Graphene oxide accelerates diabetic wound repair by inhibiting apoptosis of Ad-MSCs via Linc00324/miR-7977/STK4 pathway

Zhe Ji1 | Jian Wang1 | Shuai Yang1 | Shengjun Tao1 | Caiqi Shen1 | Hanxiao Wei1 | Qiang Li1 | Peisheng Jin1,2,3

Department of Plastic Surgery, Affiliated Hospital of Xuzhou Medical University, Xuzhou, P.R. China
2Jiangsu Center for the Collaboration and Innovation of Cancer, Xuzhou Medical University, Xuzhou, P.R. China
3Jiangsu Cancer Biotherapy Institute, Xuzhou Medical University, Xuzhou, P.R. China

Correspondence
Qiang Li and Peisheng Jin, Department of Plastic Surgery, Affiliated Hospital of Xuzhou Medical University, Huaihai West Road, Xuzhou, Jiangsu, P.R. China.
Email: fallin1983@163.com and 100000401006@xzhmu.edu.cn

Abstract
Many studies have shown that graphene oxide (GO) promotes proliferation and differentiation of a variety of stem cells. However, its effect on adipose-derived mesenchymal stem cell (Ad-MSCs) apoptosis is still unclear. Apoptosis is a significant factor affecting stem cell-based treatment of diabetic wounds. Therefore, we explored the effect of GO on Ad-MSC apoptosis and diabetic wound healing. In this study, qRT-PCR was used to detect Ad-MSC expression of LncRNAs, miRNAs, and mRNAs under high-glucose environment. RNA immunoprecipitation (RIP), RNA pull-down, and luciferase assays were used to detect interactions of specific lncRNAs, miRNAs, and mRNAs. The effects of GO on Ad-MSC apoptosis were explored by flow cytometry, TUNEL assay, and Western blot. A diabetic wound model was used to explore the function of Linc00324 on Ad-MSC reparative properties in vivo. As a result, GO inhibited high glucose-induced apoptosis in Ad-MSCs, and Linc00324 contributed to the anti-apoptotic effect of GO. RIP and RNA pull-down confirmed that Linc00324 directly interacted with miR-7977, functioning as a miRNA sponge to regulate expression of the miR-7977 target gene STK4 (MST1) and downstream signaling pathways. In addition, GO reduced the apoptosis of Ad-MSCs in wounds and promoted wound healing. Taken together, these findings suggest GO may be a superior auxiliary material for Ad-MSCs to facilitate diabetic wound healing via the Linc00324/miR-7977/STK4 pathway.

KEYWORDS
adipose-derived mesenchymal stem cells, diabetic wound, graphene oxide, Linc00324, miR-7977

Abbreviations: Ad-MSCs, adipose-derived mesenchymal stem cells; AGO2, Argonaute RISC catalytic component 2; DAPI, 4,6-diamidino-2-phenylindole; EGF, epidermal growth factor; FISH, telomere fluorescence in situ hybridization; HE, hematoxylin-eosin; LncRNAs, long noncoding RNAs; miRNA, microRNA; qRT-PCR, quantitative real-time PCR; STK4, serine/threonine-protein kinase 4; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; VEGF, vascular endothelial growth factor.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made. © 2022 The Authors. The FASEB Journal published by Wiley Periodicals LLC on behalf of Federation of American Societies for Experimental Biology.
1 | INTRODUCTION

The development of chronic diabetic wounds is related to many factors. Stem cells play an important role in wound repair in the human body. At present, various stem cells, including Ad-MSCs, have been used to treat diabetic wounds. Ad-MSCs can mobilize and chemotact to the injured site after tissue damage, which contributes to wound repair. However, apoptosis of Ad-MSCs increases in high-glucose conditions, which impairs their wound-healing effects.

GO, as an important derivative of graphene, has been widely used in disease detection and regenerative medicine. Prior studies have demonstrated that GO-related materials promote stem cell differentiation. GO film shows the strong affinity for human adipose-derived stem cells (hASCs), and GO film is an efficient substratum for the adhesion, proliferation, and differentiation of hASCs. At present, the role of GO in the apoptosis of stem cells remains unclear in many studies. GO has been reported to inhibit the apoptosis of mouse stem cells and enhance their functional diversity. However, small GO flakes and highly reduced rGO decreased MSCs proliferation and induced their apoptosis. Apoptosis impairs the efficacy of stem cells in the treatment of diabetic wounds. We postulated that GO could alleviate high glucose-induced apoptosis of stem cells and thus promote the reparative effects of stem cells in diabetic wound healing.

Large noncoding RNAs (LncRNAs) are known as RNA between their genes longer than 200 bp and do not encode protein. LncRNAs have well-characterized roles in cell proliferation, apoptosis, and invasion. Among many LncRNAs, Linc00324 is related to cell proliferation, invasion, and migration, but its role in cell apoptosis is still unclear. miRNA is a noncoding RNA with a length of 19–24 nt that exists in eukaryotes. In recent studies, LncRNAs have been shown to act as competing endogenous RNAs (ceRNAs) to bind miRNAs. For example, Linc00324 acts as a ceRNA to promote proliferation, migration, and invasion of colorectal cancer cells by targeting miR-214-3p. MEG3 acts as a ceRNA to regulate ischemic neuronal death by targeting the miR-21/PDCD4 signaling pathway. However, no prior studies have evaluated the role and mechanism of Linc00324 in Ad-MSCs.

