary antibody (1:10,000, catalog no: ZB-2301, ZSGB-BIO, Beijing, China), which was diluted by 0.01 M tris buffered saline. Subsequently, the target bands were visualized using a chemiluminescence kit (Millipore, MA, USA) and quantified by the Tanon-5200 Image Analyzer (Tanon, Shanghai, China) with normalization to GAPDH [25].

Statistical analysis

SPSS software version 26.0 (SPSS Inc, Chicago, USA) and GraphPad Prism version 7.0 (GraphPad Software Inc, San Diego, USA) was used to conduct data analysis. After performing the Shapiro–Wilk normality test, the data were presented as mean ± standard deviation and compared by Student’s t-test for two groups, as well as one-way analysis of variance for multiple groups. Statistical significance was defined as a p-value below 0.05.

Results

Determination of optimum concentration and time of AS

The cytotoxicity assay of AS with different concentrations and treatment durations in HTR-8/Svneo cells is indicated in Figure 1. Under the same concentration of AS, the cell viability with a treatment time for 48 h was significantly higher than those for 12 and 24 h, suggesting AS with a treatment time of 48 h had a good promotion effect on cell proliferation ($p<0.05$). In addition, after AS treatment for

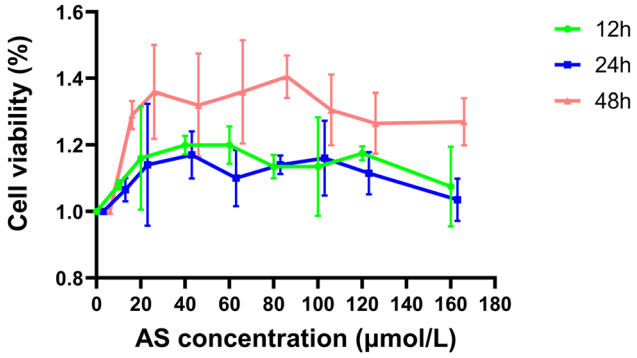

Figure 1: Cytotoxicity assay of as with different concentrations and treatment times. The optimum concentration and treatment time of as in HTR-8/Svneo cells were 80 μM and 48 h, respectively. Cell viability (%) was calculated as follows: $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$ %. The data were presented as mean and standard deviation. AS, asiaticoside.

48 h, the cell viability was generally increased with AS concentration from 0 to 80 μM, while cell viability was significantly decreased under the treatment with 100 μM AS, suggesting that AS of 80 μM not only showed no cytotoxicity effect in HTR-8/Svneo cells but also promoted cell proliferation ($p<0.05$). Therefore, the optimum concentration and time of AS on HTR-8/Svneo cells were 80 μM and 48 h, respectively, which had no cytotoxicity and were used in the follow-up experiments.

Effect of AS on mRNA level of miR-184

As shown in Figure 2A, the mRNA level of miR-184 in the mimic group was higher compared to the control group ($p<0.05$), but the mRNA level of miR-184 in the mimic NC group showed no significant difference to the control group ($p>0.05$), suggesting the success of transfection. After AS treatment, the mRNA levels of miR-184 in the AS, mimic + AS, and mimic NC + AS groups were higher than those in the control, mimic, and mimic NC groups, respectively ($p<0.05$), indicating the inhibition effect of AS on mRNA level of miR-184 in HTR-8/Svneo cells.

Effect of AS and miR-184 on cell viability

As shown in Figure 2B, the cell viability in the mimic group was significantly lower compared to the control group ($p<0.05$), but there was no significant difference in cell viability between the mimic NC group and the control group ($p>0.05$), suggesting the inhibition effect of miR-184 on cell proliferation. After AS treatment, the cell viability in the AS group was higher than that in the control group, and the cell viability in the mimic + AS group was also higher than that in the mimic group ($p<0.05$), suggesting the proliferation promotion effect of AS, which could reverse the inhibition effect of miR-184.

