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RESEARCH

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Human umbilical cord mesenchymal stem cell-derived exosomal miR-455-3p targets CAMK2N1 to improve trophoblast cell injury in gestational diabetes mellitus

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Abstract

Objective We aimed to probe the effect of microRNA (miR)-455-3p derived from human umbilical cord mesenchymal stem cell exosomes (hucMSCs-Exos) in targeting calcium/calmodulin-dependent protein kinase 2 inhibitor 1 (CAMK2N1) to mitigate trophoblast cell injury in gestational diabetes mellitus (GDM).

Methods hucMSCs were cultured, and Exos were isolated and characterized using transmission electron microscopy and Western blot (WB) for exosomal surface markers CD63, TSG101, and Calnexin. An in vitro GDM model was established by exposing human trophoblast cells (HRT-8/SVneo) to high glucose (HG), followed by intervention with Exos or overexpression of CAMK2N1. RT-qPCR or WB was applied to test miR-455-3p and CAMK2N1 expression levels. CCK-8 assay was adopted to assess cell proliferation, Transwell assay was applied to test cell migration, and flow cytometry was implemented to assess cell apoptosis. Bioinformatics websites and a dual-luciferase reporter gene assay were conducted to verify the targeting relationship between miR-455-3p and CAMK2N1.

Results The successfully isolated Exos expressed the exosomal markers CD63 and TSG101. Treatment with hucMSCs-Exos and hucMSCs-Exos carrying miR-455-3p mimic enhanced the migration and proliferation of HG-induced HRT-8/SVneo cells while reducing cell apoptosis. In contrast, the miR-455-3p inhibitor and overexpression of CAMK2N1 reversed these protective effects, leading to decreased cell migration and proliferation and increased apoptosis. Furthermore, bioinformatics analysis and experimental validation confirmed that miR-455-3p directly targeted and negatively regulated CAMK2N1.

Conclusion miR-455-3p derived from hucMSCs-Exos exerts a protective effect against trophoblast cell injury in GDM by targeting and downregulating CAMK2N1.

Keywords Human umbilical cord mesenchymal stem cell, Exosome, MicroRNA-455-3p, Calcium/calmodulin-dependent protein kinase 2 inhibitor 1, Trophoblast cell, Gestational diabetes mellitus

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Introduction

Gestational diabetes mellitus (GDM) is the most common metabolic complication during pregnancy, characterized by glucose intolerance that first emerges or is detected during pregnancy. This condition is concerned with adverse pregnancy outcomes and long-term health risks for both the mother and child [1]. As the fastest-growing from of diabetes, GDM has become a escalating public health concern, impacting between 2 and 38% of pregnant women worldwide [2, 3]. Additionally, trophoblast cells, which are essential for placental development in early pregnancy, can suffer dysfunction in GDM, leading to impaired embryo implantation and pregnancy-related complications such as recurrent spontaneous abortion [4]. Therefore, finding effective therapeutic strategies is vital to protect maternal and fetal health.

Human umbilical cord mesenchymal stem cells (hucMSCs) have self-renewal and multipotential differentiation properties, making them valuable for regenerative medicine. Their non-invasive collection and low immunogenicity further enhance their clinical utility. Recently, hucMSCs have been extensively explored for therapeutic application [5]. Exosomes (Exos) derived from hucMSCs have emerged as a promising tool for disease treatment due to their role in intercellular communication and ability to regulate various biological processes [6]. These extracellular vesicles, measuring 40–100 nm in diameter and with a density of 1.13–1.19 g/mL, contain diverse biomolecules, including proteins, messenger RNAs (mRNAs), microRNAs (miRNAs) and deoxyribonucleic acid (DNA). By delivering these biomolecules to recipient cells, Exos modulate their biochemical properties and cellular functions. Compared with other MSCs-Exos, hucMSC-Exos offer advantages such as non-invasive collection, a high proliferation rate, and low immunogenicity, making them widely used in regenerative medicine and disease treatment [7]. Recent studies have highlighted the involvement of exosomal miRNAs in the pathogenesis of GDM. miRNA-455-3p (miR-455-3p) belongs to a highly conserved miRNA family expressed across various species [8]. Previous research has identified miR-455-3p as a direct target of circ_0000491, demonstrating that the inhibition of miR-455-3p reverses the suppressive effects of circ_0000491 in high glucose (HG)-treated SV40-MES13 cells. Knockdown of circ_0000491 has been shown to alleviate HG-induced fibrosis, inflammation, apoptosis, and oxidative stress via the miR-455-3p/Hmgb1 axis in SV40-MES13 cells [9]. Whereas, studies investigating the specific role of miR-455-3p in GDM remains limited, underscoring the novelty of this study. Calcium/calmodulin-dependent kinase 2 (CAMK2) is a key regulator of intracellular calcium signaling and is involved in activating downstream pathways that control various cellular functions. Dysregulation of CAMK2 activity has been implicated in multiple disorders, including cancer, metabolic disorders, and inflammatory conditions, suggesting that CAMK2 inhibitors could have therapeutic potential [10]. Overexpression of inhibitor 1 of protein phosphatase 1 attenuates hyperglycemia-induced cardiomyocyte injury by correcting CaMK2δ alternative splicing disorders, inhibiting CaMK2 oxidation, decreasing reactive oxygen species accumulation, and inhibiting necrosis [11]. CAMK2 inhibitor 1 (CAMK2N1) and CAMK2N2 (also known as CAMKIINα and β) are endogenous inhibitors of CAMK2 [12]. Although studies directly linking CAMK2N1 to specific diseases are scarce, its close relationship with CaMK2I suggests that it may play a role in various disorders involving cell signaling pathways. In the present study, a bioinformatics analysis identified a putative miR-455-3p binding site in the 3′-untranslated region (UTR) of CAMK2N1, suggesting a potential regulatory interaction. Based on this evidence, we hypothesized that hucMSC-derived exosomal miR-455-3p could target CAMK2N1, thereby alleviating trophoblast cell injury in GDM.

