Circ_FOXP1 promotes the growth and survival of high glucose-treated human trophoblast cells through the regulation of miR-508-3p/SMAD family member 2 pathway

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Abstract. Gestational diabetes mellitus (GDM) is a health risk for pregnant women and infants. Emerging evidence suggests that the deregulation of circular RNAs (circRNAs) is associated with the progression of this disorder. The objective of this study was to investigate the role of circ_FOXP1 in GDM. Cell models of GDM were established by treating human trophoblast cells with high glucose (HG). The expression of circ_FOXP1, miR-508-3p and SMAD family member 2 (SMAD2) mRNA was detected by quantitative real-time PCR (qPCR). Cell proliferation was assessed by EdU assay and MTT assay, and cell cycle and cell apoptosis were determined by flow cytometry assay. The protein levels of proliferation- and apoptosis-related markers and SMAD2 were measured by western blot. The relationship between miR-508-3p and circ_FOXP1 or SMAD2 was validated by dual-luciferase reporter assay or pull-down assay. The expression of circ_FOXP1 was downregulated in HG-treated HTR-8/SVneo cells. Circ_FOXP1 overexpression promoted HG-inhibited HTR-8/SVneo cell proliferation and suppressed HG-induced HTR-8/SVneo cell cycle arrest and apoptosis. Circ_FOXP1 positively regulated the expression of SMAD2 by targeting miR-508-3p. MiR-508-3p was overexpressed in HG-treated HTR-8/SVneo cells, and its overexpression reversed the effects of circ_FOXP1 overexpression. MiR-508-3p inhibition also alleviated HG-induced HTR-8/SVneo cell injuries, while the knockdown of SMAD2 abolished these effects. Collectively, circ_FOXP1 promotes the growth and survival of HG-treated human trophoblast cells through the miR-508-3p/SMAD2 pathway, hinting that circ_FOXP1 was involved in GDM progression.

Key words: Circ_FOXP1, MiR-508-3p, SMAD family member 2, Gestational diabetes mellitus, Trophoblast

GESTATIONAL DIABETES MELLITUS (GDM) is defined as diabetes in patients in the second or third trimester of pregnancy who have no history of diabetes [1]. It is a common complication of pregnancy, characterized by impaired glucose tolerance due to pancreatic beta cell dysfunction [2]. Mechanistic and epidemiological studies have shown that GDM is implicated in genetic and environmental factors, suggesting the complexity of the etiology of GDM [3]. GDM is a potential risk to the health of both the mother and the fetus. Therefore, the exploitation of new and effective strategies to prevent and treat this disorder is important.

With the development of RNA sequencing technology, an increasing number of circular RNAs (circRNAs) that are abnormally expressed in patients with GDM catch the field of view [4, 5]. The dysregulation of circRNAs reveals that circRNAs participate in the progression of GDM, which arouses the public’s concern. CircRNAs

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are famous for their unique loop-closed structures, presenting high stability compared to linear transcripts [6]. CircRNAs are gradually emerging as new biomarkers to predict the initiation of GDM [7]. For example, circ_0054633 was overexpressed in blood of GDM patients and its high expression was associated with glycosylation index [7]. Unfortunately, the role of numerous circRNAs is still largely unknown in this disorder. Circ_0008234 was previously shown to be downregulated in placenta tissues of GDM patients (–7.11 fold-change compared to control) [5]. Circ_0008234 is produced by “back-splicing” approach from forkhead box P1 (FOXP1), widely known as circ_FOXP1. It has been demonstrated to be implicated in the progression of cancers, such as gallbladder cancer [8]. Though it has been shown that circ_FOXP1 is downregulated in GDM patients, the function of circ_FOXP1 in the development of GDM is unclear.

