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High glucose regulates the cells dysfunction of human trophoblast HTR8/SVneo cells by downregulating *GABRP* expression

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Abstract

Background. In response to the high-glucose environment in patients with gestational diabetes mellitus (GDM), trophoblast cells undergo a series of pathological changes. Gamma-aminobutyric acid type A receptor subunit pi (*GABRP*) is involved in the development of pregnancy-related diseases and regulation of blood glucose.

Objectives. To explore the relationship between *GABRP* and hyperglycemia stimulation in GDM patients, and to provide preliminary experimental evidence for whether *GABRP* has the potential as a molecular target for the treatment of GDM.

Materials and methods. Within 30 min after birth, placental samples were taken from 20 GDM patients and 20 pregnant women without GDM. Human chorionic trophoblast HTR-8/SVneo cells were utilized for in vitro experimental investigation. We explored changes in *GABRP* expression in placental samples and HTR-8/Svneo cells using western blot and quantitative reverse transcription polymerase chain reaction (RT-qPCR). Cells in the high-glucose treatment group were exposed to medium containing 25 mM glucose. To explore the relationship between *GABRP* and high-glucose stimulation, *GABRP* was overexpressed in HTR-8/SVneo cells. We monitored the cell viability, invasion and migration abilities using Cell Counting Kit-8 (CCK-8), transwell and scratch assays, respectively.

Results. We found that *GABRP* expression was significantly reduced in placental samples from GDM patients. Furthermore, high-glucose treatment decreased the expression level of *GABRP* in HTR-8/SVneo cells. High-glucose stimulation reduced the cell viability, invasion and migration abilities. *GABRP* overexpression reversed the biological dysfunction of the cells induced by high-glucose stimulation.

Conclusions. Hyperglycemia in GDM patients downregulates the expression of *GABRP*, and overexpression of *GABRP* promotes the viability, migration and invasive ability of HTR8-/SVneo cells.

Key words: gestational diabetes mellitus, *GABRP*, HTR8/SVneo trophoblast cell, cellular function

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Background

Gestational diabetes mellitus (GDM), defined as abnormal glucose tolerance that is first detected during pregnancy, is a common disease. The development of GDM has short- and long-term adverse effects on maternal and fetal health.[1,2] The placenta is a temporary but extremely important organ formed during pregnancy, which serves as the only nutrient transport channel between the mother and the fetus.[3] A healthy maternal environment promotes the growth, development and maturity of the placenta, thereby ensuring its normal function. Trophoblast cells are important constituent cells of the placenta.[4] During placental development, extravillous trophoblast cells with multiple cellular functions migrate and invade into the maternal decidua. Trophoblast cells are in direct contact with maternal tissues and are the first to be affected by changes in maternal blood glucose. In response to the high-glucose environment, trophoblast cells undergo a series of pathological changes. The impairment in trophoblast cells can also cause pathological changes in the placenta.

There are abnormally expressed genes in the placental tissue of GDM patients, and these genes may lead to placental dysfunction by regulating the function of trophoblast cells. Gamma-aminobutyric acid type A receptor subunit pi is the pi subunit of the inhibitory neurotransmitter gamma-aminobutyric acid (*GABA*) A receptor (*GABRP*). In addition to being expressed in nervous system, *GABRP* is also expressed in peripheral tissues, such as digestive tract, uterus and ovary.[5] Previous studies have found that *GABRP* is upregulated in the placenta during the first trimester.[6,7] Lu et al. found that upregulating the expression of *GABRP* in cells can significantly affect the invasion and apoptosis of placental trophoblast cells.[7] These results suggest that *GABRP* may participate in the occurrence and development of pregnancy-related diseases by regulating trophoblast function. Purwana et al. found that *GABA* increases β-cell mass and improves glucose homeostasis by binding to *GABRP*.[8]

In this study, we hypothesized that there might be a potential association between changes in *GABRP* expression and GDM. We investigated the expression level of *GABRP* in the placental tissue from patients with GDM. The HTR-8/SVneo trophoblast cells were cultured in vitro, and the effects of high-glucose culture on the expression level of *GABRP* and their cell functions were detected.

Objectives

The purpose of this study was to determine the relationship between *GABRP* and hyperglycemia stimulation in GDM patients, and to provide preliminary experimental evidence for whether *GABRP* has the potential as a molecular target for the treatment of GDM.