Materials and methods

Culture of hucMSCs

hucMSCs at passage 3 were obtained from iCell Bioscience Inc. (HUM-iCell-e011, China). The cells were cultured in DMEM/F12 (Gibco, A4192001, USA) supplemented with 10% Exo-depleted fetal bovine serum (FBS) (Gibco, A2720801, USA). As previously described, hucMSCs at passages 4 to 6 were utilized for subsequent experimental procedures. The culture medium was further supplemented with 100 ng/mL of penicillin and 100 U/mL of streptomycin. The cells were maintained under normoxic conditions (21% O_2, 75% N_2, and 5% CO_2) at 37 °C.

Isolation and identification of hucMSCs-derived Exos

To isolate Exos, dead cells and debris were first removed through a series of centrifugation steps. The supernatant was then processed using a 100 kDa ultrafiltration tube (Millipore, Billerica, MA, USA) and subsequently subjected to ultracentrifugation at 1,000,000 g. The supernatant was discarded, and the resulting precipitate was resuspended in phosphate-buffered saline (PBS), filtered through a 0.22 μm filter (Millipore), and kept at − 80 °C for subsequent use. Transmission electron microscopy (TEM) was implemented to observe the morphology of the Exos. The protein content of the

concentrated Exo suspension was quantified using a BCA protein assay kit (23,227, Thermo Fisher Scientific, USA). Western blot (WB) analysis was conducted to confirm the presence of exosomal surface markers CD63, TSG101, and Calnexin.

Nanosight nanoparticle tracking analysis (NTA)

To determine the size distribution of the Exos, 20 μg of Exos were resuspended in 1 mL of PBS and vortexed for 1 min to ensure uniform distribution. The sample was then analyzed using a NTA Analyzer (Malvern Instruments Ltd., UK) to measure the Exo size and concentration.

Exo fluorescent labeling and cellular uptake assay

To confirm the internalization of hucMSC–Exos by HRT-8/SVneo cells, Exos were labeled with PKH26 (red fluorescence dye) (Sigma-Aldrich, Germany) and incubated with the cells at a concentration of 100 μg/mL for 24 h at 37 °C. After incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min.

For colocalization analysis, the fixed cells were permeabilized with 0.5% Triton X-100 for 10 min and incubated overnight at 4 °C with a primary antibody against LAMP1 (lysosomal marker, 1:2000, Cell Signaling Technology). After washing, the cells were further incubated with Alexa Fluor 488-conjugated secondary antibody (1:500, Invitrogen) for 1 h at room temperature. Nuclei were counterstained with DAPI, and Exo uptake and colocalization with LAMP1 were analyzed using a laser scanning confocal microscope (Olympus FLUOVIEW FV3000).

Culture and treatment of human trophoblast cells

The human trophoblast cell line HRT-8/SVneo was bought from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium encompassing 10% FBS (Thermo Fisher, Waltham, MA, USA) and maintained in a 37 °C incubator with 5% CO_2. To stimulate the hyperglycemic environment associated with GDM, cells were grown in the HG medium containing 25 mM glucose, whereas the normal control group was maintained in normal glucose conditions (5 mM) [13].

An expression vector encoding CAMK2N1 was constructed and coloned into the pcDNA3.1 vector (oe-CAMK2N1), with the empty pcDNA3.1 vector serving as a negative control (oe-NC). Both vectors were synthesized by GenePharma (Shanghai, China). The miR-455-3p mimic and its corresponding NC were established by Ribo Biotechnology Co., Ltd. (Guangzhou, China). Lipofectamine 2000 reagent was utilized to transfect miR-455-3p inhibitor, inhibitor NC, oe-NC, and oe-CAMK2N1 into HRT-8/SVneo cells. Additionally, miR-455-3p mimic and mimic NC were transfected into hucMSCs, followed by the isolation of Exos from the modified hucMSCs. For co-culture experiments, Exo protein content (100 μg/mL) was added to HG-induced HRT-8/SVneo cells and incubated for 24 h.

Cell counting kit-8 (CCK-8) assay

After 48 h of cell treatment, HRT-8/SVneo cells were inoculated into 96-well plates, and cell proliferation was assessed using a CCK-8 kit (Beyotime, Shanghai, China). At different time points (24 h, 48 h, and 72 h), CCK-8 reagent (10 μL) was dispensed to each well. After incubation under light-protected conditions, the optical density was measured at 450 nm using a microplate reader (Molecular Devices, CA, USA).

Transwell assay

A Transwell experiment was conducted to assess cell migration ability. The upper chamber of the Transwell insert was pre-coated with serum-free DMEM, ensuring an even distribution of the gel, and incubated at 37 °C for 1 h. Then, 200 μL of cell suspension (resuspended in serum-free medium) was added to the upper chamber. The lower chamber was filled with 500 μL of medium containing 25% FBS to create a chemotactic gradient, After incubation, the inserts were carefully removed and washed with PBS. The non-migrated cells on the upper side of the membrane were removed using a wet cotton swab. The migrated cells on the lower side of the membrane were fixed with 4% paraformaldehyde for 30 min, washed three times with PBS, and stained with hematoxylin for 10 min. Following another PBS wash, five randomly selected fields were imaged under a light microscope to quantify the number of migrated cells.

