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RESEARCH ARTICLE

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Exosomes derived from human adipose-derived stem cells alleviate hepatic ischemia–reperfusion (I/R) injury through the miR-183/ALOX5 axis

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Abstract

Ischemia–reperfusion (I/R) injury is a crucial factor causing liver injury in the clinic. Recent research has confirmed that human adipose-derived stem cells (ADSCs) can differentiate into functional hepatocytes. However, the mechanism of the effects of ADSCs in the treatment of liver injury remains unclear. The characteristics of ADSCs were first identified, and exosome-derived ADSCs were isolated and characterized. The function and mechanism of action of miR-183 and arachidonate 5-lipoxygenase (ALOX5) were investigated by functional experiments in HL-7702 cells with I/R injury and in I/R rats. Our data disclosed that exosome release from ADSCs induced proliferation and inhibited apoptosis in HL-7702 cells with I/R injury. The effect of miR-183 was similar to that of exosomes derived from ADSCs. In addition, ALOX5, as a target gene of miR-183, was involved in the related functions of miR-183. Moreover, in vivo experiments confirmed that miR-183 and exosomes from ADSCs could improve liver injury in rats and inhibit the MAPK and NF-κB pathways. All of these findings demonstrate that exosomes derived from ADSCs have a significant protective effect on hepatic I/R injury by regulating the miR-183/ALOX5 axis, which might provide a therapeutic strategy for liver injury.

KEYWORDS

arachidonate 5-lipoxygenase, exosomes, human adipose-derived stem cells, ischemia–reperfusion (I/R) injury, miR-183

1 | BACKGROUND

The liver is the largest internal organ in mammals and has multiple functions, such as protein synthesis, detoxification, and glycogen storage.[1,2] Because the liver needs a rich oxygen supply to maintain normal physiological function, it is highly susceptible to ischemia–reperfusion (I/R) injury.[3] Liver I/R injury is mainly caused by liver transplantation, severe trauma, hemorrhagic shock, etc.[4,5] I/R injury can reduce liver function in metabolism and detoxification, cause microcirculation disorders and even liver failure, reduce the success rate of surgery, and affect

Abbreviations: ADSCs, adipose-derived stem cells; ALOX5, arachidonate 5-lipoxygenase; IR, ischemia–reperfusion; NC, negative control.

Yi Gong and Haisu Dai contributed equally to this work.

disease prognosis.[6,7] Therefore, further exploration of safe and effective methods to enhance the resistance of cells and improve liver regeneration is the key to reducing hepatic I/R injury.

Adipose-derived stem cells (ADSCs) are a type of pluripotent stem cell with high self-renewal ability and adipogenic, osteogenic, chondrogenic, and myogenic differentiation potential.[8] Studies have proven that ADSCs have the ability to differentiate into hepatocytes with hepatocyte functions, such as glycogen synthesis, urea formation, expression of hepatocellular-specific genes, and cytochrome P450 enzyme activity.[9,10] Current studies have shown that ADSCs can be applied to treat liver diseases such as hepatitis, liver fibrosis, and liver failure.[11–13] Among current approaches, ADSC transplantation is an effective method to promote the recovery of normal function and liver regeneration after large-scale injury.[14] It has been reported that ADSCs can secrete cytokines and growth factors associated with liver regeneration.[15,16] In animal models, ADSC transplantation showed a protective effect against liver injury and stable integration into diseased liver tissue.[14,17] However, the key mechanism of ADSCs in the treatment of liver injury is unclear.

Exosomes are lipid bilayer vesicles with diameters of 50–150 nm that are formed in the cytoplasm and released into the extracellular environment.[18] Exosomes have been recognized as a novel mechanism of cell–cell communication and have protective effects on acute renal tubular injury, liver fibrosis, nerve growth, and cancer.[19–21] Studies have also confirmed that ADSC-derived exosomes play essential roles in tissue repair and immune regulation.[22] In addition, exosomes have stable properties, are easy to store and transport, and pose no risk of immune rejection or tumor formation.[12] Therefore, exosomes are expected to be a new therapeutic strategy. However, the paracrine mechanism of ADSC-derived exosomes in liver injury has not been fully elucidated.

MicroRNA (miRNA), a highly conserved non-coding small RNA, has been extensively studied in various diseases.[23] MiRNAs are key regulators of gene expression in organisms, and exosomes carry abundant nucleic acids, including miRNAs, that can prevent protein expression processes through translation blockage or mRNA degradation.[24] Several studies have reported that exosome-carried miRNAs can affect the process of liver injury.[25–27] Among them, miR-183 was reported to accelerate hepatocellular carcinoma progression.[28,29] Furthermore, miR-183 can alleviate liver reperfusion injury through the apoptotic pathway.[6] However, whether exosomes derived from ADSCs can affect the liver injury process by regulating miR-183 has not been reported.

In this study, we first constructed the in vitro and in vivo I/R injury models based on previous researches.[30,31] Besides, we isolated and identified exosomes from authenticated ADSCs to elucidate the roles of specific ADSC-derived exosomes in liver I/R injury. Furthermore, we also investigated whether ADSC-derived exosomes can affect liver I/R injury through regulation of miR-183, and the potential downstream target genes of miR-183.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human ADSCs were obtained from Cellular Engineering Technologies, and its induction was consistent with previous research.[32] HL-7702 cells were purchased from Shanghai JinYuan Organism (http://www.ssrcc.com.cn/product/?id=181). ADSCs and HL-7702 cells were cultured in 10% fetal bovine serum (FBS)-containing DMEM at 37°C and 5% CO_2. Cultured ADSCs were observed under a microscope.