MicroRNAs (miRNAs) are small molecule RNAs, with extensive regulatory properties. The interactions between miRNA and circRNA are widely mentioned because certain circRNAs exert “sponge-like” effects on miRNAs to inhibit the expression and function of miRNAs [9]. Accumulating evidence suggested that miR-508-3p was closely implicated in the biological processes of various cancers [10, 11]. MiR-508-3p was previously shown to be highly expressed in placental tissues of GDM patients [12]. Bioinformatics tool circinteractome [13] probes that miR-508-3p is a potential target of circ_FOXP1, hinting the binding between them. It is unclear whether circ_FOXP1 plays “sponge” effects on miR-508-3p.

It is canonical that miRNAs target the 3’ untranslated region (3’UTR) of target genes to inhibit their expression. The identification of target genes contributes to the understanding of the networks of miRNA. By means of bioinformatics tool starbase [14], SMAD family member 2 (SMAD2) is predicted as a target of miR-508-3p. SMAD2 played a vital role in preeclampsia and affected the biological behaviors of trophoblast cells [15]. However, the role of SMAD2 in GDM is largely unaddressed.

High glucose (HG) is one of the major characteristics of GDM, resulting in impaired trophoblast function, thereby suppressing normal development of the placenta. Hence, HG-induced trophoblast cells are widely used to study the molecules involved in the biological function of trophoblast cells during the pathological process of GDM. To disclose the role of circ_FOXP1 in the progression of GDM to provide additional therapeutic strategies, we constructed GDM models in vitro by treating human trophoblast cells with HG. The expression of circ_FOXP1 in HG-treated trophoblast cells and the function of circ_FOXP1 on the proliferation and survival of HG-treated trophoblast cells were determined. Moreover, the role of circ_FOXP1 associated with the miR-508-3p/SMAD2 axis was clarified to partially explain the mechanism of circ_FOXP1 function in GDM.

Materials and Methods

Serum samples
Patients with GDM (n = 20) and healthy pregnant women (control; n = 13) were recruited from Xiangyang NO.1 People’s Hospital. All subjects received prenatal care at 16–19 gestational weeks, and the diagnosis of GDM was confirmed at that time. The written informed consent was provided by each subject prior to blood sample collection. GDM patients with diabetes history, multiple gestation and series complications were exclude in this study. Clinical data for GDM patients and normal controls were displayed in Table 1. Blood samples were placed at 4°C for 4 h and then centrifuged to isolate

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**Table 1**  Clinical data for the GDM patients and normal controls

| Characteristics               | GDM (n = 20)       | Control (n = 13) | p value |
|------------------------------|--------------------|------------------|---------|
| Age (year)                   | 32.33 ± 3.99       | 30.26 ± 4.58     | 0.184   |
| Gestational age (day)        | 38.57 ± 0.61       | 38.67 ± 0.49     | 0.409   |
| HbA1c (%)                    | 5.51 ± 0.76        | 4.77 ± 0.54      | 0.034*  |
| Birth weight (g)             | 3,211 ± 516.2      | 3,309 ± 500.2    | 0.152   |
| Pre-pregnancy BMI index (kg/m²) | 22.25 ± 1.99    | 21.05 ± 2.2      | 0.071   |
| Late-pregnancy BMI index (kg/m²) | 27.88 ± 1.78  | 26.23 ± 2.02     | 0.044*  |
| OGTT-0 h (mmol/L)            | 5.39 ± 1.49        | 4.35 ± 0.33      | 0.029*  |
| OGTT-1 h (mmol/L)            | 10.67 ± 3.01       | 7.21 ± 1.22      | 0.002*  |
| OGTT-2 h (mmol/L)            | 9.43 ± 3.59        | 6.17 ± 1.02      | 0.004*  |

Notes: * p < 0.05. BMI: body mass index.
serum samples, followed by storage at –80°C. The Ethics Committee of Xiangyang NO.1 People’s Hospital approved our study.

**Cell model of GDM**

Human trophoblast cells (HTR-8/SVneo) provided by Procell (Wuhan, China) were cultured in RPMI1640 medium containing 10% FBS and 5% CO2. Cell model of GDM was constructed by treating HTR-8/SVneo cells with HG (30 mM; Sigma-Aldrich, St. Louis, MO, USA) for 24 h as previously described [16-18]. Cells in the control group were treated with normal glucose (5.5 mM).