Flow cytometry

Apoptosis was measured by flow cytometry using the Annexin V-FITC/PI Apoptosis Detection Kit (Bestbio, China). Briefly, HRT-8/SVneo cells from each experimental group were harvested, washed, and stained with Annexin V-FITC and PI in a dark environment, and then were tested by flow cytometry (BD FACS Calibur, BD Biosciences, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Cells were lysed with Trizol reagent (Invitrogen, Carlsbad, CA, USA) to extract the total RNA. The spectrophotometer was employed to test RNA concentration and purity. Subsequently, PrimeScript RT reagent (TaKaRa, Shiga, Japan) was adopted to reverse transcribe RNA into cDNA. Quantitative polymerase

chain reaction (qPCR) was performed using the SYBR-Green I Premix Kit (Invitrogen) on a 7300 Real-Time Fluorescent Quantitative PCR System (Applied Biosystems, USA) to quantify the relative expression levels of target RNAs. The relative gene expression levels were quantified by the $2^{-\Delta\Delta CT}$ method, with U6 and GAPDH serving as internal references. The primers applied for RT-qPCR are presented in Table 1.

WB assay

RIPA lysis buffer (Solarbio) with protease inhibitor PMSF (Solarbio) was employed to obtain proteins from the cells, and the BCA protein assay (Vazyme) was applied to test the total protein concentration. The extracted proteins were mixed proportionally with the loading buffer (Life Technologies) and subjected to boiling at 100 °C for 8 min. Based on protein concentration, equal amounts of protein samples were were loaded onto a 10% SDS-PAGE gel for electrophoretic separation. The proteins on the gel were subsequently transferred to a PVDF membrane (Millipore) and blocked in 5% skimmed milk for 90 min. Subsequently, the membrane underwent overnight incubation with primary antibodies against CD63 (1: 1000), anti-TSG101 (1: 2000), anti-CAMK2N1 (1: 1000), anti-Calnexin (1: 2000) and anti-GAPDH (1: 5000). After washed with buffer, the membrane underwent incubation with secondary antibody goat anti-rabbit IgG (1: 5000, Abcam) at ambient temperature for 2 h. Following three additional washes, an enhanced chemiluminescence (ECL) solution was applied to detect protein signals. Finally, the gray values of protein bands were quantitatively analyzed using Image J software.

Dual-luciferase reporter gene

A potential miR-455-3p binding site in the 3'-UTR of CAMK2N1 was found using StarBase (https://rnasysu.com/encori/). A dual-luciferase reporter gene was employed to validate the interaction between miR-455-3p and CAMK2N1. The mutant (MUT) 3'-UTR and wild-type (WT) sequences of CAMK2N1 3'-UTR were cloned into the luciferase reporter vector PMIR-RB-REPORT (RiboBio). These recombinant vector was co-transfected with miR-455-3p mimic or mimic NC into HRT-8/SVneo cells using Lipofectamine 2000 (Invitrogen). Relative luciferase activity was assayed by applying a dual-luciferase reporter system (Promega, Wisconsin, USA) and normalized to Renilla luciferase activity.

Statistics

For graphical representation and statistical analysis, GraphPad Prism 9.4.0 (GraphPad Software Inc.) and SPSS 22.0 (SPSS Inc.) were employed. Data were exhibited as mean ± standard deviation (mean ± SD). To compare two groups, the t-test was implemented, while for comparisons involving multiple groups, one-way ANOVA followed by Tukey's post hoc test was applied. Statistical significance was set at $P<0.05$, and each experiment was conducted in triplicate.

Results

Identification of hucMSCs-Exo

hucMSCs-Exos were successfully isolated via ultracentrifugation. TEM revealed that the hucMSCs-Exos exhibited low-electron density components and maintained intact vesicular structures enclosed within the cell membrane (Fig. 1A). WB results displayed the presence of exosomal markers CD63 and TSG101 in hucMSCs-Exos, whereas Calnexin, an endoplasmic reticulum marker, was absent, indicating the successful isolation of Exos (Fig. 1B). NTA demonstrated that the isolated hucMSCs-Exos ranged in size from 80–200 nm, further confirming their identity. Next, we tested whether hucMSCs-Exo could be internalized into HRT-8/SVneo cells. The hucMSCs-Exo were labeled with PKH26 (red fluorescence dye) and co-cultured with HRT-8/SVneo cells, with lysosomes labeled using LAMP1. Confocal microscopy revealed that PKH26-labeled Exos accumulated in the perinuclear region of HRT-8/SVneo cells, with partial colocalization between PKH26-labeled Exos and LAMP1 (a lysosomal marker), indicating successful internalization of hucMSCs-Exos by trophoblast cells (Fig. 1C).

hucMSCs-Exos ameliorate trophoblast cell injury in GDM

To probe the protective effects of hucMSCs-Exos against GDM-induced trophoblast cell injury, HRT-8/SVneo cells were exposed to a HG environment to establish an in vitro GDM model, followed by

Table 1 Primer sequences

Gene	Primer sequence	
miR-455-3p	Forward: 5'-GCAGTCCATGGGCATATACAC-3'	Universal primer
CAMK2N1	Forward: 5'-GGACACCAACAACTTCTTCGGC-3'	Reverse: 5'-GTCGGTCATATTTTTCAGCACGTC-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3'	Reverse: 5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	Forward: 5'-CTATAAATTGAGCCCGCAGCC-3'	Reverse: 5'-GCCCAATACGACCAAATCCGT-3'