Effect of AS and miR-184 on cell migration

The cell migration in different groups is shown in Figure 3. Similar to the results of cell viability, the cell migration rate at 24 h in the mimic group was lower compared to the control group ($p<0.05$), but there was no significant difference between the mimic NC group and the control group ($p>0.05$), suggesting the inhibition effect of miR-184 on cell migration. After AS treatment, the cell migration rate in the AS group was higher than that in the control group, and the cell migration rate in the mimic + AS group was also higher compared to the mimic group ($p<0.05$), suggesting the

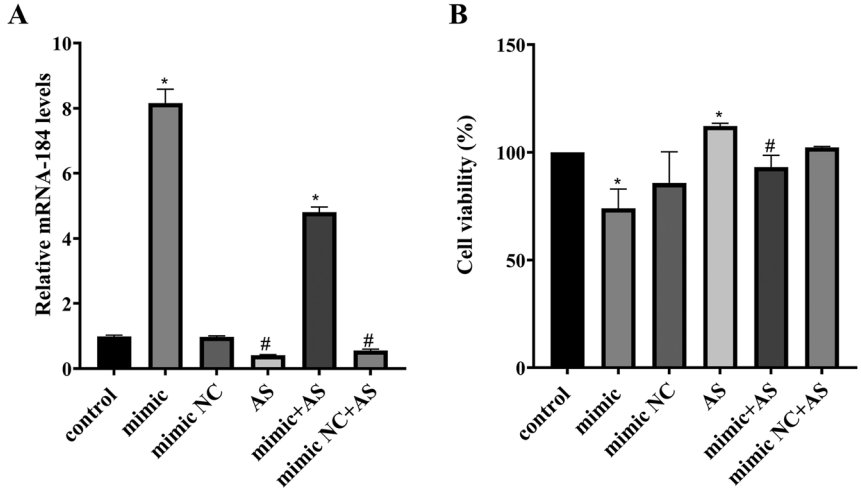

Figure 2: Comparison of mRNA level of miR-184 and cell viability among groups. (A) The comparison of relative miR-184 levels in different groups. (B) The comparison of cell viability in different groups. The data were presented as mean and standard deviation, *$p<0.05$ vs. control group, #$p<0.05$ vs. corresponding group without as treatment. NC, negative control; AS, asiaticoside.

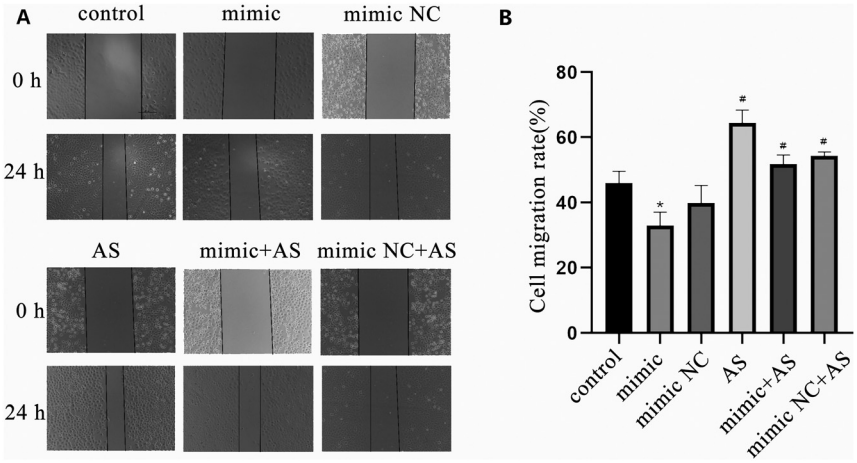

Figure 3: Comparison of cell migration among groups. (A) The representative pictures of scratch in HTR-8/Svneo cells at 0 and 24 h incubation. (B) The comparison of cell migration rates in different groups. The data were presented as mean and standard deviation, *p<0.05 vs. control group, #p<0.05 vs. corresponding group without AS treatment. NC, negative control; as, asiaticoside.

migration promotion effect of AS, which could reverse the inhibition effect of miR-184.

MMP2 and MMP9 play a role in cell migration. As shown in Figure 4, the protein expressions of MMP2 and MMP9 in the mimic group were lower than those in the control group (p<0.05), but there was no significant difference between the mimic NC group and the control group (p>0.05), suggesting miR-184 could inhibit the expressions of MMP2 and MMP9 to further affect cell migration. After AS treatment, the protein expressions of MMP2 and MMP9 in the AS, mimic + AS, and mimic NC + AS groups were higher than those in the control, mimic, and mimic NC groups, respectively (p<0.05); therefore, AS might facilitate cell migration by increasing the protein expressions of MMP2 and MMP9, and reverse the inhibition effect of miR-184 on cell migration.