Materials and methods

Clinical samples

From January 2019 to May 2020, 20 GDM patients (GDM group) were admitted to the Department of Obstetrics at the Second Affiliated Hospital of Wenzhou Medical University (Zhejiang, China). Another 20 healthy pregnant women without GDM were used as the control group. In this study, GDM cases were diagnosed according to the recommended guidelines for the diagnosis of GDM in China.[9] Placental tissue was collected from GDM patients and healthy pregnant women within 30 min of delivery. Tissue samples were cleaned with phosphate-buffered saline (PBS) and then kept in liquid nitrogen for subsequent use. The differences in age, gestational weeks, body mass index (BMI), and fasting blood glucose between the 2 groups are summarized in Table 1. The average fasting blood glucose in the GDM group was 5.04 mmol/L, which was higher than 4.16 mmol/L in the control group. The research protocol was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University (approval No. LCKY2019-287). All participants signed informed consent prior to the study.

Cell culture and treatment

Human villous trophoblasts HTR-8/SVneo cells were purchased from the Cell Bank of Chinese Academy of Sciences (Beijing, China). HTR-8/SVneo cells were grown adherently in 10% fetal bovine serum (FBS) Dulbecco's modified Eagle's medium (DMEM) medium (Life Technology, Guangzhou, China), and incubated in a humidity-saturated incubator containing 5% CO_2 at 37°C. Twenty-four hours after plating, cell culture media were starved for 24 h. Cells were divided into 2 groups: high-glucose

Table 1. Demographic and clinical characteristics of the study subjects

Parameter	Control group (n = 20)	GDM group (n = 20)	Mann–Whitney U test	p-value
Age [years]	30.58 ±3.73	31.60 ±4.09	223.000	0.547
Gestational age [weeks]	38.15 ±1.33	38.02 ±1.74	189.000	0.779
BMI [kg/m^2]	24.13 ±1.92	24.03 ±2.21	188.000	0.758
Fasting blood glucose [mmol/L]	4.16 ±0.49	5.04 ±1.09	312.500	0.002

Data were expressed as mean ± standard deviation (M ±SD); BMI – body mass index.

induction group (HG) and normal concentration glucose group (control). Cells in the HG group were exposed to medium containing 25 mM glucose, while cells in the control group were normally cultured in medium containing 5 mM glucose.

Cell transfection

To overexpress *GABRP*, HTR-8/SVneo cells were subjected to transient transfection. The *GABRP* gene was cloned into the pcDNA3.1 plasmid (Gene Pharma, Shanghai, China). Cells were divided into 3 groups: the overexpression (OE) of *GABRP* group (transfected with the pcDNA3.1-*GABRP* plasmid), the negative control (NC) group (transfected with the empty pcDNA3.1 plasmid) and the blank control group (untransfected cells). The manufacturer's instructions for Lipofectamine 3000 (Invitrogen Life Technologies, Carlsabad, USA) were properly followed throughout the transfection process. After transfection, the cell culture medium of each group was replaced. After 48 h culture, total cell RNA and protein were collected to evaluate transfection efficiency.

Cell migration assay

HTR-8/SVneo cells were seeded on the 6-well plates for cell scratch test. When the cells reached >80% confluency, the scratch line was gently scraped out with a 1 mL pipette tip, and the exfoliated cells were removed. The culture media was switched out for one that contained a high concentration of glucose. Scratch images were photographed at 0 h and 24 h using an inverted microscope (Axiovert 40 CFL; Carl Zeiss AG, Jena, Germany) and the cell migration rate was calculated.

Transwell invasion assay

An amount of 50 µL of diluted Matrigel (BD Biosciences, San Jose, USA) was evenly and carefully spread on the bottom surface of each upper chamber, and then air-dried. Cells were resuspended in FBS-free DMEM medium. Then, 200 µL of cell suspension and 700 µL of culture medium was added to the upper and lower chambers, respectively. After continuing to culture for 24 h, the transwell chamber was removed for the next experiment. The transwell chamber was fixed with 4% paraformaldehyde for 5 min and finally stained with 0.1% crystal violet for 1 min. After natural drying, 3 fields of view were randomly selected and photographed at a ×100 magnification to count the number of migrated cells. The average number of migrated cells was calculated and the above experiment was repeated 3 times.