2.2 | Cell treatment

HL-7702 cells were cultured in glucose-free RPMI 1640 medium (Gibco BRL/Life Technologies Inc.) in an oxygen-free atmosphere (95% N_2 and 5% CO_2 at 37°C) for 24 h, and then were incubated in normal RPMI 1640 medium at 37°C with 95% air and 5% CO_2 for 24 h to establish the I/R injury model. Coculture was performed using transwell chambers with a 3.0-μm pore size. The upper chamber was seeded with 1×10^5 ADSCs, while 1×10^4 HL-7702 cells were seeded in the lower chamber for 24 h.

We obtained an ALOX5-overexpressing plasmid and empty vector from GenePharma and negative control (NC) and miR-183 mimics from RiboBio. ADSCs or HL-7702 cells were transfected with NC (100 nM), miR-183 mimics (100 nM), ALOX5-overexpressing plasmid (1 μg) or empty vector (1 μg) using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

2.3 | Animals

Male Sprague–Dawley rats (230–300 g, 10–12 weeks) were obtained from the Laboratory Animal Center of Sun Yat-Sen University. The animal experiments were performed after clinical evaluation under the same conditions (temperature (20 ± 2°C), 50% humidity, 12 h light/dark cycle). All animal experiments were approved by the Ethics Committee of Southwest Hospital, Third Military Medical University (Army Medical University).

2.4 | Rat model of hepatic I/R

The rat model of hepatic I/R was established based on previous studies.[33,34] Briefly, rats were anesthetized with isoflurane. Partial (70%) liver arterial/portal venous blood was interrupted by a traumatic clip crossing the portal triad above the right lateral lobe. After 60 min of ischemia, the clip was removed to allow reperfusion for 24 h. After anesthesia, liver tissue was collected and a blood sample was taken from the aorta. Sham animals underwent the same surgical procedure without vascular occlusion. To induce miR-183 overexpression, the miR-183 agomir (GenePharma) was injected into the rats via the tail vein at 30 mg/kg. Rats injected with agomir control were used as negative controls.

2.5 | Detection of ADSC surface antigens

ADSCs were collected and counted (1×10^6/ml). Then, 100 μl of cell suspension was mixed with FITC-CD31, FITC-CD34, FITC-CD45, FITC-CD90, FITC-CD105, FITC-CD29, FITC-CD73, FITC-HLA-DR at 4°C for 60 min in the dark. After washing with PBS, the cells were resuspended in 500 μl of PBS. Then, the positive rate of cell surface antigen in each group was examined by flow cytometry.

2.6 | Oil red O staining

The harvested ADSCs (1×10^5/ml) were incubated with adipogenic differentiation medium (Millipore) for 16 days. After washing with PBS, ADSCs were fixed using 4% formaldehyde for 30 min and treated with oil red O dye for 30 min. The staining effect was observed under a microscope.

2.7 | Alizarin red staining

Harvested ADSCs (1×10^5/ml) were grown in osteogenic differentiation medium (Millipore) for 2 weeks. After washing and fixing, ADSCs were stained with Alizarin red dye for 5 min. The staining effect was observed under a microscope.

2.8 | Exosome extraction

As described in a previous study,[35] ADSCs were washed with PBS three times, and the culture media were replaced with EBM-2 containing 0.1% fatty acid-free bovine serum albumin (Sigma-Aldrich). After 48 h of incubation, the medium was collected, filtered through a 0.22-μm filter (EdLab), and used for assays as ADSCs-cm. Exosomes were extracted using ExoQuick-TCExosome Precipitation Solution (System Biosciences). Briefly, the appropriate volume of ExoQuick-TCExosome Precipitation Solution was added to the CM and refrigerated overnight. The sample was centrifuged at 1500 rpm for 30 min and then at 3000 rpm for 5 min at 4°C. The exosome pellet was resuspended in approximately 300 μl of cell medium and incubated for 72 h with recipient cells. Exosome-depleted medium was obtained by ultracentrifugation at 25 000 rpm for 90 min.

ADSCs were starved in serum-free medium for 24 h and then transfected with NC or miR-183 mimics. Twenty-four hours later, serum-free medium was replaced with complete medium. Then, exosomes were collected by the above method.

2.9 | RT-qPCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA using SuperScript II (Vazyme). Amplification reactions were conducted using SYBR Green Mix (Promega). Amplification data were obtained with ABI Prism 7900 (Applied Biosystems) and analyzed using the $2^{-\Delta\Delta Ct}$ method. U6 was used as references for miRNAs. The primer sequences of miR-183 used in our study were as follows: F, 5′- ACACTCCAGCT GGGTATGGCACTGGTAGAAT-3′; R, 5′- CTCAACTG GTGTCGTGGA-3′.