Effect of AS and miR-184 on AKT phosphorylation

As indicated in Figure 5, the AKT protein expression was similar among groups; however, the p-AKT protein expression was quite different. In quantitative analysis, the relative protein expression of p-AKT/AKT in the mimic group was decreased compared to the control group (p<0.05), but there was no significant difference between the mimic NC group and the control group (p>0.05), showing the inhibition effect of miR-184 on AKT phosphorylation. However, the relative protein expressions of p-AKT/AKT in the AS, mimic + AS, and mimic NC + AS groups were increased in comparison with the corresponding groups without AS treatment (p<0.05), suggesting the promotion effect of AS on AKT phosphorylation, which could reverse the inhibition effect of miR-184.

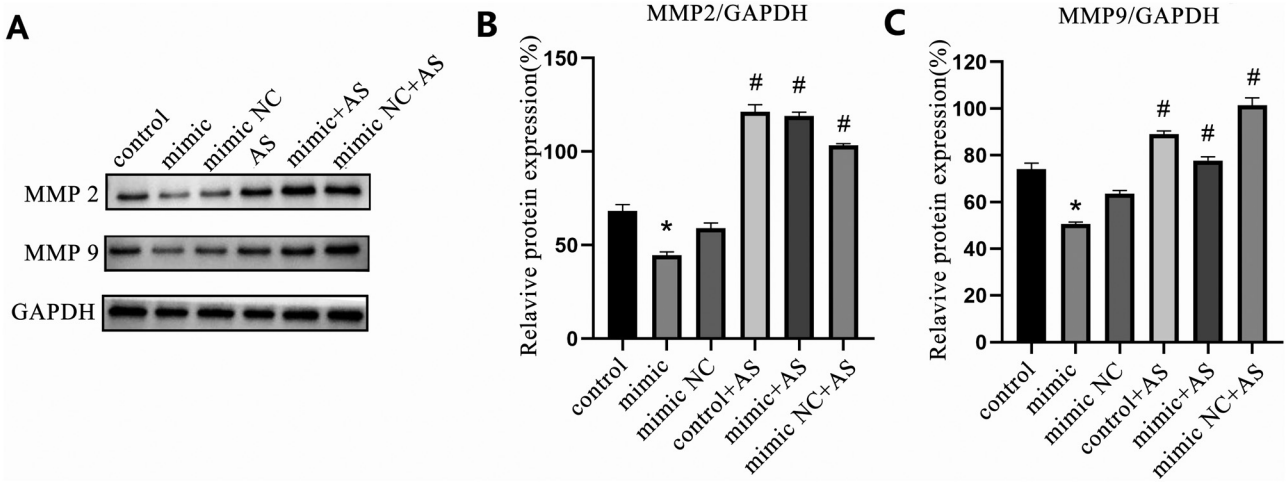

Figure 4: Comparison of protein expressions of MMP2 and MMP9 among groups. (A) The representative pictures of western blotting of MMP2, MMP9 and GAPDH in HTR-8/Svneo cells in different groups. (B) The relative protein expressions of MMP2 to GAPDH in HTR-8/Svneo cells in different groups. (C) The relative protein expressions of MMP9 to GAPDH in HTR-8/Svneo cells in different groups. The data were presented as mean and standard deviation, *p<0.05 vs. control group, #p<0.05 vs. corresponding group without AS treatment. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC, negative control; AS, asiaticoside; MMP, matrix metalloproteinase.