Western blot analysis

After treatment, the cells were washed and lysed. Protein sample concentrations were measured to adjust loaded samples to contain the same concentration of protein. Protein samples were added to the wells of the gel for separation, and the separated proteins are transferred to polyvinylidene difluoride (PVDF) membranes by electrotransfer. The washed membrane was blocked in blocking buffer for 2 h at room temperature (RT). The corresponding primary antibodies were added to the washed membrane and incubated overnight at 4°C. The following primary antibodies were used in this study: *GABRP* (1:1000; Invitrogen) and *β-actin* (1:1000; Beyotime Biotechnology, Shanghai, China). After washing, the appropriate secondary antibody (1:2000; Boster, Wuhan, China) was added to the membrane and incubated at room temperature for 1 h. The fluorescent signal of the bands was detected and the gray value was analyzed using ImageJ software (National Institutes of Health, Bethesda, USA).

Quantitative real-time PCR

Placental tissue samples and cell plates were taken out, and the total RNA was isolated using Trizol method. The following tests were conducted strictly in line with the kit's manufacturer's instructions (Takara, Shiga, Japan). Beta-actin served as an internal benchmark. The relative mRNA expression of *GABRP* was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences relevant to the study are included in Table 2.

Table 2. Sequences of quantitative reverse transcription polymerase chain reaction (RT-qPCR) primers

Gene name	Primer	Sequence 5′ > 3′
β-actin	forward	GCTGTGCT ATCCCTGTACGC
	reverse	TGCCTCAGGGCAGCGGAACC
GABRP	forward	TTTCTCAGGCCCAATTTTGGT
	reverse	GCTGTCGGAGGTATATGGTGG

Statistical analyses

The experimental data were expressed as mean ± standard deviation (M ±SD). IBM SPSS v. 25.0 statistical software IBM Corp., Armonk, USA)was used for statistical analysis. The student's t-test or one-way analysis of variance (ANOVA) was used for comparison between groups. A p-value <0.05 indicates that the difference is statistically significant.

Results

GABRP expression decreased in placental tissues of GDM patients

As shown in Fig. 1, the expression levels of *GABRP* protein and mRNA in the placenta of GDM patients were lower than those of healthy pregnant women ($p < 0.001$).