In this study, we observed that Ad-MSC apoptosis is increased in high-glucose conditions, which is alleviated by GO via Linc00324. Linc00324 decoys miR-7977 as a sponge RNA and regulates Ad-MSC apoptosis through the miR-7977/STK4 axis. Thus, GO promotes diabetic wound healing by alleviating cell apoptosis, and it is a potential therapeutic modality for diabetic wounds.

2 | MATERIALS AND METHODS

2.1 | Antibodies

Bcl-2, STK4, BIM, Bax, AGO2, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2 | Isolation and culture of human Ad-MSCs

All the methods were conducted as described in our previous studies. Human Ad-MSCs were isolated from subcutaneous adipose tissue obtained from nonobese human donors (body mass index <25, age range 20–35 years) undergoing abdominal dermolipectomy with informed consent (Plastic Surgery Department, the Affiliated Hospital of Xuzhou Medical University). We obtained the ethics approval for isolating human Ad-MSCs from the Medical Ethic Committee of the Affiliated Hospital of Xuzhou Medical University (XYFY2018-KL027). Passage 3–5 cells of all seven donors were mixed equally and used as a pool for the following experiments.

2.3 | Cell co-cultivation

The GO dispersion was provided by Xian Feng Nanotechnology (Nanjing, China), the thickness of GO in the dispersion was 1–6 layers, the lateral size of it was 0.5–5 μm, and water was solvent of GO dispersion. GO dispersions with final concentrations of 0.1, 0.2, and 0.4 mg/ml were added to Ad-MSC culture medium, and cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (TransGen, Beijing, China) and cultured in a humidified incubator with 5% CO₂ at 37°C. The results of previous experiments showed that apoptosis rate was higher at 80 mM glucose, but this concentration was too far from the blood glucose threshold of diabetic patients. Thus, we used the concentration of 20 mM for in vitro experiments. The model diagram is shown in Figure S1.

2.4 | CM-Dil staining

CM-Dil (Invitrogen, USA) labels cells by binding to lipid molecules of the membrane. After the cells were digested (1 x 10⁷), the supernatant was centrifugally discarded and washed with PBS buffer once. The cells were then incubated at 37°C for half an hour with CM-Dil living cell stain.
(1 μmol/L), and then, they were shaken and mixed for 15 min at 4°C. After centrifugation, the supernatant was discarded. We used 1 ml of PBS to blow the precipitated cells for forming a cell suspension before injection into nude mice and detected by the NightOwl LB 983 imaging system (Berthold, GER).

### 2.5 Western blot

Cells were harvested and subjected to total protein extraction at designated time points. Equal amounts of proteins were isolated on a 12% SDS-PAGE gel and transferred to 0.45 μm pore size PVDF membrane (Millipore, Billerica, MA, USA) to analyze protein expression by Western blot analysis. After blocking with 3% bovine serum albumin, the membrane was incubated with primary antibodies (Bcl-2, STK4, BIM, Bax, AGO2, and β-actin) at 4°C overnight. On the following day, the membranes were probed with a horseradish peroxidase (HRP)-labeled goat anti-rabbit/mouse IgG. Bound antibodies were detected by ECL Plus Western Blot Substrate (Thermo Fisher, Waltham, MA, USA) and visualized with a ChemiDoc™ Imaging System (Biorad, Hercules, CA, USA). Band density was quantified by ImageJ Software (Wayne Rasband, National Institutes of Health, MD). The relative amount of each protein was determined by normalizing the densitometry value of interest to that of the loading control.

### 2.6 Flow cytometry

Treated cells were digested, centrifuged, and washed with PBS twice. We placed 1 × 10^5 Ad-MSCs in a tube and added 200 μl of the binding buffer; then, Ad-MSCs were stained with 5 μl PE Annexin V/7-AAD (BD Roche, MA, USA) and visualized with a ChemiDoc™ Imaging System (Biorad, Hercules, CA, USA). Band density was quantified by ImageJ Software (Wayne Rasband, National Institutes of Health, MD). The relative amount of each protein was determined by normalizing the densitometry value of interest to that of the loading control.

### 2.7 TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (in situ cell death detection kit; Roche Diagnostics) was used to measure Ad-MSC apoptosis. After fixing with 4% paraformaldehyde, Ad-MSCs were treated with 0.1% penetration solution, and cells were then washed with PBS. Briefly, Ad-MSCs of 4 × 10^5 were incubated with TdT and fluorescein-labeled dUTP at 37°C for 45 min. Apoptotic cells were observed and photographed using a fluorescence microscope (Olympus IX83, USA).

### 2.8 Bioinformatics analysis

Interaction between LncRNAs and miRNAs was predicted using StarBase (http://starbase.sysu.edu.cn), Chipbase (https://www.chipbase.co.za/), and LncRNAdb (http://lncrnadb.org/). miRNA target genes were predicted using miRDB (http://www.mirdb.org/). Next, we identified the intersection of miRNAs and mRNAs. Based on the screening of LncRNA–miRNA and miRNA–mRNA pairs, KEGG is a practical database resource to elucidate advanced functional and biological molecular systems, especially macromolecular datasets from genome sequencing and other high-throughput experimental technologies.28 Pathway analysis was performed to explore significant pathways of differentially expressed genes using KEGG (http://www.genome.jp/kegg/).