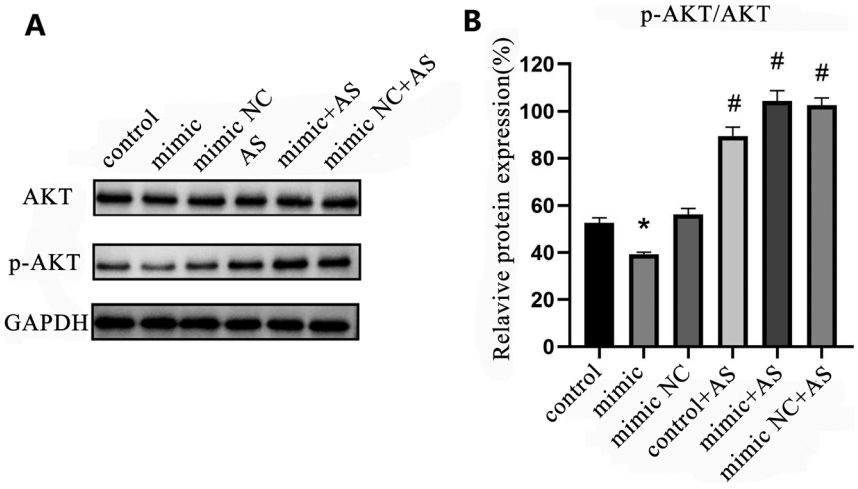

Figure 5: Comparison of protein expressions of AKT and p-AKT among groups. (A) The representative pictures of western blotting of AKT, p-AKT and GAPDH in HTR-8/Svneo cells in different groups. (B) The relative protein expressions of AKT to p-AKT in HTR-8/Svneo cells in different groups. The data were presented as mean and standard deviation, *p<0.05 vs. control group, #p<0.05 vs. corresponding group without as treatment. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NC: negative control; AS: asiaticoside; AKT: protein kinase B.

Discussion

GDM can cause severe damage to mothers or their offsprings. It has been revealed that miRNAs play an important role in a wide range of biologic and pathologic processes, and their abnormal expression is closely associated with reproductive system diseases [12]. For example, miR-184 has been found to induce early spontaneous abortion and is speculated to be involved in the development of GDM [12]. HTR-8/Svneo cells are immortalized human chorionic trophoblast line and are commonly used as an extravillous trophoblast model. Therefore, we introduced HTR-8/Svneo cells to explore the role of miR-184 on GDM. It has been reported that miR-184 is related to cancer biology. Tan et al. have indicated that miR-184 delays cell proliferation, migration, and invasion in prostate cancer [26]. In the study of Rao and Lu, miR-184 can restrain the proliferation, migration, and invasion of lung adenocarcinoma cells by targeting C1QTNF6 [27]. Trophoblast cells have similarities in properties of proliferation, migration, and invasion with cancer cells, suggesting analogous underlying mechanisms between them in the regulation of cell functions and behaviors [28]. Our results indicated that the overexpression of miR-184 suppressed the cell viability and migration of HTR-8/Svneo cells, which is consistent with the previous findings in cancer cells. MMPs are critical for the degradation of extracellular matrix and basement membrane, therefore, participate in various physiological and pathological processes [29]. MMPs secreted by trophoblast cells play a role in cell migration and invasion, as well as successful embryo implantation, which has also attracted wide interest in gynecological and obstetric diseases [4]. It has been found that miR-184 could suppress the protein expressions of MMP2 and MMP9 in the glioma U87MG cell line and breast cancer MCF-7 cell line, echoing our results that the overexpression of miR-184 reduced the protein expressions of MMP2 and MMP9 in HTR-8/Svneo cells [30]. It is reported that *in vitro* treatment of MMP2 and MMP9 can significantly promote invasion and migration of mouse trophoblastic cells; in this way, the inhibition effect of miR-184 on the expression of MMP2 and MMP9 might further affect cell migration and invasion in our study [31]. AKT is reported to induce multiple processes, including glucose uptake, cell survival, proliferation, metabolism, and translation; meanwhile, phosphorylation is an essential posttranslational modification for its kinase activity [32]. The protein expression of p-AKT has been indicated to be markedly decreased in human limbal epithelial keratinocytes expressing miR-184 compared with controls [33]. Our results showed that the overexpression of miR-184 could significantly decrease the relative protein expression of p-AKT/AKT in HTR-8/Svneo cells, showing the inhibition effect of miR-184 on AKT phosphorylation, which is in agreement with the previous study. Cell proliferation and migration are the growth foundations of trophoblast cells, which can affect the development of GDM [8]. Therefore, the inhibition effect of miR-184 on cell proliferation, migration, and invasion induces the maldevelopment of the placenta tissues, further resulting in the development of GDM. These results provide possible evidence that miR-184 is a potential therapeutic target, and the treatment with molecules or drugs targeting miR-184 may contribute to the prevention of

GDM. Currently, the available research about the effect of miR-184 on gestational diabetes is rather little; therefore, the results of this study enrich the investigation about the role of miR-184 as a potential therapeutic target in GDM.