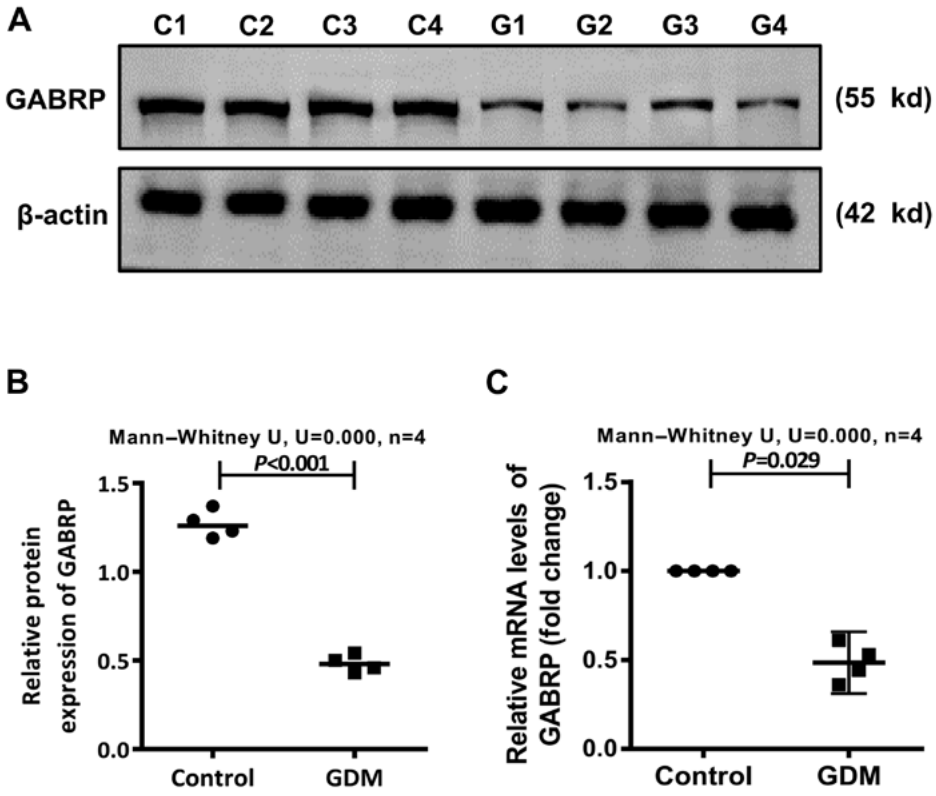

Fig. 1. Gamma-aminobutyric acid type A receptor subunit pi (*GABRP*) expression decreased in placental tissues of gestational diabetes mellitus (GDM) patients. A. Representative western blot bands of *GABRP*. Beta-actin served as an internal control; B. The ratio of *GABRP/β-actin* was used to quantify protein levels; C. The mRNA level of *GABRP* in placental tissue was assessed using quantitative reverse transcription polymerase chain reaction (RT-qPCR). Each experiment was repeated 3 times and the data were expressed as mean ± standard error of the mean (M ±SEM)

***p < 0.001 compared to control.

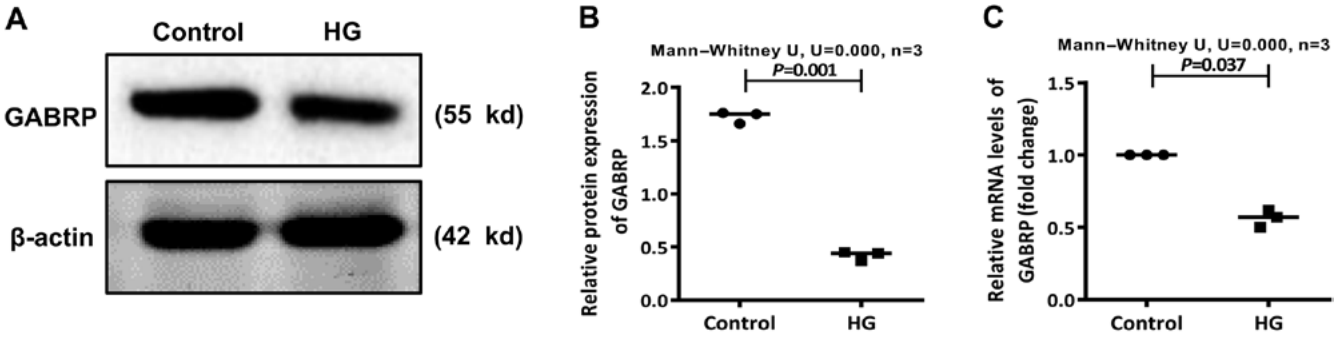

Fig. 2. High-glucose stimulation reduced the expression of gamma-aminobutyric acid type A receptor subunit pi (*GABRP*) in HTR-8/SVneo cells. A. Representative western blot bands of *GABRP*. Beta-actin served as an internal control; B. The ratio of *GABRP/β-actin* was used to quantify protein levels; C. The mRNA level of *GABRP* in cells was assessed using quantitative reverse transcription polymerase chain reaction (RT-qPCR)

**p < 0.01; ***p < 0.001 compared to control.

That is, the expression of *GABRP* in the placental tissue samples of GDM patients is reduced.

High-glucose stimulation reduced the expression of *GABRP* in HTR-8/SVneo cells

After 25 mM glucose treatment, the expression of *GABRP* protein was lower than that of the control group ($p < 0.01$; Fig. 2A,B). Similarly, the expression of *GABRP* mRNA was decreased in HTR-8/SVneo cells cultured with high-glucose ($p < 0.001$; Fig. 2C). Overall, high-glucose (25 mM) treatment decreases *GABRP* expression in cells.

High-glucose stimulation inhibited cell viability, invasion and migration

To investigate whether high-glucose stimulation can modulate cellular function, we examined the cell viability, invasion and migration abilities using Cell Counting Kit-8 (CCK-8), transwell and scratch assays. As shown in Fig. 3A, when compared to the control group, there was no difference in the cell viability in the 24 h group ($p > 0.05$), whereas it decreased in the 48-h and 72-h groups ($p < 0.001$). From the results of tranwell and scratch assays ($p < 0.01$; Fig. 3B–E), it can be seen that after high-glucose culture, the invasion and migration ability decreased. The above results indicate that high-glucose stimulation can affect the cellular function.