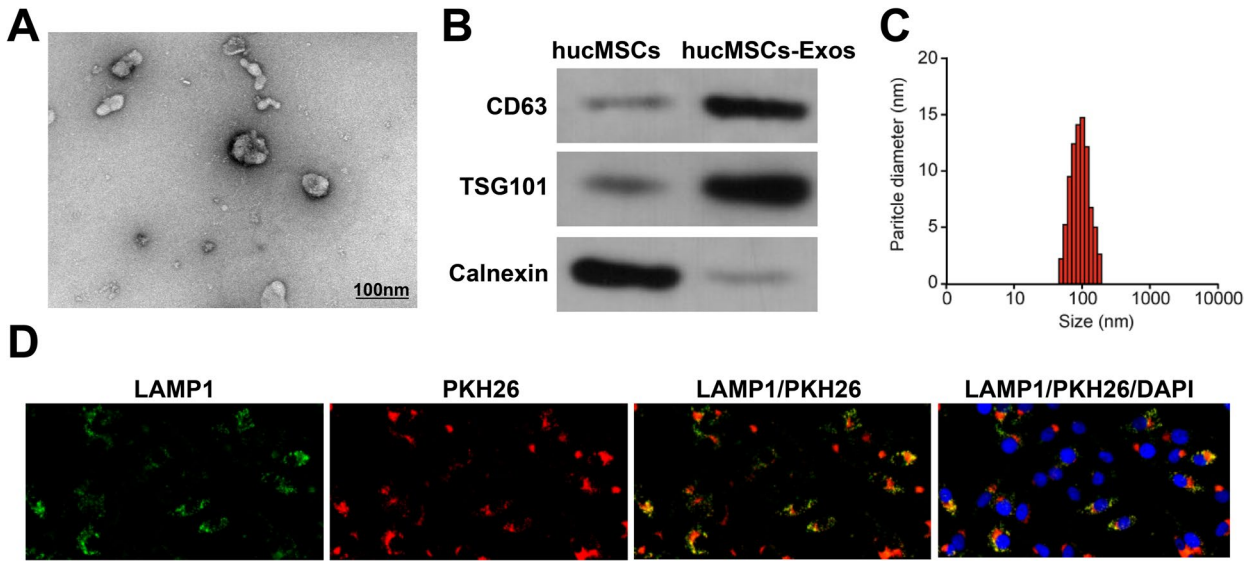

Fig. 1 Identification of hucMSCs-Exo. **A** Transmission electron microscopy observation of isolated hucMSCs-Exo; **B** Western blot detection of hucMSCs-Exo surface markers; **C** NTA detection of diameter distribution and concentration of extracellular vesicles; **D** Exo uptake assay observation of hucMSCs-Exo internalization into HRT-8/SVneo cells. All experiments were repeated more than three times

co-cultivation with hucMSCs-Exos for 24 h. The functional assays uncovered that compared with the Control group, HRT-8/SVneo cells in the HG-induced group (HG group) exhibited decreased migration and proliferationand raised apoptosis. However, after intervention with hucMSCs-Exos, cell migration and proliferation enhanced, and cell apoptosis diminished (Fig. 2A–C). RT-qPCR results indicated that by comparison with the Control group, miR-455-3p expression in the HG group was reduced, while after treatment with hucMSCs-Exos, its expression was elevated (Fig. 2D).

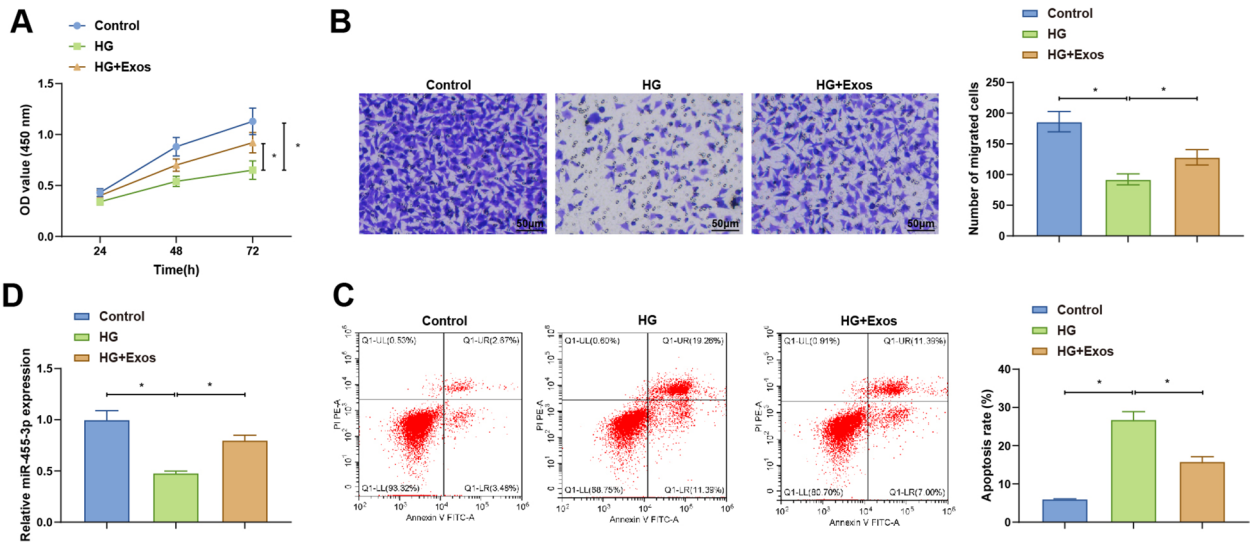

Fig. 2 hucMSCs-Exos ameliorate trophoblast cell injury in GDM. **A** CCK-8 assay for cell proliferation; **B** Transwell assay for cell migration; **C** Flow cytometry for cell apoptosis; **D** RT-qPCR for miR-455-3p expression level. Analysis of Variance (ANOVA) was adopted for comparisons among multiple groups, with Tukey's test as the post hoc test. * indicates $P < 0.05$. All experiments were repeated more than three times