### 2.9 Establishment of lentivirus packaging and stable cell lines

Stable cell lines were established as described previously.29 Ad-MSCs were infected with control, Linc00324KD (Linc00324 knockdown), miR-7977 inhibitors (miR-7977 knockdown), vector, Linc00324OE (Linc00324 overexpression), or miR-7977 mimic (miR-7977 overexpression) lentiviruses to stably knock down or overexpress Linc00324 and miR-7977. Forty-eight hours after infection, the cells were continuously provided with medium supplemented with 2.5 μg/ml puromycin (Sigma, St. Louis, MO, USA). Surviving cells were developed into stable cell lines expressing control, Linc00324KD, miR-7977 inhibitors, vector, Linc00324OE, or miR-7977 mimics.

### 2.10 RNA pull-down experiment

Sense or antisense Linc00324 was biotin-labeled by the Biotin RNA Labeling Mix (Thermo Fisher Scientific, MA, USA) and transcribed in vitro by T7 RNA polymerase (Thermo Fisher Scientific). Subsequently, the biotin-labeled RNAs were added into cell lysates with the refolding buffer and went through 4 h incubation under 4°C. This procedure was followed by the mixture with streptavidin agarose beads (Thermo Fisher Scientific). Following washing for three times, the beads were boiled, and then, the precipitated mRNA or proteins were examined by quantitative real-time PCR (qRT-PCR) or Western blot analysis, respectively.
2.11 | Establishment of wound model in diabetic nude mice

All the procedures were approved by the Animal Care and Use Committee of Xuzhou Medical University (project number: L20210226430). A total of 42 Female BALB/C nude mice (22–24 g, 4–6 weeks old) were used in this experiment, and their cleanliness was specific-pathogen-free grade. After fasting (free drinking water) for 12 h, body weight was measured and 2% streptozocin was intraperitoneally injected at a dose of 150 mg/kg. Mice with serum glucose level greater than 16.7 mmol/L for at least 4 weeks were considered diabetic. After the animals were anesthetized, a full-thickness skin wound of 1.5 cm was made on their backs.

2.12 | RNA binding protein immunoprecipitation

Ad-MSCs were co-transfected with pcDNA3.1-MS2, pcDNA3.1-MS2-Linc00324, pcDNA3.1-MS2-Linc00324-mut or pcDNA3.1-MS2-ATB (no miR-7977 binding site), and pMS2-GFP. After 48 h, cells were used to perform RNA immunoprecipitation (RIP) experiments using a GFP antibody (Abcam, CA, USA), IgG antibody (as negative antibody, Millipore, Bedford, MA, USA), and the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) following the instructions of the manufacturer. The products were purified and enriched to detect the target microRNAs by real-time PCR. For anti-AGO2 RIP, Ad-MSCs were transfected with miR-7977 or microRNA-negative control. After 48 h, cells were used to perform RIP experiments using an AGO2 antibody (Millipore) as previously described.

2.13 | Fluorescence in situ hybridization (FISH)

GenePharma synthesized FITC- and Cy3-labeled probes were used to detect Linc00324 and miR-7977, respectively. Probe signal was detected using a fluorescence in situ hybridization kit (GenePharma, Shanghai, China) in accordance with the instructions of the manufacturer. Images were recorded digitally using a Leica TCS SP8 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany).

2.14 | qRT-PCR

As mentioned above, RNA was extracted and prepared for qRT-PCR. A Prime Script reverse transcriptase kit (Takara, Dalian, China) was used to reverse-transcribe total RNA to cDNA in accordance with the protocol of the manufacturer. qRT-PCR was conducted using a Roche-Light Cycler 96 sequence detector (Roche, Germany) with SYBR Green chemical reagents. The specific primers are provided in Table S1.

2.15 | Evaluation of wound closure

A total of 42 nude diabetic mice were divided into seven groups with six mice in each group. They were in the blank group (injected with PBS), the control group (injected with untreated Ad-MSCs), the Linc00324OE group (injected with Linc00324-overexpressing Ad-MSCs), and the Linc00324KD group (injected with Linc00324-downexpressing Ad-MSCs). The other groups were GO + control group (injected with GO and untreated Ad-MSCs), GO + Linc00324OE group (injected with GO and Linc00324-overexpressing Ad-MSCs), and GO + Linc00324KD group (injected with GO and Linc00324-downexpressing Ad-MSCs). Before harvesting, the size of the wounded area on each mouse’s back was measured with a ruler and recorded on parfocal digital photographs taken on days 0, 7, and 14 after injecting. Each mouse was injected with 10^7 cells in the Ad-MSC group, Linc00324-overexpression Ad-MSC group, and Linc00324-knockdown Ad-MSC group. Each mouse was injected with 10^7 cells and 0.5 mg GO in the GO + Ad-MSC group, GO + Linc00324-overexpression Ad-MSC group, and GO + Linc00324-knockdown Ad-MSC group. Three mice were randomly selected from each group for data measurement. Wound closure rate was measured as follows:

\[
\text{Wound closure index (\%) = (1 - unhealed wound area/ original wound area)} \times 100\%.
\]

2.16 | Statistical analysis

Results are representative of experiments repeated at least in triplicate. All the quantitative data were expressed as mean ± standard error of the means (SEM). Statistical analysis was performed using SPSS version 22.0 (SPSS Inc, Chicago, IL). One-way analysis of variance (ANOVA) and post hoc tests were conducted to compare the differences among multiple groups. The differences between two groups were determined using the Student’s t test. \( p < .05 \) was considered to be statistically significant.