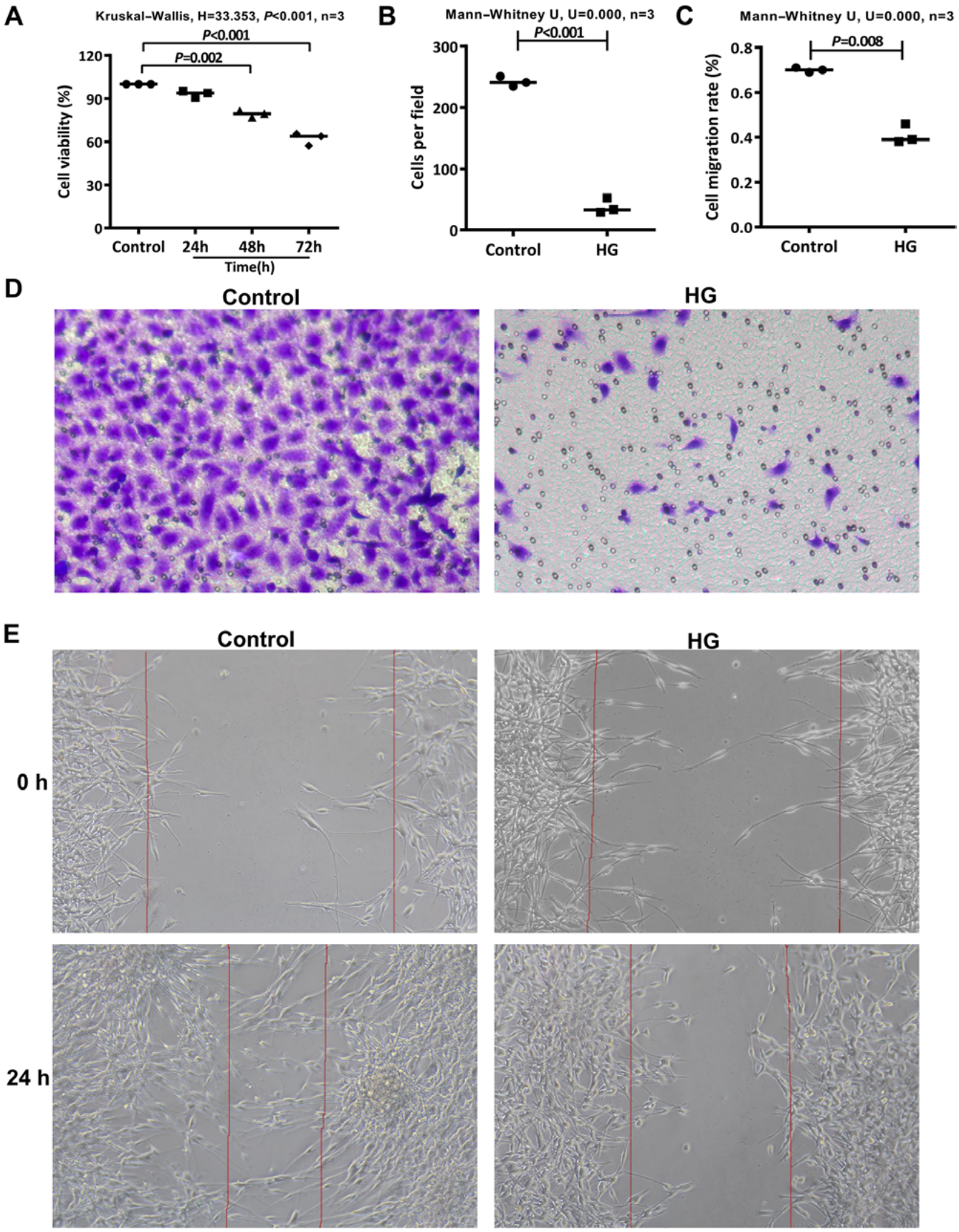

Fig. 3. High-glucose stimulation inhibited cell viability, invasion and migration of HTR-8/SVneo cells. A. The cells viability of HTR-8/SVneo cells at different timepoints (24 h, 48 h or 72 h) cultured in high glucose was detected with Cell Counting Kit-8 (CCK-8) assay; B. Quantification of invasive cell number; C. Graph indicating the rate of cell migration; D. Images of the transwell invasion assay; E. Representative photographs of scratch wounds at point 0 and 24 h of the cell migration assay (magnification ×100). Red lines depict the edges of the wound