**Quantitative real-time PCR (qPCR)**

Total RNA was conventionally isolated using Trizol reagent (Sangon Biotech, Shanghai, China), and its quality was checked using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription assay was performed using TruScript First Strand cDNA Synthesis Kit (Norgenbiotek, Ontario, Canada) or microScript microRNA cDNA Synthesis Kit (Norgenbiotek) as appropriate. Quantitative reaction was performed using SYBR Green SuperMix (Invitrogen). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH or U6 as an internal reference. Primer sequences were displayed as below:

- circ_FOXP1, F 5'-ACAGCTCTCCATCCACACT-3', and R 5'-TGAGCTCGAAAACTTGGT-3';
- FOXP1, F 5'-CAGCGGAACCCGAAAGTTTG-3', and R 5'-GAGTCCGGGGAGGGAGTAG-3';
- miR-508-3p, F 5'-GCGTGATTGTAGCCTTTTGG-3', and R 5'-AGTGAGGGTCCGAGGTATT-3';
- SMAD2, F 5'-TGGGGACTGAGTACCAAA-3', and R 5'-GGGATACCTGGAGACGACCA-3';
- GAPDH, F 5'-GGAGTCCACTGGCTTTCA-3', and R 5'-GGTTCACACCCATGACGGC-3';
- U6, F 5'-CTCGCTTCGGCAGCACATATACT-3' and R 5'-ACGCTTCAGGAATTTGCGTTC-3'.

**RNase R and actinomycin D treatment**

To verify the existence and stability of circ_FOXPI, RNase R and actinomycin D were utilized. Simply put, total RNA was treated with RNase R (3 U/μg; Epicenter Technologies, Madison, WI, USA) for 30 min at 37°C incubator. Actinomycin D (2 μg/mL; Sigma-Aldrich) was used to treat cells in culture medium for different time (0, 6, 12 or 24 h), and the treated cells were collected for RNA isolation. After treatment with RNase R or actinomycin D, qPCR was performed to determine the expression of circ_FOXPI and its linear transcript.

**CircRNA location**

Cytoplasmic RNA or nuclear RNA from HTR-8/SVneo cells was separated isolated using the PARIS kit (Invitrogen). Each RNA sample was used for qPCR to determine the expression level of circ_FOXPI, using GAPDH as a control in cytoplasm and U6 as a control in nucleus.

**Cell transfection**

Circ_FOXPI overexpression vector pCD5-cir-circ_FOXPI (circ_FOXPI) and blank vector (Vector) constructed by Geneseed (Guangzhou, China). MiR-508-3p mimic (miR-508-3p), miR-508-3p inhibitor (anti-miR-508-3p) and their negative control (miR-NC or anti-miR-NC) were provided by Ribobio (Guangzhou, China). Small interference RNA targeting SMAD2 (si-SMAD2) and its negative control (si-NC) were synthesized by Ribobio. Oligonucleotides or vectors were transfected into HTR-8/SVneo cells using Lipofectamine 3000 reagent (Invitrogen).

**EdU assay**

By using EdU Imaging Kit (Life Technologies, Gaithersburg, MD, USA), the proliferative capacity of cells was assessed. Simply put, cells plated into 96-well plates (4 × 10^3 cells/well) were cultured at 60%–70% confluence. Cells in each well were then labeled with 20 μM EdU solution for 2 h and next fixed using 3.7% paraformaldehyde for 20 min in the dark. The staining was stopped using anti-EdU working solution, and cell nuclei was stained by 4',6-diamidino-2-phenylindole (DAPI). The number of EdU-positive cells was assessed using a fluorescent microscope (Olympus, Tokyo, Japan).

**MTT assay**

Cells plated into 96-well plates (4 × 10^3 cells/well) were cultured for different time (0, 12, 24 or 72 h). Then, cells in each well were treated with MTT reagent (Sangon Biotech) for another 4 h. Formazan was dissolved using DMSO. The absorbance at 570 nm was measured under a microplate reader (Thermo Fisher Scientific).