3 | RESULTS

3.1 | GO alleviated high glucose-induced Ad-MSC apoptosis

First, flow cytometry was used to identify extracted Ad-MSCs based on their surface markers. CD105, CD90,
and CD44 were positive, while CD106, CD31, and CD34 were negative (Figure S2), which demonstrates that the extracted cells were Ad-MSCs. We detected increased apoptotic Ad-MSCs in high-glucose conditions (Figures S3–S5). This finding is consistent with our previous results. After co-culturing GO with Ad-MSCs, we used flow cytometry to detect Ad-MSC apoptosis. We found a significantly reduced apoptosis rate in the same high-glucose environment after co-cultivation (Figure S6A,B). Subsequently, we used a TUNEL kit for apoptosis detection and obtained similar results (Figure S6C,D). Furthermore, the reduction in the number of apoptotic cells was more obvious with the increase in GO concentration. Western blot revealed that the expression of proapoptotic molecules BAX, BIM, and STK4 decreased with the increase in GO, and the expression of antiapoptotic protein BCL-2 rose with the increment in GO (Figure S6E,F). This result confirms that GO could reduce apoptosis of Ad-MSCs in a high-glucose environment.

3.2 | GO inhibited the expression of Linc00324 in Ad-MSCs

In our preliminary experiment, cell sequencing was used to screen out the highly variable Linc00324 under high-glucose environment (Figure S13). After the cell co-cultivation with GO, the content of Linc00324 was the most variable among them, but its role in cell apoptosis remains unclear. Therefore, we selected Linc00324 to explore its role in cell apoptosis. We found that the expression of Linc00324 in Ad-MSCs decreased after the GO concentration was increased during co-cultivation (Figure 1A,B). Thus, we chose to establish cell lines with downregulated and overexpressed Linc00324 (Figure 1C,D) and verified them. We found that the apoptosis rate of the downregulated Linc00324 group was significantly reduced under high-glucose conditions, while the overexpression group showed the opposite result (Figures 1E–I, S7). We used a cell line overexpressing Linc00324 to co-culture with GO to further verify whether GO regulates cell apoptosis through Linc00324. We found that cell apoptosis did not decrease (Figure 1J–M). Therefore, Linc00324 was one of the factors involved in GO regulation of apoptosis.

3.3 | Linc00324 decoyed miR-7977 as a sponge RNA

We performed qPCR to detect the location of Linc00324 for determining the mechanism by which Linc00324 regulates Ad-MSC apoptosis. Linc00324 was mainly located in the cytoplasm (Figure 2A,B), which indicates that it may have the function of competing with endogenous RNA and can serve as a molecular sponge for miRNA. A search of the database revealed five miRNAs with the highest binding scores (Figure 2C). We designed qPCR primers for the five miRNAs to determine whether they are regulated by Linc00324. Notably, miR-7977 was significantly downregulated in the Linc00324 overexpression group, but it was upregulated in the Linc00324 knockdown group (Figure 2D). Subsequently, we used RNA-FISH to detect the cellular localization of Linc00324 and miR-7977. miR-7977 was also primarily located in the cytoplasm (Figure 2E). We also predicted the possible binding sites of Linc00324 in miR-7977 and constructed wild-type (WT) and mutant (MUT) luciferase reporter genes, including firefly and Renilla luciferase sequences (Figure 2F). The miR-7977 mimic reduced Linc00324 WT reporter levels, but it did not affect Linc00324 MUT reporter levels (Figure 2G).

Endogenous binding between miR-7977 and Linc00324 was assessed by RIP and qPCR analysis. Linc00324 RIP in Ad-MSCs was significantly enriched for miR-7977 compared with the empty vector (MS2) Linc00324 with a mutation in the miR-7977 targeting site (Linc00324-mut). The MS2 and another lncRNA-ATB without a predetermined miR-7977 targeting site vector had no change (Figure 2H). Linc00324 was significantly enriched for miR-7977 when biotin-labeled Linc00324 was used to pull down miR-7977 in vitro, while no change had occurred in others. These findings further confirm endogenous binding between miR-7977 and Linc00324 (Figure 2I).

miRNA inhibits the expression of its target via Argonaute RISC catalytic component 2 (AGO2). Thus, we used an RNA pull-down assay to determine whether Linc00324 binds AGO2. As demonstrated by Western blot, Linc00324 could bind to AGO2 compared with antisense control (Figure 2J). RIP analysis was also performed to assess the interaction between Linc00324 and miR-7977. Enrichment of Linc00324 in the anti-AGO2 group confirmed binding between Linc00324 and miR-7977 (Figure 2K,L). Anti-AGO2 RIP was performed in Ad-MSCs overexpressing miR-7977 to further evaluate the binding mechanism of Linc00324 and miR-7977. Cells transfected with miR-7977 were specifically enriched with endogenous Linc00324 by AGO2 (Figure 2M), which implies that miR-7977 could be a Linc00324-carrying miRNA. We also measured Linc00324 expression in the miR-7977 mimic and inhibitor groups, which reveals that Linc00324 expression decreased in the miR-7977 mimic group but increased in the miR-7977 inhibitor group (Figure 2N).
These findings support the notion that Linc00324 acts as a sponge for miR-7977.

### 3.4 miR-7977 targeted STK4 to inhibit Ad-MSC apoptosis

Linc00324 promotes apoptosis by targeting miR-7977 given that they have a competitive endogenous relationship. miR-7977 mimics and inhibitors were transferred into the cells to further study the role of miR-7977 in apoptosis. Flow cytometry (Figure 3A,B) and TUNEL (Figure 3C,D) revealed decreased apoptosis in the miR-7977 mimic group under high-glucose stress, while the miR-7977 inhibitor group exhibited increased apoptosis. Western blot results revealed that BAX, BIM, and STK4 expression decreased with miR-7977 mimic transfection under high-glucose stress, while BCL-2 expression increased (Figure 3E,F). These findings suggest that miR-7977 alleviates Ad-MSC apoptosis. We predicted the downstream targets of miR-7977 via miRDB microRNA target prediction to elucidate the cytoprotective mechanism of miR-7977 (Figure 4A).