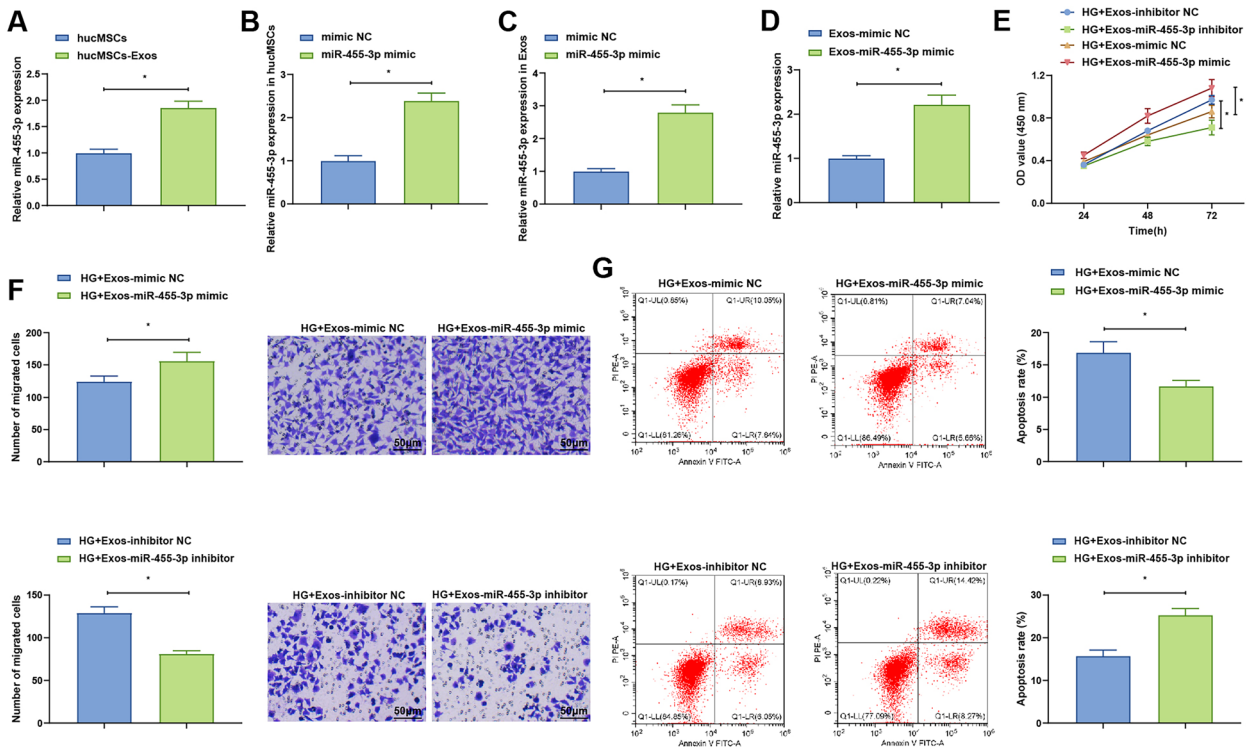

Fig. 3 miR-455-3p derived from hucMSCs-Exos improves trophoblast cell injury in GDM. **A** qRT-PCR for miR-455-3p expression level in hucMSCs-Exos; **B** RT-qPCR for miR-455-3p expression level in hucMSCs transfected with miR-455-3p mimic; **C** qRT-PCR for miR-455-3p expression level in Exos isolated from miR-455-3p mimic-transfected hucMSCs; **D** RT-qPCR for miR-455-3p expression level in HRT-8/SVneo cells co-cultured with Exos-miR-455-3p mimic; **E** CCK-8 assay for cell proliferation; **F** Transwell assay for cell migration; **G** Flow cytometry for cell apoptosis. The t-test was used for comparisons between two groups, and ANOVA was used for comparisons among multiple groups, with Tukey's test as the post hoc test. * indicates $P < 0.05$. All experiments were repeated more than three times