**p < 0.01; ***p < 0.001 compared to control.

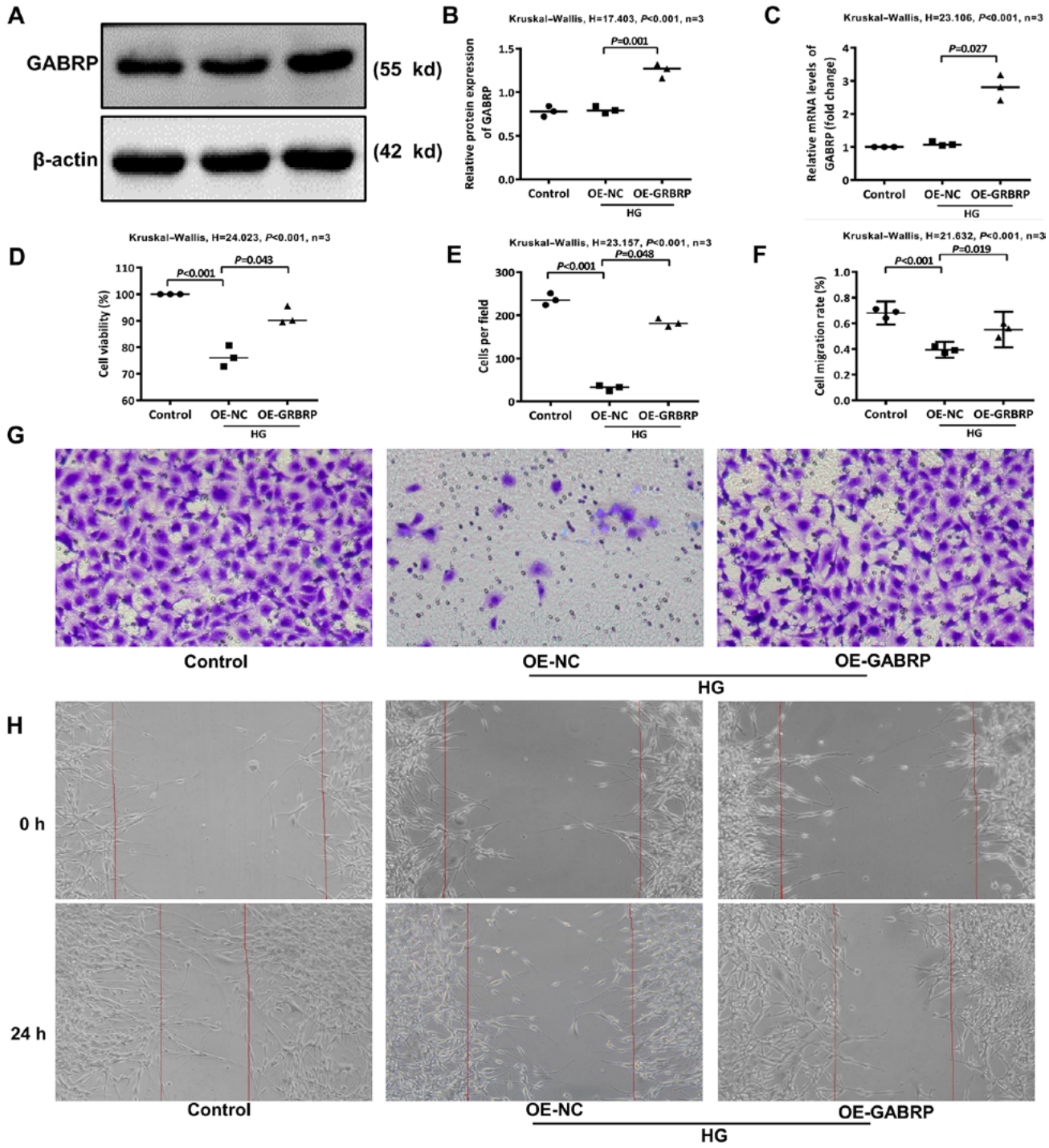

Fig. 4. Gamma-aminobutyric acid type A receptor subunit pi (*GABRP*) overexpression reversed the effect of high glucose stimulation on the biological function of HTR-8/SVneo cells. A. Representative protein bands showing the protein expression of *GABRP*. Beta-actin was used as a loading control; B. The ratio of GABRP/β-actin was used to quantify protein levels; C. The relative mRNA expression level of *GABRP*; D. Cell viability of each group detected using the Cell Counting Kit-8 (CCK-8) assay; E. Number of transwell cells was quantified; F. Cell migration rates were assessed using the scratch test method; G. Representative image of transwell; H. Representative images of the scratch test

$^{**}p < 0.01$; $^{***}p < 0.001$ compared to control; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ compared to control.