We selected STK4, also known as MST1, because it is a known regulator of apoptosis. The 3′UTR sequence of STK4 was cloned into the psiCHECK™-2 vector and an MUT 3′-UTR reporter that had no miR-7977 binding site was constructed to determine if miR-7977 targets STK4 (Figure 4B). Introduction of miR-7977 diminished luciferase activity of STK4 3′-UTR reporter, while MUT STK4 3′-UTR reporter gene activity remained unchanged (Figure 4C). qPCR and Western blot experiments identified that miR-7977 downregulated STK4 mRNA and protein (Figure 4D–F). We then co-treated the miR-7977 inhibitor group with the STK4 inhibitor XMU-MP-1. Flow cytometry analysis showed that XMU-MP-1 alleviated apoptosis induced by miR-7977 inhibitors under high-glucose stress (Figure 4G,H). This result is consistent with the TUNEL analysis (Figure 4I,J) and Western blot (Figures 4K, S8) findings. Overall, these findings suggest that miR-7977 directly targeted the STK4 3′-UTR, which downregulated STK4 expression and inhibited Ad-MSC apoptosis in high-glucose stress.

### 3.5 Linc00324 regulated high glucose-induced Ad-MSC apoptosis via the miR-7977/STK4 axis

We further investigated whether the miR-7977/STK4 axis is involved in Linc00324 regulation of apoptosis under high-glucose stress. We co-transfected Ad-MSCs with miR-7977 mimics and a Linc00324 overexpression vector. Flow cytometry revealed that miR-7977 mimics significantly inhibited apoptosis caused by Linc00324 overexpression (Figure 5A,B). TUNEL (Figure 5C,D) and Western blot analyses (Figures 5E and S9) revealed consistent results. We also used miR-7977 inhibitors and Linc00324 knockdown to co-transfect Ad-MSCs. The miR-7977 inhibitor group reversed the decrease in apoptosis caused by Linc00324 knockdown (Figures 5F–J, S10). Overall, these findings suggest that Linc00324 regulates high glucose-induced Ad-MSC apoptosis through miR-7977/STK4.

### 3.6 GO inhibited cell apoptosis and promoted wound healing in diabetic nude mice

We established a wound repair model in diabetic nude mice to further assess the effect of GO on Ad-MSC-mediated wound healing in diabetic animals. A wound with a diameter of 1.5 cm was created. PBS was injected into the blank control group. We injected treated cells stained with the fluorescent dye CM-Dil into the skin of the wound margin of each group of nude mice by intradermal injection. Cell survival rate was evaluated after 7 days. The GO+Linc00324KD group had the highest survival rate (Figure 6A,B). We then evaluated the wound-healing effect on nude mice for 14 days. We observed better results in the GO group than in the blank control group, and the GO+Linc00324KD group had the most robust wound healing effect (Figure 6C,D). Hematoxylin–eosin and Masson staining of tissue sections confirmed that the GO+Linc00324KD group had the most robust wound healing (Figure 6E,F). We further used tissue immunofluorescence to detect angiogenesis (CD31) and inflammation...
(TNF-α) in the wound tissue. We found high CD31 expression in the downregulated and GO groups, while TNF-α expression was low, which suggests good wound healing (Figure 6G–I). We used wound tissue to perform an ELISA test for detecting cytokines related to wound healing. The results showed that the expression levels of EGF, VEGF, and IL10 in the GO+ Linc00324KD group that promoted wound healing were higher than those in other groups, while the IL6 that delayed wound healing was lower than those in other groups (Figure 6I). The in vivo results suggested that GO could inhibit stem cell apoptosis in diabetic nude mice, which promoted wound healing.
FIGURE 3  miR-7977 inhibited the apoptosis of Ad-MSCs. (A) Flow cytometry was used to detect the apoptosis of Ad-MSCs. (B) Statistical analysis of flow cytometry in a high glucose environment. (C) The TUNEL assay was used to detect the apoptosis of Ad-MSCs. (D) Statistical analysis of the TUNEL assay under high glucose environment. (E) Western blot experiment detected apoptosis-related proteins BAX, BIM, STK4, and BCL-2. (F) Statistical results of western blot. Scale bar = 50 μm. *p < .05, **p < .01, ***p < .001.
DISCUSSION

Diabetic wounds are a significant unmet clinical need, and they represent a major difficulty in care and management of diabetic patients. Prior reports have demonstrated that graphene and stem cells promote the repair of skin wounds. Graphene provides protection against microbial invasion and minimizes the incidence of sepsis, while MSCs can secrete various factors with therapeutic effects on skin damage repair and tissue regeneration. However, no prior studies have evaluated the effect of GO in the treatment of diabetic wounds. Therefore, considering GO had efficient substratum for the adhesion, proliferation, and differentiation of stem cells, as mentioned above, apoptosis is related to wound repair, and GO can reduce apoptosis under certain conditions. So we used to suitable concentration GO to repair diabetic wounds.