**Western blot assay**

The primary antibodies, including anti-PCNA (ab92552), anti-Bel-2 (ab182858), anti-Bax (ab32503), anti-SMAD2 (ab40855), were purchased from Abcam (Cambridge, MA, USA). Total protein from cells was extracted by treating cells with RIPA lysis buffer (Sangon Biotech). After quantification, the equal amount of protein was loaded on 10% SDS-PAGE, and the separated protein was transferred onto PVDF membranes. Membranes carrying proteins were blocked by 5% skim milk and incubated with the primary antibodies, followed
by the exposure with the HRP-labeled secondary antibody (ab205718; Abcam). The signaling on membranes was appeared using ECL kit (Sangon Biotech).

**Flow cytometry assay**

For cell cycle analysis, cells were digested by trypsin and resuspended in PBS. Cells were fixed overnight using 70% ethanol at 4°C. Then, cells were washed with PBS and stained with PI + RNase A working buffer (Beyotime, Shanghai, China). Cell DNA content at different stages was determined by a flow cytometer (Beckman Coulter, Miami, FL, USA).

To detect cell apoptosis, cells after treatment were examined using Cell Apoptosis Kit with Annexin V FITC and PI (Invitrogen). All procedures were conducted in line with the manufacturer’s protocol. The number of apoptotic cells was counted using a flow cytometer (Beckman Coulter).

**Dual-luciferase reporter assay**

The binding site between miR-508-3p and circ_FOXP1 or SMAD2 3’UTR was analyzed by circinteractome (https://circinteractome.nia.nih.gov/) [13] or starbase (http://starbase.sysu.edu.cn/) [14]. According to the binding site, the mutant sequence fragment of circ_FOXP1 or SMAD2 was designed. Dual-luciferase reporter plasmids, including circ_FOXP1-WT, circ_FOXP1-MUT, SMAD2-WT or SMAD2-MUT, were constructed by Sangon Biotech, which contained wild or mutant miR-508-3p binding site. MiR-508-3p mimic or miR-NC were transfected with circ_FOX1-WT, circ_FOX1-MUT, SMAD2-WT or SMAD2-MUT into HTR-8/SVneo cells. After culturing for 48 h, cells were collected to detect luciferase activity using Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA).

**Pull-down assay**

The probes of circ_FOX1-WT, circ_FOX1-MUT and scramble sequence (NC) were labeled by biotin by Ribobio. Pull-down assay was performed in line with the protocol from the Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific). RNA complex was captured by biotin probe-coated streptavidin magnetic beads and isolated for qPCR analysis.

**Statistical analysis**

Data were collected from three independent experiments. The data were then processed and analyzed using GraphPad Prism 7 (GraphPad Inc., La Jolla, CA, USA), and value was presented as mean ± standard deviation (S.D.). Difference comparison in different groups was conducted using Student’s t-test or ANOVA. P < 0.05 indicated that the difference is statistically significant.

**Results**

**Circ_FOX1 was downregulated in GDM serum samples and HG-treated trophoblast cells**

The expression of circ_FOXP1 was pronouncedly decreased in serum samples from GDM patients relative to non-GDM controls (Fig. 1A). GDM models in vitro were established by treating HTR-8/SVneo cells with HG. The data showed that the expression of circ_FOXP1 expression was strikingly declined in HG-treated HTR-8/SVneo cells in a dose-dependent manner (Fig. 1B). Circ_FOXP1 was produced from the exon11-exon14 regions of FOXP1 mRNA, with 587 bp in length (Fig. 1C). RNase R digestion assay showed that circ_FOXP1 was resistant to RNase R digestion, while linear FOXP1 was degraded by RNase R digestion (Fig. 1D). In addition, circ_FOXP1 expression was hardly affected by actinomycin D, while linear FOXP1 expression was notably depleted by actinomycin D (Fig. 1E). Moreover, circ_FOXP1 was mainly located in the cytoplasm but not in the nucleus (Fig. 1F). The data suggested the downregulation of circ_FOXP1 in HG-treated trophoblast cells and its high stability.