2.10 | Cell proliferation assay

Treated HL-7702 cells (3×10^4 cells) were seeded into a 24-well plate and mixed with 200 μl of EdU (Invitrogen, Cat. No. A10044) in medium (1:1000) for a 2-h incubation at 37°C with 5% CO_2. Then, the cells were harvested and resuspended in PBS. Then, the cells were fixed with 4% formaldehyde for 30 min, neutralized with 2 mg/ml glycine for 5 min, and permeabilized with 0.5% Triton X-100 for 10 min. EdU-positive cells were identified by flow cytometry (Applied Biosystems).

2.11 | Western blotting analysis

Total protein was extracted from treated HL-7702 cells, rat liver tissues, and exosomes using RIPA buffer (Beyotime Biotechnology) supplemented with 1% protease inhibitors (Pierce). After quantification with a bicinchoninic acid (BCA, Thermo Fisher Scientific) assay, the protein (40 μg)

was separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). After blocking, the membranes containing the target protein were incubated with primary antibodies overnight at 4°C and then with the appropriate secondary antibody (Abcam, ab288151) for 1 h. Enhanced chemiluminescence (ECL, Millipore) was applied for visualization. The primary antibodies included rabbit polyclonal to Bax (1:1000, Abcam, ab263897), rabbit polyclonal to Bcl2 (1:1000, Abcam, ab196495), rabbit polyclonal to caspase 3 (1:1000, Abcam, ab4051), rabbit polyclonal to TSG101 (1 μg/ml, Abcam, ab30871), rabbit polyclonal to CD9 (1:1000, Abcam, ab223052), rabbit polyclonal to CD63 (1:1000, Abcam, ab216130), goat polyclonal to ALOX5 (1:1000, Abcam, ab53514), rabbit polyclonal to p-p65 (1:1000, Abcam, ab194726), rabbit polyclonal to p-JNK (1:1000, Abcam, ab4821), rabbit polyclonal to p-p38 (1:1000, Abcam, ab4822), rabbit monoclonal to p-ERK (1:1000, Abcam, ab223500), and rabbit polyclonal to GAPDH (1:2500, Abcam, ab9485).

2.12 | Transmission electron microscopy

Exosomes extracted from ADSCs were suspended in 0.2% paraformaldehyde (Sigma-Aldrich), and 3 μl of the suspension was added to Formvar/carbon-coated grids (Electron Microscopy Sciences) at room temperature for 5 min. Then, the samples were dyed with 1.75% uranyl acetate. After drying, the exosomes were observed using transmission electron microscopy (HITACHI H-7500, Hitachi).

2.13 | Dynamic light scattering (DLS)

Nanosizer™ technology (Malvern Instruments) and Zetasizer software (Malvern) were applied for the detection of exosome size.

2.14 | Isolation of RNA and protein from exosomes

Based on the manufacturer's instructions, a total exosome RNA and protein isolation kit (Invitrogen) was adopted to extract RNA and protein from exosomes.

2.15 | DiD labeling of exosomes

Referring to previous studies,[36,37] exosome uptake was analyzed using Vybrant® DiD (Life Technologies) at room temperature for 10 min.

2.16 | Enzyme-linked immunosorbent assay (ELISA)

In accordance with the kit instructions, aspartate transaminase (AST), alanine transaminase (ALT), and lactate dehydrogenase (LDH) levels were examined using the respective kits. The AST ELISA kit (Cat. no. C0010-2-1) and ALT ELISA kit (Cat. no. C009-2-1) were obtained from Jiancheng Bioengineering Institute; the LDH ELISA kit (Cat. no. C0016) was purchased from Beyotime Biotechnology.

2.17 | Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Liver tissue sections of rats in each group were dewaxed, and apoptosis was examined using a DeadEnd™ Fluorometric TUNEL System kit (Promega, Cat. no. G3250) according to the manufacturer's instructions.

2.18 | Immunohistochemistry (IHC) assay

After routine dewaxing and hydration, 3% H_2O_2 was added to liver tissue sections at room temperature for 10 min. After thermal antigen repair, the sections were blocked using 5% BSA at 37°C for 30 min and incubated with rabbit monoclonal ALOX5 antibody (1:200, Abcam, ab169755) overnight at 4°C, followed by secondary antibody (Abcam, ab150077) for 30 min. After diaminobenzidine (DAB) staining for 10 min, the samples were dehydrated, cleared, and then sealed with neutral gum. Images were acquired using a microscope.

2.19 | Dual-luciferase reporter assay

The ALOX5 luciferase reporter plasmid was obtained from VIPOTION Biotechnology. HL-7702 cells were cotransfected with the ALOX5 luciferase reporter plasmid and miR-183 mimics, and the experiment was conducted using the Dual-Luciferase® Reporter Assay System (Promega) based on the specifications.

2.20 | Statistical analysis

Measurement data with a normal distribution are expressed as the mean ± SD. SPSS 21.0 statistical software was used for data processing and analysis using *t* tests or

one-way analysis of variance (ANOVA) with Duncan's test. $p < .05$ indicated that the differences were significant.