miR-455-3p derived from hucMSCs-Exos improves trophoblast cell injury in GDM

miR-455-3p has been previously identified as a key component of hucMSCs-Exos [14]. Our results unveiled that miR-455-3p expression in hucMSCs-Exos was higher versus that in hucMSCs (Fig. 3A). To confirm the role of miR-455-3p in the beneficial impact of hucMSCs-Exos on trophoblast cell injury in GDM, hucMSCs were transfected with a miR-455-3p mimic to upregulate miR-455-3p expression, and Exos were subsequently isolated from the transfected cells. RT-qPCR results disclosed that miR-455-3p expression in both transfected hucMSCs and their isolated Exos were enhanced (Fig. 3B, C). Additionally, HRT-8/SVneo cells co-cultured with Exos from miR-455-3p mimic-transfected hucMSCs also exhibited elevated miR-455-3p expression, confirming successful miR-455-3p transfer via Exos (Fig. 3D). Furthermore, we established an in vitro model by inducing HRT-8/SVneo cells with HG and intervened with hucMSCs-Exos-miR-455-3p mimic. hucMSCs-Exos carrying miR-455-3p mimic significantly promoted trophoblast cell proliferation and migration, while reducing apoptosis (Fig. 3E–G). To further validate the essential role of miR-455-3p in mediating the protective effects of hucMSCs-Exos, HRT-8/SVneo cells were transfected with a miR-455-3p inhibitor before being treated with hucMSCs-Exos. Experimental results revealed that miR-455-3p inhibition reversed the protective effects of hucMSCs-Exos, leading to decreased cell proliferation and migration whle increased apoptosis (Fig. 3E–G). These findings strongly support the hypothesis that hucMSCs-Exos protect trophoblast cells from HG-induced injury primarily through miR-455-3p regulation.

miR-455-3p targets and regulates CAMK2N1

Bioinformatics analysis identified a putative miR-455-3p binding site within the 3'-UTR of CAMK2N1, suggesting a potential regulatory interaction (Fig. 4A). To validate this interaction, a dual-luciferase reporter assay was performed. The results demonstrated a significant reduction in relative luciferase activity in HRT-8/SVneo cells co-transfected with

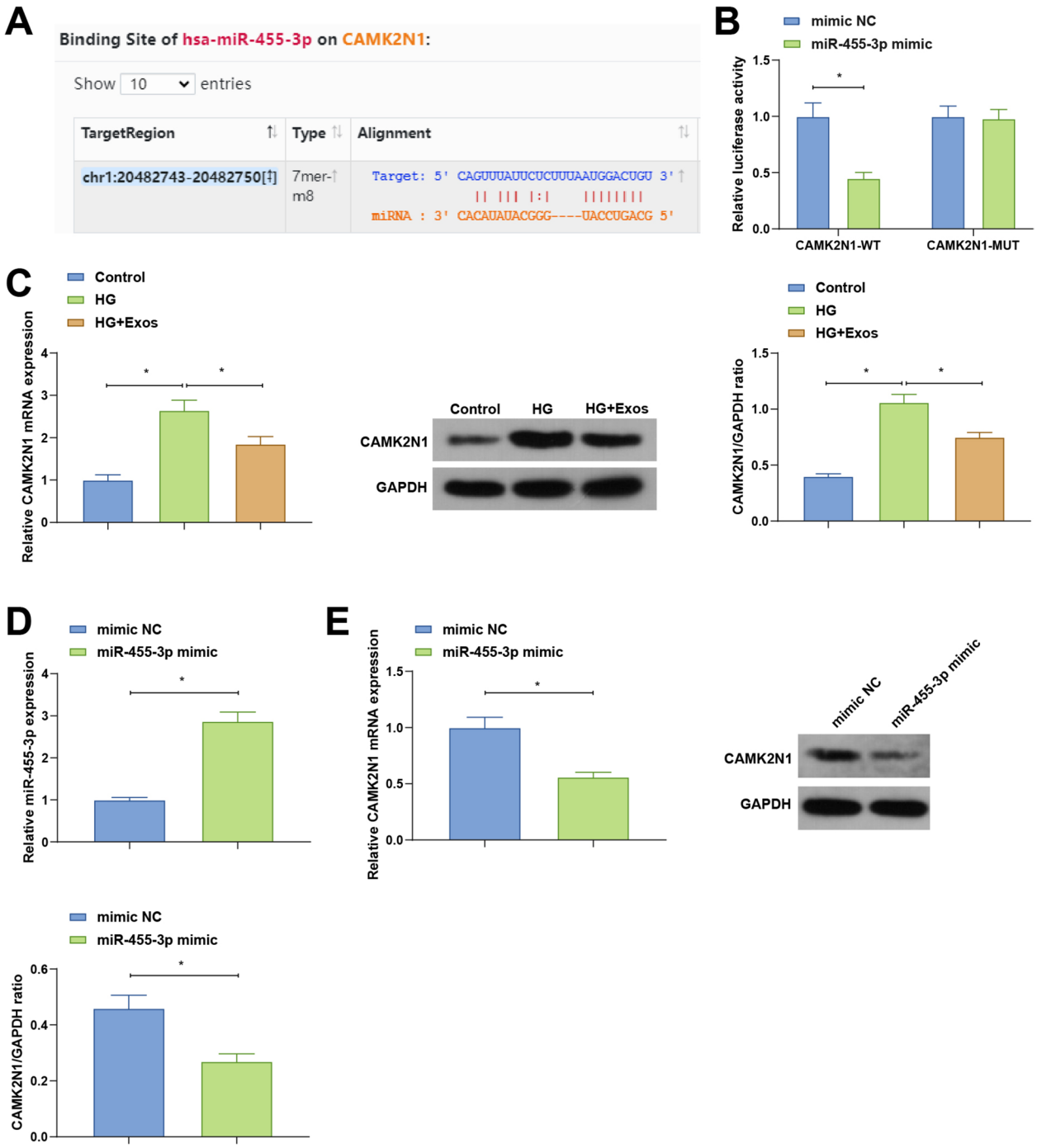

Fig. 4 miR-455-3p targets and regulates CAMK2N1. **A** Bioinformatics website prediction of the binding site between miR-455-3p and CAMK2N1; **B** Dual luciferase reporter gene assay verifying the targeting relationship between miR-455-3p and CAMK2N1; **C** RT-qPCR and WB detection of CAMK2N1 mRNA and protein expression levels in HRT-8/SVneo cells after HG treatment or exosome intervention; **D** RT-qPCR detection of miR-455-3p expression levels in HRT-8/SVneo cells after miR-455-3p overexpression; **E** RT-qPCR and WB detection of CAMK2N1 mRNA and protein expression levels in HRT-8/SVneo cells after miR-455-3p overexpression; The t-test was used for comparisons between two groups, with * indicating $P < 0.05$. All experiments were repeated more than three times