**Circ_FOX1 overexpression promoted HG-treated trophoblast cell proliferation and inhibited cell cycle arrest and cell apoptosis**

The endogenous level of circ_FOXP1 was notably increased in HTR-8/SVneo cells transfected with circ_FOX1, while the expression of FOXP1 mRNA was not changed (Fig. 2A). For cell proliferative capacity detection, EdU assay presented that HG treatment notably reduced the number of EdU-positive cells, while circ_FOXP1 overexpression recovered the number of EdU-positive cells (Fig. 2B). MTT assay presented that OD value was decreased in HG-treated HTR-8/SVneo cells but recovered in HG-treated HTR-8/SVneo cells with circ_FOX1 overexpression at 3 days post-transfection (Fig. 2C). The expression of proliferation-related marker, PCNA, was notably decreased in HG-treated HTR-8/SVneo cells but restored by the overexpression of circ_FOX1 (Fig. 2D). For cell cycle analysis, the data from flow cytometry presented that HG treatment induced cell cycle arrest at the G0/G1 phase, while circ_FOX1 overexpression alleviated this arrest (Fig. 2E). Moreover, flow cytometry assay manifested that circ_FOX1 overexpression largely alleviated HG-induced HTR-8/SVneo cell apoptosis (Fig. 2F). The expression of Bel-2 was reduced in HG-treated HTR-8/SVneo cells but recovered by circ_FOX1 overexpression, while the expression of Bax was enhanced in
HG-treated HTR-8/SVneo cells but repressed by circ_FOX1 overexpression (Fig. 2G). Overall, circ_FOX1 overexpression promoted HG-treated trophoblast cell proliferation and inhibited cell cycle arrest and cell apoptosis.

**MiR-508-3p served as a target of circ_FOX1**

Several target miRNAs of circ_FOX1 were predicted by circinteractome. MiR-508-3p aroused much attention because miR-508-3p was widely involved in human diseases, and its role had not been declared in gestational diabetes mellitus. Circ_FOX1 possessed a special binding site with miR-508-3p (Fig. 3A). Dual-luciferase reporter assay verified the binding between miR-508-3p and circ_FOX1 because the transfection of miR-508-3p and circ_FOX1 notably reduced luciferase activity in cells (Fig. 3B). Pull-down assay presented that miR-508-3p could be largely enriched by bio-circ_FOX1-WT probe compared to bio-circ_FOX1-MUT probe or bio-NC (Fig. 3C). The expression of miR-508-3p was strikingly suppressed by circ_FOX1 overexpression (Fig. 3D). MiR-508-3p expression was elevated in HG-treated HTR-8/SVneo cells (Fig. 3E). All data indicated that miR-508-3p was a target of circ_FOX1.

**Circ_FOX1 alleviated HG-induced trophoblast cell injuries by targeting miR-508-3p**

The expression of miR-508-3p was strikingly decreased in HTR-8/SVneo cells transfected with circ_FOX1 but partially recovered in cells transfected with circ_FOX1 + miR-508-3p (Fig. 4A). Rescue experiment was performed to determine the interactions between circ_FOX1 and miR-508-3p. EdU assay and MTT assay revealed that HG-inhibited HTR-8/SVneo cell proliferative capacity was effectively recovered by circ_FOX1 overexpression but repressed by the reintroduction of miR-508-3p (Fig. 4B and 4C). The expression of PCNA was partially enhanced in HG-treated HTR-8/SVneo cells transfected with circ_FOX1 but reduced in cells transfected with circ_FOX1 + miR-508-3p (Fig. 4D). In addition, the restoration of miR-508-3p promoted cell cycle arrest that was alleviated by circ_FOX1 overexpression alone in HG-treated HTR-8/SVneo cells (Fig. 4E). HG-induced HTR-8/SVneo cell apoptosis was largely suppressed by circ_FOX1 overexpression but repressed by miR-508-3p enrichment (Fig. 4F). The expression of Bcl-2 was enhanced by circ_FOX1 overexpression but repressed by miR-508-3p restoration in HG-treated HTR-8/SVneo cells, while the expression of Bax was opposite to Bcl-2 expression (Fig. 4G). The data suggested that circ_FOX1 alleviated HG-induced trophoblast cell injuries by targeting miR-508-3p.