GABRP overexpression reverses the effects of high-glucose stimulation on cellular function

To explore the relationship between *GABRP* and high-glucose stimulation, *GABRP* was overexpressed by transfection. As shown in Fig. 4A–C, the levels of *GABRP* protein and mRNA expression were considerably higher in the OE-*GABRP* group compared to the OE-NC group ($p < 0.001$). The cell viability, invasion and migration abilities were altered after *GABRP* overexpression. Comparing with the HG+OE-NC group, the cellular function of HG+OE-*GABRP* group was

improved ($p < 0.01$; Fig. 4D–H). In conclusion, overexpression of *GABRP* reverses the functional impairment induced by high-glucose stimulation.

Discussion

Before the fetus matures, the placenta is an important place for the fetus to absorb nutrients from the mother. In addition, the placenta is also a crucial endocrine organ, and the development of the placenta affects the outcome of pregnancy.[10,11] Trophoblast cells are the main constituent cells of the placenta and serve important functions.[12] During placental development, trophoblast cells secrete a large number of hormones and cytokines.[13,14] When the cellular function of trophoblasts is abnormal, it will cause a variety of pregnancy diseases. The biological behavior of trophoblasts and related regulatory mechanisms are key physiological events to maintain placental homeostasis.[15] However, an imbalance of these processes can impair placental function under pathological conditions. In this study, the cell viability, invasion and migration abilities were inhibited after high-glucose stimulation.

Patients with GDM are have hyperglycemia, which increases the incidence of macrosomia.[16] When hyperglycemia is severe, it is more likely to lead to early miscarriage, defects in placenta formation, intrauterine growth retardation, and malformations.[17] This study found that the viability of trophoblast cells cultured in high glucose in vitro decreased. The results are consistent with the study by Zhang et al.[18] High-glucose-induced reduction in viability of trophoblast cells is involved in the pathogenesis of GDM. However, in clinical practice, the majority of GDM patients exhibit excessive placental tissue growth and give birth to macrosomic infants. It is reasonable to speculate that this difference may be related to the concentration of glucose used to culture cells in vitro. The concentration of glucose used in this experiment is 25 mmol/L, which is mainly used for in vitro cellular studies of type 2 diabetes.[19,20] Patients with GDM are usually considered to have mild type 2 diabetes.[21] People with type 2 diabetes before pregnancy are more likely to have adverse pregnancy outcomes.[22,23] There is no clear cutoff value for hyperglycemia leading to adverse pregnancy outcomes, but it is positively correlated with the degree of elevated blood glucose.[24] Therefore, the conclusions of this study are more applicable to GDM patients with poor blood sugar control.

Islet cells secrete *GABA* at a relatively constant rate, which is regulated by the metabolic state of the cells.[25] Purwana et al. showed that *GABA* participates in the improvement of human β-cell mass by combining with *GABRP*, which may be beneficial for the treatment of diabetes.[8] In the reproductive system, there are many studies on the role of *GABRP* in the uterus. Lu et al. found that changing the expression of *GABRP* in cells can significantly affect the invasion function and apoptosis behavior of trophoblast cells.[7] In this study, by comparing the expression of *GABRP* in the placenta of GDM and normal pregnant women, it was found that the expression of *GABRP* in the former was abnormally reduced. Meanwhile, the expression level of *GABRP* was decreased in HTR-8/SVneo cells cultured under high-glucose conditions. After overexpressing *GABRP*, the biological behavior of trophoblast cells altered. The results showed that *GABRP* could reverse the functional impairment of HTR-8/SVneo cells induced by high-glucose stimulation.

Limitations

We speculated that the high-glucose environment may inhibit cellular function by downregulating the expression of *GABRP*. It is suggested that *GABRP* differentially expressed in clinical samples of GDM patients may be involved in adverse pregnancy outcomes. The upstream and downstream signaling molecules of *GABRP* are not clear. Therefore, further mechanistic exploration and etiological studies are urgently needed to confirm.

Conclusions

The state of hyperglycemia in GDM patients downregulates the expression of *GABRP*, and overexpression of *GABRP* promotes the viability, migration and invasive ability of HTR8-SV/neo cells.

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