3 | RESULTS

3.1 | Characteristics of ADSCs

To collect exosomes from ADSCs, we purchased and identified ADSCs. According to the reports, CD90, CD105, CD29, and CD73 are the canonical markers of ADSCs.[38,39] And we first analyzed the surface markers of ADSCs by flow cytometry, and the data indicated that ADSCs were negative for CD31, CD34, CD45, and HLA-DR and positive for CD90, CD105, CD29, and CD73 (Figure 1A). Besides, research showed that ADSCs could be identified by Oil red O staining (adipogenic induction), Alizarin red staining (osteogenic induction), and Alcian blue staining (chondrogenic induction).[40,41] Microscopically, the cells grew in a spindle-like shape (Figure 1B). Oil red O staining results indicated that there were small cytoplasmic lipid droplets in ADSCs, suggesting the adipogenic potential of ADSCs (Figure 1C). Alizarin red staining results showed that calcium mineral deposits were present in large numbers in the ADSCs, indicating the osteogenic potential of ADSCs (Figure 1D). The chondrogenic potential of ADSCs was observed by Alcian blue staining (Figure 1E). In contrast to previous study[32] and the dyeing results HL-7702 cells, we also successfully identified ADSCs based on their specific characteristics.

3.2 | Inhibition of exosomes strongly downregulates miR-183 expression, suppresses proliferation, and induces apoptosis in HL-7702 cells after I/R injury

Subsequently, we further investigated whether exosomes could affect cell functions associated with liver injury, including proliferation and apoptosis. An exosome inhibitor (GW4869) was adopted to treat co-cultured ADSCs and HL-7702 cells with I/R injury. RT-qPCR data confirmed that miR-183 levels were greatly reduced in the I/R injury group relative to the control group, while the reduction in miR-183 levels could be reversed by coculture of ADSCs and HL-7702 cells with I/R injury; interestingly, GW4869 could also strongly downregulate miR-183 expression in HL-7702 cells with I/R injury after coculture with ADSCs (Figure 2A). Next, by flow cytometry, we discovered that the decrease in cell proliferation (EdU-positive cells) was significantly attenuated by coculture of ADSCs and HL-7702 cells with I/R injury, while this increase in the proliferation of HL-7702 cells with I/R injury after coculture with ADSCs was markedly reversed by GW4869 ($p < .01$, Figure 2B). Additionally, we observed that the apoptosis results were opposite to the cell proliferation results in each group; that is, the coculture of ADSCs clearly suppressed the apoptosis of HL-7702 cells with I/R injury, which was also markedly reversed by GW4869 (Figure 2C). In addition, as displayed in Figure 2D, the coculture of ADSCs dramatically downregulated caspase 3 and Bax expression and upregulated Bcl2 expression in HL-7702 cells with I/R injury, while these protein changes mediated by coculture of ADSCs were again notably reversed by GW4869 (Figure 2D). In summary, these data demonstrated that inhibition of exosomes in ADSCs strongly reversed the proliferation induction and apoptosis inhibition mediated by coculture of ADSCs and HL-7702 cells with I/R injury.

3.3 | Extraction and identification of exosomes produced by ADSCs

To further verify the effects of exosomes produced by ADSCs on HL-7702 cells after I/R injury, we extracted and identified exosomes from ADSCs. The TEM images demonstrated the morphology of the extracted vesicles (Figure 3A), and DLS data showed that these vesicles mainly ranged from 80 to 120 nm in size (Figure 3B). In addition, western blotting data confirmed that the expression of exosome markers (TSG101, CD9, and CD63) was markedly elevated in the extracted exosomes but absent in untreated ADSC culture supernatant (Figure 3C). Therefore, our data manifested that the exosomes were successfully extracted. We also confirmed that after coculture, DiD-labeled exosomes were transferred to HL-7702 cells (Figure 3D).

3.4 | Exosomes derived from ADSCs dramatically enhance proliferation and inhibit apoptosis in HL-7702 cells after I/R injury by upregulating miR-183 expression

Next, we explored the impact of exosomes and miR-183 on the proliferation and apoptosis of HL-7702 cells after I/R injury. Exosomes from miR-183-overexpressing ADSCs were used to treat HL-7702 cells after I/R injury. RT-qPCR data indicated that the miR-183 level was clearly decreased in the I/R injury group relative to the blank group, and the decrease in miR-183 level was significantly reversed by exosomes in HL-7702 cells with I/R injury; moreover, miR-183 overexpression in exosomes strongly upregulated miR-183 expression in HL-7702 cells with