High-glucose stress increases apoptosis of stem cells, which weakens their therapeutic effect on diabetic wounds. Indeed, we found that Ad-MSC apoptosis increased under high-glucose stress (Figures S3–S5). Thus, we added GO to cultured Ad-MSCs under high-glucose stress. GO alleviated Ad-MSC apoptosis under high-glucose stress (Figure S6). We evaluated the relationship between GO and Lnc RNA, which has been shown to regulate apoptosis in a number of recent studies, to

**FIGURE 4** miR-7977 targeted STK4 to regulate apoptosis of Ad-MSCs. (A) The predicted target STK4 of miR-7977 found in the miRDB database. (B and C) miR-7977 bound STK4 for dual luciferase assays were shown. (D-F) The protein and mRNA expression of STK4 was verified by Western blot and qRT-PCR after transferring miR-7977 mimics and inhibitors into Ad-MSCs. (G) After transferring the miR-7977 inhibitors and adding STK4 inhibitor XMU-MP-1 to Ad-MSCs, the apoptosis of the cells was detected by flow cytometry. (H) Statistical analysis of flow cytometry under high glucose environment. (I) The apoptosis of the cells was detected by TUNEL assay. (J) Statistical analysis of the TUNEL assay under high glucose environment. (K) Western blot experiment detected apoptosis-related proteins BAX, BIM, STK4, and BCL-2. Scale bar = 50μm. *p < .05, **p < .01, ***p < .001.
further explore the regulatory mechanisms of this anti-apoptotic effect. For example, the Lnc IGF2AS regulated high glucose-induced apoptosis in human retinal pigment endothelial cells. In diabetic cardiomyopathy, LncRNA H19 downregulation activated the oxidative response in diabetic rats. These findings prompted us to further study the basis of GO suppression of stem cell apoptosis. We found that Linc00324 expression decreased...
significantly after co-cultivation. Subsequently, Linc00324 promoted cell apoptosis in a high-glucose environment. We added GO to Linc00324-overexpressing cells to verify the involvement of Linc00324 in GO regulation of cell apoptosis, and this addition reversed the anti-apoptotic effect of GO.

LncRNAs have different functions due to different distributions. In our previous experiments, we sequenced Ad-MSCs in normal condition and treated with high glucose. We found several groups of LncRNAs with large differences, such as Linc00324. After we treated cells with GO, we verified the RNA expression of these groups and found that Linc00324 had obvious changes. As a result, it was used in further analysis. Preliminary test results are shown in Figure S13. We conducted localization experiments to further explore the role of Linc00324 in the regulation of cell apoptosis. Linc00324 was mainly distributed in the cytoplasm, and cytoplasmic LncRNA primarily operates via ceRNA. Then, we found through bioinformatics prediction that miR-7977 has a Linc00324 binding site. miRNAs interact with the AGO protein family to suppress translation or degrade mRNA. Next, we combined the RIP and RNA pull-down experiments to demonstrate the ability of Linc00324 to bind to the AGO2 protein. The luciferase reporter assay further verified that Linc00324 could inhibit miR-7977 expression. We performed qRT-PCR in the miR-7977 inhibitor group. The results showed that Linc00324 was increased, which confirms that Linc00324 decoyed miR-7977 as a sponge RNA.

miRNAs are involved in the regulation of post-transcriptional gene expression in cell biology. In blood diseases, miR-7977 is believed to be transferred to bone marrow mesenchymal stromal cells (MSCs) and reduce the proliferative capacity of MSC stem/progenitor cells by suppressing PCBP1 protein, which leads to acute myeloid leukemia and myelodysplastic-syndrome normal hematopoietic dysfunction. According to Chen, miR-7977 is closely related to the occurrence and development of lung cancer, and it can suppress tumor cell invasion and migration. We studied the role of miR-7977 in Ad-MSC apoptosis given that Linc00324 sponged miR-7977 as a ceRNA and found that miR-7977 reduced apoptosis.

We sought to further elucidate the role of miR-7977 in the regulation of Ad-MSC apoptosis; miR-7977 has been reported to possibly regulate Hippo-YAP signaling pathway through STK4. Our study was further extended on this basis. Whether GO regulates adipose-derived stem cells through Linc00324 via this signaling pathway remains unclear. STK4 is a serine/threonine kinase, and it plays a key role in organ size control and tumor suppression by limiting proliferation and promoting apoptosis given that it is a central component of the Hippo signaling pathway. We verified that Linc00324 regulates Ad-MSC apoptosis through miR-7977/STK4, which elucidates the mechanism by which GO alleviates Ad-MSC apoptosis under high-glucose stress. Subsequently, animal experiments verified the role of GO in reducing apoptosis and promoting wound healing. The GO+ Linc00324KD group had the most robust wound healing among all of the animal groups.

5 | CONCLUSION

GO decreases expression of Linc00324 while Linc00324 increases cell apoptosis in a high-glucose environment via miR-7977 as a ceRNA. STK4 also acts as target to regulate apoptosis of Ad-MSCs. Therefore, GO is an emerging biomaterial for diabetic wound healing and has good therapeutic prospect.

ACKNOWLEDGMENT

The authors thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

FUNDING INFORMATION

This study was supported by grants from the National Natural Science Foundation of China (82072819), the National Natural Science Foundation of Jiangsu Province (BK20201155), China Postdoctoral Science Foundation (2019T120462), Jiangsu Postdoctoral Science Foundation (2019K155), and Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX20_2456, KYCX20_2480).
DISCLOSURES
The authors declare that they do not have any competing financial interests.