**SMAD2 was a target of miR-508-3p, and circ_FOX1 enhanced SMAD2 expression by targeting miR-508-3p**

Further bioinformatics analysis from starbase identified that SMAD2 was one of the targets of miR-508-3p,
and miR-508-3p directly bound to SMAD2 3'UTR (Fig. 5A). Dual-luciferase reporter assay showed that the transfection of miR-508-3p and SMAD2-WT markedly reduced luciferase activity in cells (Fig. 5B), which confirmed the binding between miR-508-3p and SMAD2 3'UTR. The expression of miR-508-3p was increased in HTR-8/SVneo cells transfected with miR-508-3p but declined in HTR-8/SVneo cells transfected with anti-miR-508-3p (Fig. 5C), while the expression of SMAD2 at both mRNA and protein levels was reduced in HTR-8/SVneo cells transfected with miR-508-3p but promoted in HTR-8/SVneo cells transfected with anti-miR-508-3p (Fig. 5D and 5E). The expression of SMAD2 mRNA and protein was notably declined in HG-treated HTR-8/SVneo cells (Fig. 5F and 5G). Furthermore, we found that the expression of SMAD2 mRNA and protein was strikingly enhanced in HTR-8/SVneo cells transfected with circ_FOXP1 but partially declined in HTR-8/SVneo cells transfected with anti-circ_FOXP1.
cells transfected with circ_FOXP1 + miR-508-3p (Fig. 5H and 5I). These data indicated that circ_FOXP1 targeted miR-508-3p and thus relieved the inhibition of miR-508-3p on SMAD2.

**MiR-508-3p depletion alleviated HG-induced trophoblast cell injuries by promoting SMAD2**

The expression of SMAD2 mRNA and protein was significantly enhanced in HTR-8/SVneo cells transfected with anti-miR-508-3p but partially repressed in HTR-8/SVneo cells transfected with anti-miR-508-3p + si-SMAD2 (Fig. 6A and 6B). Then, rescue experiment was conducted in these cells to determine the interplay between miR-508-3p and SMAD2. In function, the data from EdU assay and MTT assay presented that HG treatment-inhibited cell proliferative capacity was recovered by miR-508-3p inhibition but repressed by the reintroduction of SMAD2 (Fig. 6C and 6D). The expression of PCNA was largely recovered in HG-treated HTR-8/SVneo cells transfected with anti-miR-508-3p alone but partly repressed in HG-treated HTR-8/SVneo cells transfected with anti-miR-508-3p + si-SMAD2 (Fig. 6E). Flow cytometry assay presented that HG-induced cell cycle arrest and cell apoptosis were alleviated by the inhibition of miR-508-3p, while the reintroduction of SMAD2 partially promoted cell cycle arrest and cell apoptosis (Fig. 6F and 6G). The expression of Bcl-2 was enhanced in HG-treated HTR-8/SVneo cells transfected with anti-miR-508-3p alone but reduced by anti-miR-508-3p + si-SMAD2 transfection, while the expression of Bax was repressed in HG-treated HTR-8/SVneo cells transfected with anti-miR-508-3p alone but recovered by anti-miR-508-3p + si-SMAD2 transfection (Fig. 6H). These data manifested that miR-508-3p depletion alleviated HG-induced trophoblast cell injuries by promoting SMAD2.