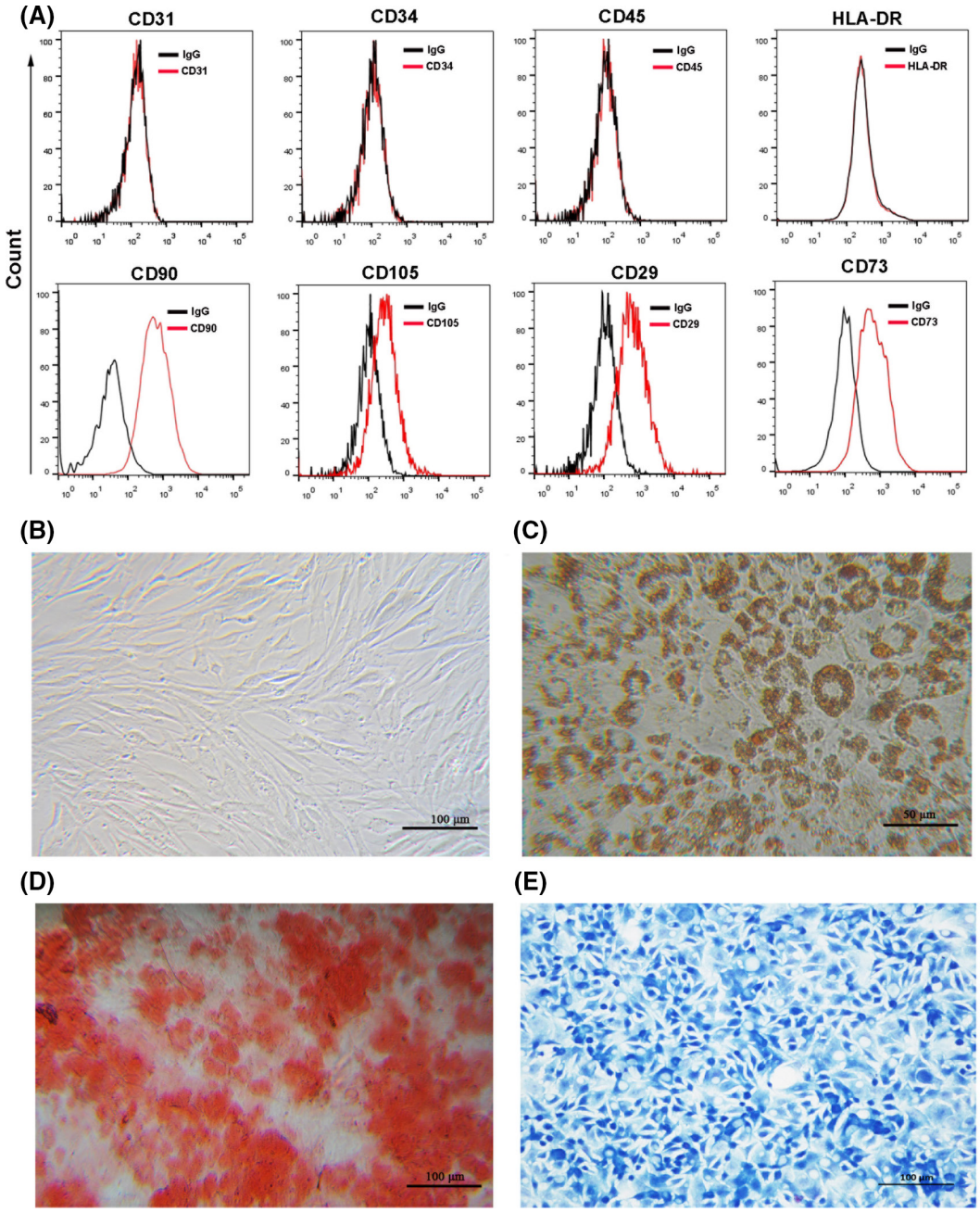

FIGURE 1 Characteristics of ADSCs. (A) Flow cytometry was applied to assess the phenotype of ADSCs through the identification of relevant surface markers, including CD31, CD34, CD45, CD90 CD105, CD29, CD73, and HLA-DR. (B) ADSC morphology was determined under a microscope. Magnification, ×200; Scale bar = 100 μm. (C) The formation of intracellular lipid droplets was observed by oil red staining in ADSCs. Magnification, ×200; Scale bar = 50 μm. (D) The formation of mineralized nodules was evaluated by Alizarin red staining in ADSCs Magnification, ×200; Scale bar = 100 μm. (E) Chondrogenic potential of ADSCs was evaluated by Alcian blue. Magnification, ×200; Scale bar = 100 μm. Each experiment was performed at least three times.

I/R injury (Figure 4A). Functionally, similar to coculture with ADSCs, exosomes alone significantly promoted the proliferation of HL-7702 cells with I/R injury, which was further enhanced by miR-183 overexpression (Figure 4B). TUNEL staining results also verified that the apoptosis of HL-7702 cells with I/R injury was dramatically inhibited by exosomes, and exosome-mediated inhibition of apoptosis was further enhanced by miR-183 overexpression (Figure 4C). In addition, western blotting analysis revealed that exosomes markedly downregulated caspase 3 and Bax expression and upregulated Bcl2 expression in HL-7702 cells with I/R injury, and changes in the expression of these proteins were further strengthened by miR-183 overexpression (Figure 4D). Overall, we demonstrated that exosomes derived from ADSCs could alleviate hepatic I/R injury by upregulating miR-183 expression in vitro.