DATA AVAILABILITY STATEMENT
The datasets and resources generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

AUTHORS’ CONTRIBUTIONS
PSJ and QL conceived and designed experiments. ZJ, JW, and SY participated in experiments and drafted the manuscript. ZJ contributed to sample collection and interpretation of the data. SJT, CQS, and HXW performed statistical analysis. PSJ revised the manuscript. All of the authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
All the animal experiments and all of the protocols were authorized by the Guide for Animal Care and Use Committee of Xuzhou Medical University (L20210226430).

CONSENT FOR PUBLICATION
Not applicable.

ORCID
Peisheng Jin https://orcid.org/0000-0002-2239-0156

REFERENCES
1. Barman PK, Koh TJ. Macrophage dysregulation and impaired skin wound healing in diabetes. Front Cell Dev Biol. 2020;8:528.
2. Dong X, Yang L. Inhibition of fatty acid binding protein 4 attenuates gestational diabetes mellitus. Prostaglandins Leukot Essent Fatty Acids. 2020;161:102179.
3. Dekoninck S, Blanpain C. Stem cell dynamics, migration and plasticity during wound healing. Nat Cell Biol. 2019;21:18-24.
4. Tyeb S, Shiekh PA, Verma V, Kumar A. Adipose-derived stem cells (ADSCs) loaded gelatin-sericin-laminin cryogels for tissue regeneration in diabetic wounds. Biomacromolecules. 2020;21:294-304.
5. Dhole NR, Kaushik K, Das A. Cxcr6-based mesenchymal stem cell gene therapy potentiates skin regeneration in murine diabetic wounds. Mol Ther. 2020;28:1314-1326.
6. Moradi A, Zare F, Mostafavinia A, et al. Photobiomodulation plus adipose-derived stem cells improve healing of ischemic infected wounds in type 2 diabetic rats. Sci Rep. 2020;10:1206.
7. Huang R, Wang J, Chen H, et al. The topography of fibrous scaffolds modulates the paracrine function of Ad-MSCs in the regeneration of skin tissues. Biomater Sci. 2019;7:4248-4259.
8. Li Q, Xia S, Yin Y, Guo Y, Chen F, Jin P. miR-5591-5p regulates the effect of ADSCs in repairing diabetic wound via targeting AGEs/AGER/JNK signaling axis. Cell Death Dis. 2018;9:566.
9. Novoselov KS, Geim AK, Morozov SV, et al. Electric field effect in atomically thin carbon films. Science. 2004;306:666-669.
10. Xu S, Liu Y, Wang T, Li J. Positive potential operation of a cathodic electrogenerated chemiluminescence immunosensor based on luminol and graphene for cancer biomarker detection. Anal Chem. 2011;83:3817-3823.
11. Choe G, Oh S, Seok JM, Park SA, Lee JY. Graphene oxide/alginate composites as novel bioinks for three-dimensional mesenchymal stem cell printing and bone regeneration applications. Nanoscale. 2019;11:23275-23285.
12. Zambrano-Andazol I, Vazquez N, Chacon M, et al. Reduced graphene oxide membranes in ocular regenerative medicine. Mater Sci Eng C Mater Biol Appl. 2020;114:111075.
13. Fu C, Pan S, Ma Y, Kong W, Qi Z, Yang X. Effect of electrical stimulation combined with graphene-oxide-based membranes on neural stem cell proliferation and differentiation. Artif Cells Nanomed Biotechnol. 2019;47:1867-1876.
14. Kim TH, Lee KB, Choi JW. 3D graphene oxide-encapsulated gold nanoparticles to detect neural stem cell differentiation. Biomaterials. 2013;34:8660-8670.
15. Garcia-Alegria E, Iliut M, Stefanska M, et al. Graphene oxide promotes embryonic stem cell differentiation to haematopoietic lineage. Sci Rep. 2016;6:25917.
16. Kim J, Choi KS, Kim Y, et al. Bioactive effects of graphene oxide cell culture substratum on structure and function of human adipose-derived stem cells. J Biomed Mater Res A. 2013;101:3520-3530.
17. Sekula-Stryjewski M, Noga S, Dzwigonska M, et al. Graphene-based materials enhance cardiomyogenic and angiogenic differentiation capacity of human mesenchymal stem cells in vitro—focus on cardiac tissue regeneration. Mater Sci Eng C Mater Biol Appl. 2021;119:111614.
18. Cheng YH, Cheng SJ, Chen HH, Hsu WC. Development of injectable graphene oxide/laponite/gelatin hydrogel containing Wharton’s jelly mesenchymal stem cells for treatment of oxidative stress-damaged cardiomyocytes. Colloids Surf B Biointerfaces. 2022;209:112150.
19. Kovanezic I, Gelfand R, Lin G, et al. Stem cells from a female rat model of type 2 diabetes/obesity and stress urinary incontinence are damaged by in vitro exposure to its dyslipidemic serum, predicting inadequate repair capacity in vivo. Int J Mol Sci. 2019;20:4044.
20. Conlon TM, John-Schuster G, Heide D, et al. Inhibition of LTbetaR signalling activates WNT-induced regeneration in lung. Nature. 2020;588:151-156.
21. Ren C, Hu X, Zhou Q. Graphene oxide quantum dots reduce oxidative stress and inhibit neurotoxicity in vitro and in vivo through catalase-like activity and metabolic regulation. Adv Sci (Weinh). 2018;5:1700595.