**Discussion**

The risk of GDM is that babies are typically excessive birth weight or macrosomia [19]. To provide more strategies for GDM diagnosis and treatment, we investigated the role of circ_FOXP1 in the pathological model of GDM. The results presented that the expression of circ_FOXP1 was remarkably decreased in HG-treated HTR-8/SVneo cells transfected with anti-FOXO1, while the expression of circ_FOXP1 was alleviated by the inhibition of miR-508-3p, while the reintroduction of SMAD2 partially promoted cell cycle arrest and cell apoptosis (Fig. 6F and 6G). The expression of Bcl-2 was enhanced in HG-treated HTR-8/SVneo cells transfected with anti-miR-508-3p alone but reduced by anti-miR-508-3p + si-SMAD2 transfection, while the expression of Bax was repressed in HG-treated HTR-8/SVneo cells transfected with anti-miR-508-3p alone but recovered by anti-miR-508-3p + si-SMAD2 transfection (Fig. 6H). These data manifested that miR-508-3p depletion alleviated HG-induced trophoblast cell injuries by promoting SMAD2. Previous studies demonstrated that HG inhibited the
invasiveness of human trophoblast cells during implantation and placentation [20]. Besides, HG-induced trophoblast cell viability inhibition and cell apoptosis are crucial events in GDM, leading to the function loss of trophoblast cells [21]. Therefore, HG-induced HTR-8/SVneo cells were widely used as cell models of GDM pathological conditions in numerous studies [22, 23]. The data from circRNA expression profiles reveal that numerous circRNAs are differently expressed in umbilical cord blood exosomes, placenta tissues or placental villi of GDM patients [4, 24, 25], suggesting the wide expression of circRNAs. Nonetheless, the function of circRNAs in HG-treated HTR-8/SVneo cells was still lacking. We chose circ_FOXP1 whose expression was strikingly declined in placenta tissues of GDM patients [5]. Consistent with these data, our study discovered that circ_FOXP1 expression was also decreased in HG-treated HTR-8/SVneo cells. Functional analysis showed that HG-induced HTR-8/SVneo cell proliferation inhibition, cell cycle arrest and cell apoptosis were largely alleviated by circ_FOXP1 overexpression, suggesting that circ_FOXP1 enrichment might be a strategy for the

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**Fig. 4** Circ_FOXP1 overexpression alleviated HG-induced HTR-8/SVneo cell injuries by targeting miR-508-3p. (A) The expression of miR-508-3p in HTR-8/SVneo cells transfected with circ_FOXP1 alone or circ_FOXP1 + miR-508-3p was detected by qPCR. In HG-treated HTR-8/SVneo cells transfected with circ_FOXP1 alone or circ_FOXP1 + miR-508-3p, (B and C) cell proliferation was assessed by EdU assay and MTT assay. (D) The protein level of PCNA was detected by western blot. (E and F) Cell cycle progression and cell apoptosis were determined by flow cytometry assay. (G) The protein levels of Bcl-2 and Bax were ascertained by western blot. Three independent experiments were performed, with three duplications in each experiment; Data were expressed as mean ± S.D.; * p < 0.05.
treatment of GDM. Previous studies have stated the important role of circ_FOXP1 in cancer biology through multiple biological processes [8, 26]. Our study was the first to determine the potential role of circ_FOXP1 in GDM, which provided a new perspective into the understanding of GDM pathogenesis.

It was documented that oncogene SOX9-induced circ_FOXP1 promoted the development of hepatocellular carcinoma by acting as miR-875-3p/miR-421 sponges [26]. Following this manner, we further explored the target miRNAs of circ_FOXP1. MiR-508-3p was identified to be a target of circ_FOXP1 in this study. MiR-508-3p was a tumor suppressor in various cancers, and its enrichment suppressed the malignant behaviors of cancer cells [27, 28]. In terms of GDM, it was only mentioned that the expression of miR-508-3p was notably elevated in placental tissues of GDM patients [12]. Our study further explored the function of miR-508-3p in HG-treated HTR-8/SVneo cells and found that the inhibition of miR-508-3p relieved HG-induced HTR-8/SVneo cell proliferation inhibition, cell cycle arrest and cell apoptosis. Rescue experiments showed that miR-508-3p restoration reversed the effects of circ_FOXP1 overexpression, indicating that circ_FOXP1 prevented the progression of GDM partially by depleting miR-508-3p expression.