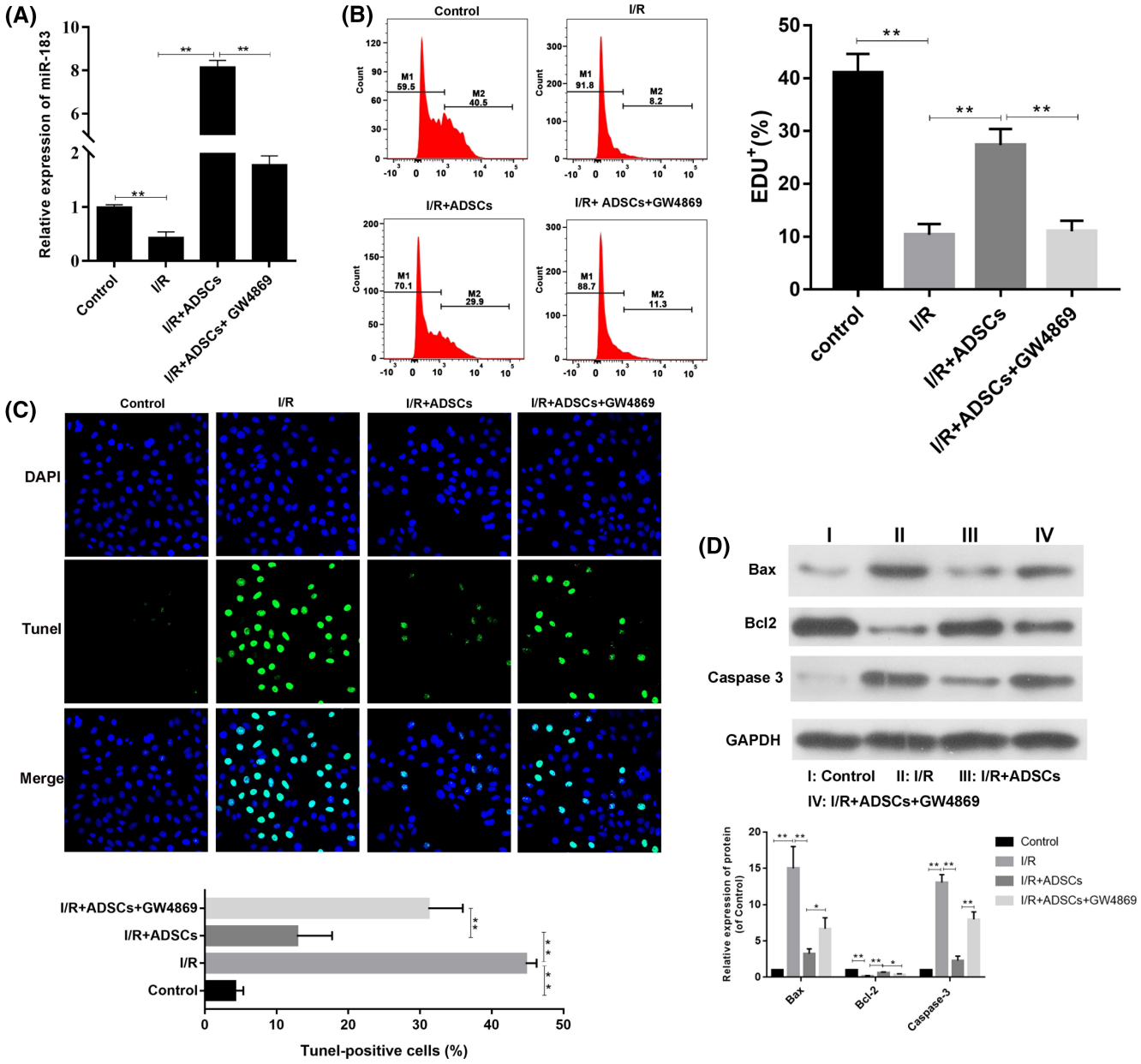

FIGURE 2 Inhibition of exosomes strongly downregulates miR-183 expression, suppresses proliferation, and induces apoptosis in HL-7702 cells after I/R injury. HL-7702 cells after I/R injury were cocultured with ADSCs and then treated with 10 M GW4869 for 48 h. (A) MiR-183 expression was quantified using RT-qPCR assay in each group. (B) Cell proliferation was assessed by flow cytometry, and the rate of EdU-positive cells was calculated. (C) TUNEL staining was conducted to evaluate cell apoptosis in each group. Magnification, ×200. And the number of TUNEL-positive cells was quantified. (D) Bax, Bcl2, and caspase 3 expressions were tested using western blotting analysis, which also was quantified. $*p < .05$, $**p < .01$. Each experiment was performed at least three times.

3.5 | Overexpression of miR-183 markedly accelerates proliferation and prevents apoptosis of HL-7702 cells after I/R injury

In addition, we further evaluated whether miR-183 could independently affect the proliferation and apoptosis of HL-7702 cells after I/R injury. First, miR-183 was notably overexpressed in HL-7702 cells after I/R injury through the transfection of miR-183 mimics (Figure 5A). Second, flow cytometry data showed that the overexpression of miR-183 dramatically accelerated the proliferation of HL-7702 cells after I/R injury (Figure 5B). Third, TUNEL staining data confirmed that the overexpression of miR-183 clearly inhibited the apoptosis of HL-7702 cells after I/R injury (Figure 5C). Finally, we found that the overexpression of miR-183 markedly reduced caspase 3 and Bax expression and elevated Bcl2 expression in HL-7702 cells with I/R injury (Figure 5D). Consequently, these data denoted that the