CAMK2N1-WT and miR-455-3p mimic, confirming that miR-455-3p directly targets CAMK2N1 (Fig. 4B). Moreover, we found that HG treatment upregulated both mRNA and protein expression levels of CAMK2N1 in HRT-8/SVneo cells, while Exo intervention or overexpression of miR-455-3p inhibited the expression of CAMK2N1 (Fig. 4C–E).

miR-455-3p derived from hucMSCs-Exos targets CAMK2N1 to ameliorate trophoblast cell injury in GDM

To explore the mechanism by which hucMSC-derived Exos exert protective effects against trophoblast cell injury in GDM, we conducted a reversal experiment by overexpressing CAMK2N1 in the presence of hucMSCs-Exos. HRT-8/SVneo cells were treated with HG and exosomes, followed by transfection with either oe-NC or oe-CAMK2N1. The results unveiled that versus the Exos-mimic NC + oe-NC group, CAMK2N1 expression level was enhanced in the Exos-mimic NC + oe-CAMK2N1 group. Similarly, compared to the Exos-miR-455-3p mimic + oe-NC group, CAMK2N1 expression level was also increased in the Exos-miR-455-3p mimic + oe-CAMK2N1 group (Fig. 5A–C). Next, results of functional analysis disclosed (Fig. 5D–F) that in contrast with the Exos-mimic NC + oe-NC group, the Exos-mimic NC + oe-CAMK2N1 group exhibited decreased cell migration and proliferation, and increased cell apoptosis. Versus the Exos-miR-455-3p mimic + oe-NC group, the Exos-miR-455-3p mimic + oe-CAMK2N1 group also showed reduced cell proliferation and migration, as well as elevated cell apoptosis. These findings indicate that overexpression of CAMK2N1 reversed the protective effect of Exos carrying the miR-455-3p mimic on trophoblast cell injury in GDM.

Discussion

GDM is traditionally defined as abnormal glucose tolerance [15] that develops or is first detected during pregnancy, posing both short- and long-term risks to both mother and fetus [16]. Besides, trophoblastic injury plays a pivotal role in the development of placental dysfunction, which is clinically significant in pregnancy complications [17]. This study focused on the mechanism of hucMSC-derived exosomal miR-455-3p and CAMK2N1 in ameliorating trophoblast injury in GDM.

Exos are known to facilitate intercellular communication by delivering biological molecules to target cells, and their role in GDM and normal pregnancy has garnered increasing research attention [18]. The results of this study displayed that HRT-8/SVneo cells exhibited reduced migration and proliferation and elevated apoptosis after HG induction compared to cells without HG induction. However, treatment with hucMSCs-Exos significantly enhanced trophoblast cell survival and reduced apoptosis, suggesting that hucMSCs-Exos play a protective role in GDM-associated trophoblast injury.

As previously reported, GDM-MSCs-derived Exos carrying miR-130b-3p inhibited human umbilical vein

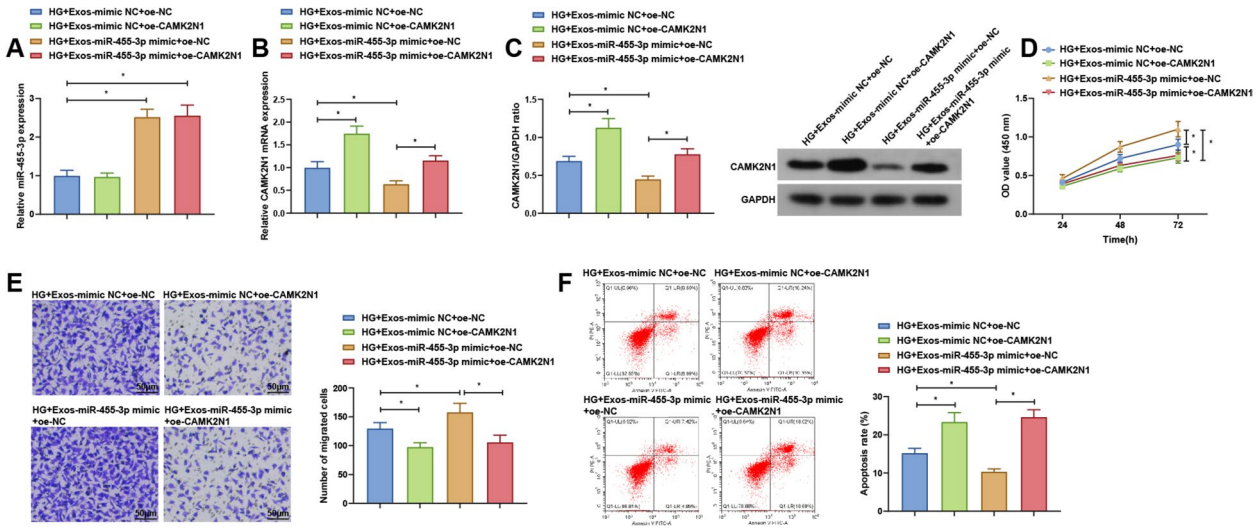

Fig. 5 miR-455-3p derived from hucMSCs-Exos targets CAMK2N1 to ameliorate trophoblast cell injury in GDM. **A** RT-qPCR detection of miR-455-3p expression levels in cells of each group; **B**, **C** RT-qPCR and WB detection of CAMK2N1 mRNA and protein expression levels in cells of each group; **D** CCK-8 detection of cell proliferation in each group; **E** Transwell detection of cell migration in each group; F. Flow cytometry detection of cell apoptosis in each group; ANOVA was used for comparisons between multiple groups, with post-hoc testing using Tukey's method, and * indicating $P < 0.05$. All experiments were repeated more than three times