AS possesses extensive bioactivity, including antioxidant, immunoregulation, anti-tumor, anti-inflammatory, and promotion of wound healing; moreover, several studies previously mentioned have proposed that AS may have potential therapeutic effects on GDM, but there is no report on the effect of AS on trophoblast cells [15–18, 34]. The opinions about the effect of AS on the biological characteristics of cells are not consistent. AS is reported to promote cell migration, attachment, and growth of human skin *in vitro* [35]. However, the migratory and invasive properties of drug-resistant myeloma cell line KM3/BTZ can be suppressed by AS via the modulation of signal transducer and activator of the transcription-3 signaling pathway [36]. Our study first explored the optimum concentration and treatment time of AS in HTR-8/Svneo cells and found that AS with a concentration of 80 μM and a treatment time for 48 h showed no cytotoxicity, even promoting cell proliferation. Whereafter, AS was indicated to promote cell proliferation and migration of HTR-8/Svneo cells. As mentioned above, MMPs are often associated with cell migration and invasion; our results indicated that AS increased the protein expressions of MMP2 and MMP9, which might further enhance cell migration. It has been reported that AS can promote AKT phosphorylation in myocardial ischemia/reperfusion animal models and mouse HL-1 cardiomyocytes with oxygen-glucose deprivation/reperfusion stimulation, which is consistent with our results that AS increased the protein expressions of p-AKT/AKT to some extent [37]. Moreover, AS also possesses a regulating effect on miRNA. For example, AS can upregulate miR-635 expression to restrain cell proliferation and migration of gastric cancer [19]. In our study, we explored the relatively unworked area about the effect of AS on miR-184 was performed, and found that AS was able to inhibit the mRNA level of miR-184 and reverse the inhibition effect of miR-184 to facilitate cell proliferation and migration, as well as increase the protein expressions of MMP2, MMP9 and p-AKT/AKT. Based on the effect of AS on miR-184, it is speculated that AS may be a promising drug to prevent and treat GDM.

As far as we know, our study first reported the effect of AS and miR-184 on trophoblast cells. However, due to the simple, superficial and preliminary study, there are still some deficiencies in this paper. Our results have demonstrated that miR-184 inhibits cell proliferation, migration, and phosphorylation of AKT, and AS is able to reverse the inhibition effect of miR-184; however, these findings rely on cell-based assays, which are not sufficient to fully prove the effect of miR-184 and AS on GDM. Therefore, in-depth studies based on *in vivo* model of GDM are necessary for further verification of the effect of miR-184 and AS. Moreover, the relationship between AS and miR-184, as well as their underlying mechanism affecting trophoblast cells or GDM, are still uncovered. Our experimental design is also relatively single and simple; hence, various research methods, including Transwell migration assay, flow cytometry for cell cycle, and apoptosis detection, should be performed to enrich our results. In addition, the generalizability (external validity) of our results is limited due to the cell-based study. These defects also point out the direction of our future research, and our findings have laid the foundation for future *in vivo* experiments to further clarify the clinical effect of AS.

Conclusions

In conclusion, we firstly explore the effect of AS and miR-184 on trophoblast cells and find that AS can reverse the inhibition effect of miR-184 on HTR-8/Svneo cells to facilitate cell proliferation, migration, and AKT phosphorylation, suggesting AS might be a novel promising therapeutic drug in GDM treatment. Moreover, in-depth studies are necessary for further verification, including our findings *in vitro* and *in vivo* models of GDM, as well as the mechanism of AS in the treatment of GDM.

Research ethics: Not applicable.
Informed consent: Not applicable.
Author contributions: Zhiqin Jia, Ya Long and Xiaolan Yu designed the research study and performed the research. Xiangyue Li, Zhilan Hu, Mingyan Wang and Xuemei Huang collected and analyzed the data. Zhiqin Jia and Ya Long wrote the manuscript. The authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Use of Large Language Models, AI and Machine Learning Tools: None declared.
Conflict of interest: The authors state no conflict of interest.
Research funding: This study was funded by the Joint project of Luzhou Science and Technology Bureau and Southwest Medical University (grant number 2019LZXNYDJ53), and the Dazhou Science and Technology Bureau Project (grant number 22YYJC0036).
Data availability: The raw data can be obtained on request from the corresponding author.

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