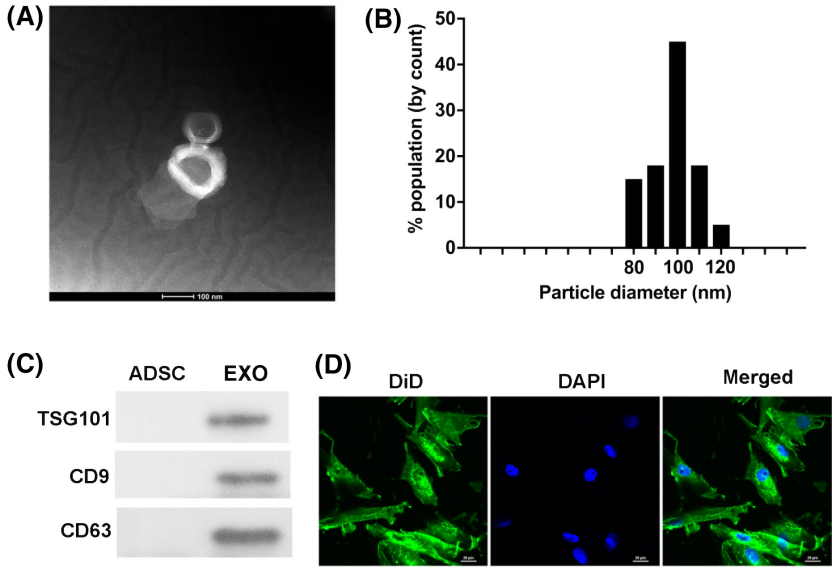

FIGURE 3 Extraction and identification of exosomes in ADSCs after miR-183 overexpression. Exosomes were extracted from ADSCs transfected with miR-183 mimics. (A) TEM was performed to observe the morphology of exosomes. Scale bar = 100 nm. (B) DLS was applied to confirm the particle diameter of exosomes. (C) The levels of exosome surface markers (TSG101, CD9 and CD63) were determined by western blotting in ADSCs and exosomes. (D) The transfer of exosomes was evaluated by immunofluorescence (IF) staining with DiD labeling in HL-7702 cells. Magnification, ×400; Scale bar = 20 μm. Each experiment was performed at least three times.

overexpression of miR-183 could also markedly alleviate hepatic I/R injury in vitro.

3.6 | ALOX5, as a target gene of miR-183, is related to the miR-183-mediated effects on the proliferation and apoptosis of HL-7702 cells with I/R injury

We further investigated the downstream target gene of miR-183. As displayed in Figure 6A, we found that the overexpression of miR-183 significantly downregulated ALOX5 expression in HL-7702 cells with I/R injury. The bioinformatics analysis results indicated the presence of binding sites of miR-183 in ALOX5 (Figure 6B). Dual-luciferase reporter results revealed that increased miR-183 levels strongly attenuated the luciferase activity of WT-ALOX5 but not that of Mut-ALOX5 (Figure 6C). More importantly, when HL-7702 cells with I/R injury were transfected with ALOX5 and/or miR-183 overexpression constructs, the data showed that the ALOX5 overexpression plasmid markedly upregulated ALOX5 expression, which was then downregulated by miR-183 mimics in HL-7702 cells with I/R injury (Figure 6D). Then, we discovered that the overexpression of ALOX5 dramatically inhibited the proliferation (Figure 6E) and facilitated the apoptosis (Figure 6F) of HL-7702 cells with I/R injury, which was partially reversed by miR-183. We also found that ALOX5 overexpression markedly increased caspase 3 and Bax expression and decreased Bcl2 expression in HL-7702 cells with I/R injury (Figure 6G). Thus, we revealed that miR-183 could attenuate hepatic I/R injury by targeting ALOX5 in vitro.

3.7 | MiR-183 or exosomes from ADSCs ameliorate liver function in I/R rats and inhibit MAPK and NF-κB pathways

Furthermore, we verified the effect of ADSC-derived exosomes and miR-183 on the liver function of rats with I/R injury. I/R rats were established and treated with exosomes and miR-183. As presented in Figure 7A, AST, ALT, and LDH levels were markedly elevated in the I/R rats relative to the control rats, and exosome introduction or miR-183 overexpression clearly reduced the AST, ALT, and LDH levels in I/R rats ($p < .01$, Figure 7A). Additionally, TUNEL staining results revealed that I/R injury led to significant enhancement of apoptosis in rats, and this enhancement of apoptosis was significantly weakened after treatment (Figure 7B). IHC results then revealed that ALOX5 expression was strongly increased in I/R rats compared to control rats, and this increase was also partially reversed by exosomes or miR-183 overexpression in the liver tissues of I/R rats (Figure 7C). Moreover, our data confirmed that the expression of ALOX5, p-p65, p-JNK, p-p38, and p-ERK was markedly upregulated in the liver tissues of I/R rats compared to