Additionally, our study proposed that miR-508-3p directly bound to SMAD2 3’UTR to inhibit SMAD2 expression. The role of SMAD2 in diabetes mellitus was frequently reported to be involved in transforming

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**Fig. 5** SMAD2 was a target of miR-508-3p. (A) The predicted binding site between miR-508-3p and SMAD2 3’UTR. (B) Their relationship was confirmed by dual-luciferase reporter assay. (C) The expression of miR-508-3p in HTR-8/SVneo cells transfected with miR-508-3p or anti-miR-508-3p was detected by qPCR. (D and E) The expression of SMAD2 mRNA and protein in HTR-8/SVneo cells transfected with miR-508-3p or anti-miR-508-3p was detected by qPCR and western blot. (F and G) The expression of SMAD2 mRNA and protein in HG-treated HTR-8/SVneo cells was detected by qPCR and western blot. (H and I) The expression of SMAD2 mRNA and protein in HTR-8/SVneo cells transfected with circ_FOXP1 alone or circ_FOXP1 + miR-508-3p was detected by qPCR and western blot. Three independent experiments were performed, with three duplications in each experiment; Data were expressed as mean ± S.D.; * p < 0.05.
growth factor beta (TGF-β)-mediated signaling pathways [29,30]. It was of great significance to explore the effects of SMAD2 in HG-treated HTR-8/SVneo cells. The expression of SMAD2 was notably reduced in HG-treated HTR-8/SVneo cells. SMAD2 knockdown recovered HG-induced HTR-8/SVneo cell dysfunctions that were relieved by miR-508-3p inhibition, which was consistent with the previous study whose results presented that the depletion of SMAD2 induced by miR-27a blocked the regular proliferation, migration and invasion of HTR-8/SVneo cells [15]. Our data indicated that miR-508-3p accelerated GDM progression by targeting SMAD2. Interestingly, circ_FOXP1 positively regulated SMAD2 expression by competitively targeting miR-508-3p and thus alleviating miR-508-3p-mediated inhibition on SMAD2 expression. The regulatory network of the circ_FOXP1/miR-508-3p/SMAD2 axis was established.

**Conclusion**

Collectively speaking, circ_FOXP1 expression was declined in HG-treated human trophoblast cells. In GDM pathological condition in vitro, low circ_FOXP1 expression inhibited trophoblast cell proliferation and cell cycle progression and promoted cell apoptosis through regulating the expression of miR-508-3p and downstream SMAD2 molecule (Fig. 7). It can be speculated from our present findings that circ_FOXP1 overexpression may be a promising strategy in the treatment of GDM via improving trophoblast cell functions.

**Declarations**

**Ethics approval and consent to participate**

The present study was approved by the ethical review committee of Xiangyang NO.1 People’s Hospital, Hubei University of Medicine. Written informed consent was obtained from all enrolled patients.
Consent for publication
Patients agree to participate in this work.

Availability of data and materials
The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Authors’ Contribution
Conceptualization and Methodology: Yuqin Huang and Hongli Xi; Formal analysis and Data curation: Wei Zhang, Ziwu Xiang and Lingyun Wang; Validation and Investigation: Hongyan Guo, Xianyu Li and Mingqun li; Writing-original draft preparation and Writing-review and editing: Mingqun li, Yuqin Huang, and Hongli Xi; Approval of final manuscript: all authors

Highlights
1. Low expression of circ FOXP1 is shown in HG-treated HTR-8/SVneo cells.
2. Circ FOXP1 overexpression promotes the proliferation and survival of HG-treated HTR-8/SVneo cells.
3. The miR-508-3p/SMAD2 is one of the regulatory networks of circ FOXP1.
4. Circ FOXP1 regulates gestational diabetes mellitus progression partly through the miR-508-3p/SMAD2 pathway.

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