endothelial cell angiogenesis, migration, and proliferation [19]. In contrast, miR-455-3p, another member of the miRNA family, has been identified as a key component of hucMSCs-Exos [8, 14]. A previous study investigating circ_0000491 in diabetic nephropathy demonstrated that miR-455-3p is a direct target of circ_0000491, and its inhibition reverses the protective effect of circ_0000491 silencing in HG-induced SV40-MES13 cells. In addition, high-mobility group box 1 (Hmgb1) was identified as a target gene of miR-455-3p, playing a protective role in HG-induced cellular damage by targeting Hmgb1 [9]. Unlike this study, our research concentrated on exploring the mechanism of action of hucMSCs-Exos-derived miR-455-3p on trophoblast cell injury associated with GDM. The findings unveiled that miR-455-3p expression in hucMSCs-Exos was higher in comparison with that in hucMSCs, while its expression in Exo was downregulated under GDM conditions; transfection of miR-455-3p mimic ameliorated the injury status of GDM trophoblast cells, supporting the hypothesis that hucMSCs-Exos-derived miR-455-3p protects trophoblasts from HG-induced injury.

Through bioinformatics analysis, we identified a putative miR-455-3p binding site within the 3'-UTR of CAMK2N1. This was experimentally confirmed by dual-luciferase reporter assays, which confirmed that CAMK2N1 was a direct target gene of miR-455-3p. Moreover, hucMSCs-Exos intervention or overexpression of miR-455-3p inhibited CAMK2N1 expression, revealing a novel regulatory mechanism by which miR-455-3p ameliorates trophoblast cell injury in GDM through CAMK2N1 suppression. To further verify this mechanism, we performed reversal experiments, demonstrating that the protective effects of hucMSCs-Exos carrying miR-455-3p mimic were reversed upon CAMK2N1 overexpression. This further confirmed the critical role of miR-455-3p in mediating trophoblast protection via CAMK2N1 regulation. In human embryonic kidney cells, hyperglycemia enhances CAMK2 phosphorylation at RyR2-S2814 residue, while inhibition of CAMK2 prevents this increase [20]. Nevertheless, research on the direct role of CAMK2N1 in GDM is scarce, and studies exploring the involvement of miR-455-3p in mitigating trophoblast cell injury in GDM by targeting CAMK2N1 are even more limited, highlighting the originality of this study.

Notably, existing studies have demonstrated that re-expression of CAMK2N1 downregulates the PI3K/AKT and MEK/ERK signaling pathways, playing a crucial role in regulating cell proliferation, survival, migration, and invasion [21]. Furthermore, research by Xiaolong Zhang et al. suggests that CAMK2N1 exerts an antitumor effect by inhibiting the Wnt/β-catenin pathway [22]. These findings indicate that CAMK2N1 functions as a tumor suppressor in multiple cancer models and has the capacity to regulate several key signaling pathways, including PI3K/AKT, MEK/ERK, and Wnt/β-catenin. Interestingly, these signaling pathways are also implicated in the pathogenesis of GDM and trophoblast function regulation. Given this, targeting CAMK2N1 may modulate the activity of these pathways, thereby improving trophoblast function in GDM patients. Thus, further investigating the downstream signaling pathways regulated by CAMK2N1 could provide a more comprehensive understanding of the molecular mechanisms underlying the role of miR-455-3p in trophoblast cell function. However, tthis study did not fully elucidate the complete mechanism of action of miR-455-3p. To gain deeper insights into its regulatory network, future research should explore the specific downstream pathways influenced by miR-455-3p via CAMK2N1.

In summary, this study has not only demonstrated the therapeutic potential of hucMSCs-Exos in alleviating trophoblast cell injury in GDM, but also provided novel insights into the mechanism by which miR-455-3p exerts its protective effects through the regulation of CAMK2N1. This discovery offers a fresh perspective on the pathogenesis of GDM and establishes a theoretical and experimental foundation for developing innovative MSC-Exos-based therapeutic strategies. However, this study has some limitations. First, we did not conduct CAMK2N1 knockdown experiments, which could provide stronger evidence for its role in trophoblast function. Second, the downstream signaling pathways of CAMK2N1 were not explored, leaving gaps in understanding its precise regulatory mechanisms in GDM. Third, this study was limited to in vitro experiments, and further validation in animal models and clinical samples is needed to confirm the translational potential of these findings. Future studies should address these limitations to provide a more comprehensive understanding of the miR-455-3p/CAMK2N1 axis in GDM and its therapeutic implications.

Author contributions

Y. Z. finished the study design. M. D. and B. Z. finished the experimental studies. R. M. finished the data analysis. J. Z. finished the manuscript editing. All authors read and approved the final version of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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