22. Ali T, Grote P. Beyond the RNA-dependent function of LncRNA genes. eLife. 2020;9:e60583.
23. Wu S, Gu Z, Wu Y, Wu M, Mao B, Zhao S. LINC00324 accelerates the proliferation and migration of osteosarcoma through regulating WDR66. J Cell Physiol. 2020;235:339-348.
24. Ni X, Xie JK, Wang H, Song HR. Knockdown of long non-coding RNA LINC00324 inhibits proliferation, migration and invasion of colorectal cancer cell via targeting miR-214-3p. Eur Rev Med Pharmacol Sci. 2019;23:10740-10750.
25. Tyc KM, Wong A, Scott RT Jr, Tao X, Schindler K, Xing J. Analysis of DNA variants in miRNAs and miRNA 3’ UTR binding sites in female infertility patients. Lab Invest. 2021;101:503-512.
26. Zhu J, Deng J, Zhang L, et al. Reconstruction of lncRNA-miRNA-miRNA network based on competitive endogenous RNA reveals functional lncRNAs in skin cutaneous melanoma. BMC Cancer. 2020;20:927.
27. Yan H, Rao J, Yuan J, et al. Long non-coding RNA MEG3 functions as a competing endogenous RNA to regulate ischemic neuronal death by targeting miR-21/PDCD4 signaling pathway. Cell Death Dis. 2017;8:3211.
28. Kanehisa M, Sato Y, Furumichi M, Morishima K, Tanabe M. New approach for understanding genome variations in KEGG. Nucleic Acids Res. 2019;47:D590-D595.
29. Xie M, Ji Z, Bao Y, et al. PHAP1 promotes glioma cell proliferation by regulating the Akt/p27/statmin pathway. J Cell Mol Med. 2018;22:3595-3604.
30. Zhang YX, Yan YF, Liu YM, et al. Smad3-related miRNAs regulated oncogenic TRIB2 promoter activity to effectively suppress lung adenocarcinoma growth. Cell Death Dis. 2016;7:e2528.
31. Turunen SP, von Nandelstadh P, Ohman T, et al. FGFR4 phosphorylates MST1 to confer breast cancer cells resistance to MST1/2-dependent apoptosis. Cell Death Differ. 2019;26:2577-2593.
32. Wear-Maggitti K, Lee J, Conejero A, Schmidt AM, Grant R, Breitbart A. Use of topical sRAGE in diabetic wounds increases neovascularization and granulation tissue formation. Ann Plast Surg. 2004;52:519-521. discussion 522.
33. Hehenberger K, Heilborn JD, Brismar K, Hansson A. Inhibited proliferation of fibroblasts derived from chronic diabetic wounds and normal dermal fibroblasts treated with high glucose is associated with increased formation of l-lactate. Wound Repair Regen. 1998;6:135-141.
34. Kim JH, Ruegger PR, Lebig EG, et al. High levels of oxidative stress create a microenvironment that significantly decreases the diversity of the microbiota in diabetic chronic wounds and promotes biofilm formation. Front Cell Infect Microbiol. 2020;10:259.
35. Lou D, Luo Y, Pang Q, Tan WQ, Ma L. Gene-activated dermal equivalents to accelerate healing of diabetic chronic wounds by regulating inflammation and promoting angiogenesis. Bioact Mater. 2020;5:667-679.
36. Nyambat B, Chen CH, Wong PC, Chiang CW, Satapathy MK, Chuang EY. Genipin-crosslinked adipose stem cell derived extracellular matrix-nano graphene oxide composite sponge for skin tissue engineering. J Mater Chem B. 2018;6:979-990.
37. Lasocka I, Jastrzebska E, Szulc-Dabrowska L, et al. The effects of graphene and mesenchymal stem cells in cutaneous wound healing and their putative action mechanism. Int J Nanomedicine. 2019;14:2281-2299.
38. Yu X, Luo Y, Chen G, et al. Long non-coding RNA IGF2AS regulates high-glucose induced apoptosis in human retinal pigment epithelial cells. IUBMB Life. 2019;71:1611-1618.
39. Li B, Zhou Y, Chen J, et al. Long non-coding RNA H19 contributes to wound healing of diabetic foot ulcer. J Mol Endocrinol. 2020;69:84.
40. Horiguchi H, Kobune M, Kikuchi S, et al. Extracellular vesicle miR-7977 is involved in hematopoietic dysfunction of mesenchymal stromal cells via poly(rC) binding protein 1 reduction in myeloid neoplasms. Haematologica. 2016;101:437-447.
41. Chen L, Cao P, Huang C, Wu Q, Chen S, Chen F. Serum exosomal miR-7977 as a novel biomarker for lung adenocarcinoma. J Cell Biochem. 2020;121:3382-3391.
42. Yoshida M, Horiguchi H, Kikuchi S, et al. miR-7977 inhibits the Hippo-YAP signaling pathway in bone marrow mesenchymal stromal cells. PLoS One. 2019;14:e0213220.
43. Peng X, Ji C, Tan L, et al. Long non-coding RNA TNRC6C-AS1 promotes methylation of STK4 to inhibit thyroid carcinoma cell apoptosis and autophagy via Hippo signalling pathway. J Cell Mol Med. 2020;24:304-316.
44. Guo F, Wang W, Song Y, et al. LncRNA SNHG17 knockdown promotes Parkin-dependent mitophagy and reduces apoptosis of podocytes through Mst1. Cell Cycle. 2020;19:1997-2006.

SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Ji Z, Wang J, Yang S, et al. Graphene oxide accelerates diabetic wound repair by inhibiting apoptosis of Ad-MSCs via Linc00324/miR-7977/STK4 pathway. The FASEB Journal. 2022;36:e22623. doi: 10.1096/fj.202201079RR