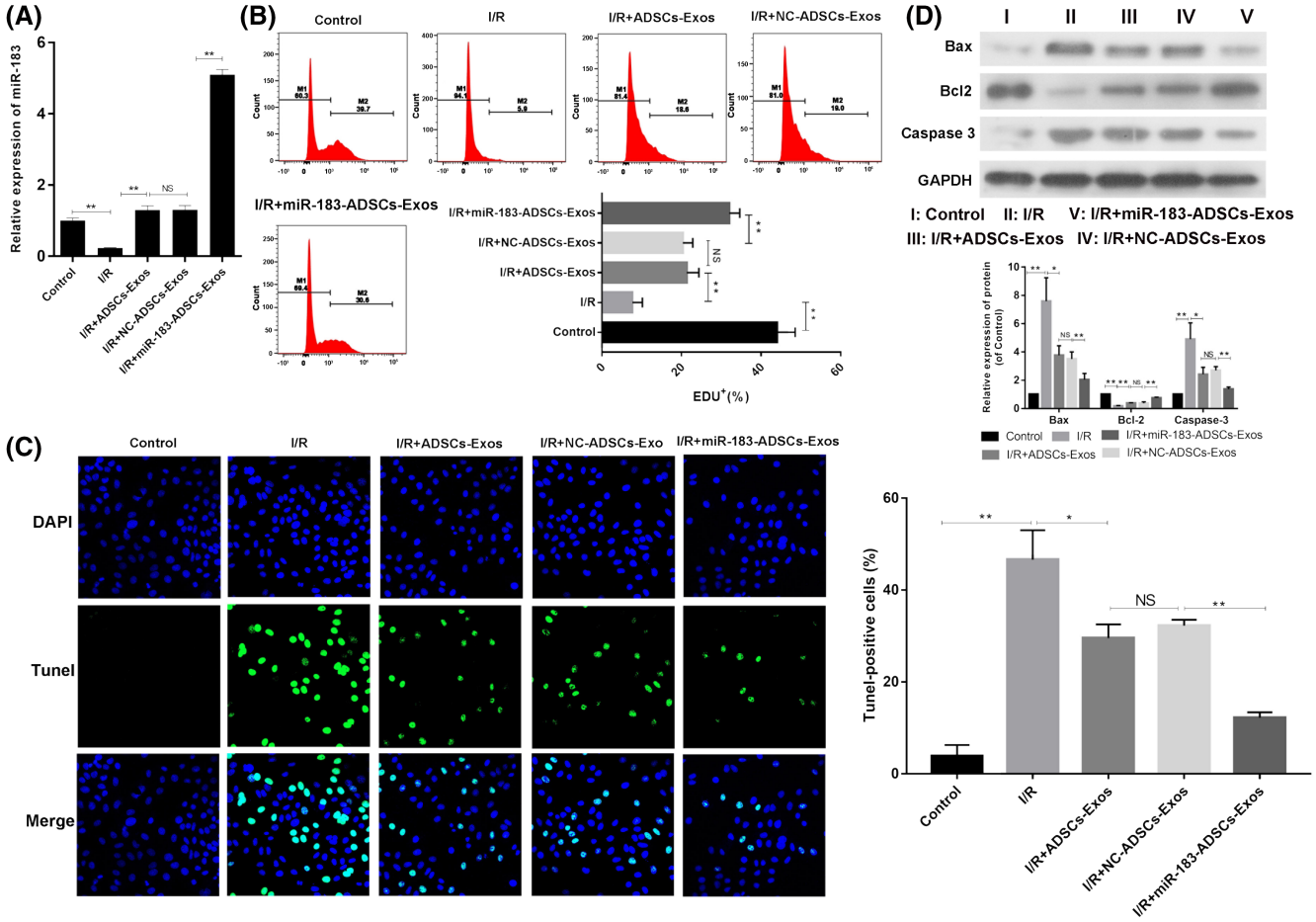

FIGURE 4 Exosomes derived from miR-183-overexpressing ADSCs dramatically enhance the proliferation and inhibit the apoptosis of HL-7702 cells after I/R injury by upregulating miR-183 expression. HL-7702 cells after I/R injury were treated with exosomes from ADSCs. (A) RT-qPCR analysis of miR-183 expression in each group. (B) EdU-positive cells in each group were monitored and counted by flow cytometry. (C) Representative images of TUNEL staining are presented for cell apoptosis, which was also quantitatively analyzed. Magnification, ×200. (D) western blotting analysis was utilized for the detection of Bax, Bcl2, and caspase 3 expressions. HL-7702 cells were stimulated with 5 μg/ml ADSC-Exos, ADSC-NC-Exo ASC-miR-183-Exos for 24 h. Each experiment was performed at least three times. $^{*}p < .05$, $^{**}p < .01$.

those of control rats, and exosomes or miR-183 overexpression markedly attenuated the upregulation of the expression of these proteins in the liver tissues of I/R rats (Figure 7D). Overall, we demonstrated that exosome introduction or miR-183 overexpression significantly improved the liver function of I/R rats.

4 | DISCUSSION

Liver I/R injury is a complex and dynamic pathophysiological process that mainly includes ischemia injury of local tissue cells and inflammation-mediated reperfusion injury.[3,42] Researchers discovered that ADSC transplantation can play a therapeutic role in liver injury by inhibiting inflammation, apoptosis, oxidative stress, and autophagy and promoting liver cell regeneration.[43–45] To further investigate the therapeutic mechanism of ADSCs in hepatic I/R injury, we obtained human ADSCs. In our study, we found that these ADSCs were negative for CD31, CD34, and CD45 and positive for CD90 and CD105. Moreover, our results denoted that the obtained ADSCs have adipogenic and osteogenic potential. ADSCs are spindle-shaped in vitro; ADSCs can express stem cell-specific surface markers (CD29, CD44, CD73, CD90, CDL05, and CDL66) but lack hematopoietic markers (CD45 and CD34) and surface markers of neovascular endothelial cells (CD31), and ADSCs have a strong collagen-forming ability.[46,47] Therefore, we successfully identified the obtained ADSCs.

As a vital component of cells, exosomes carry mRNA, small RNA, and various proteins.[48] Previous literature has reported that ADSC-derived exosomes can promote the

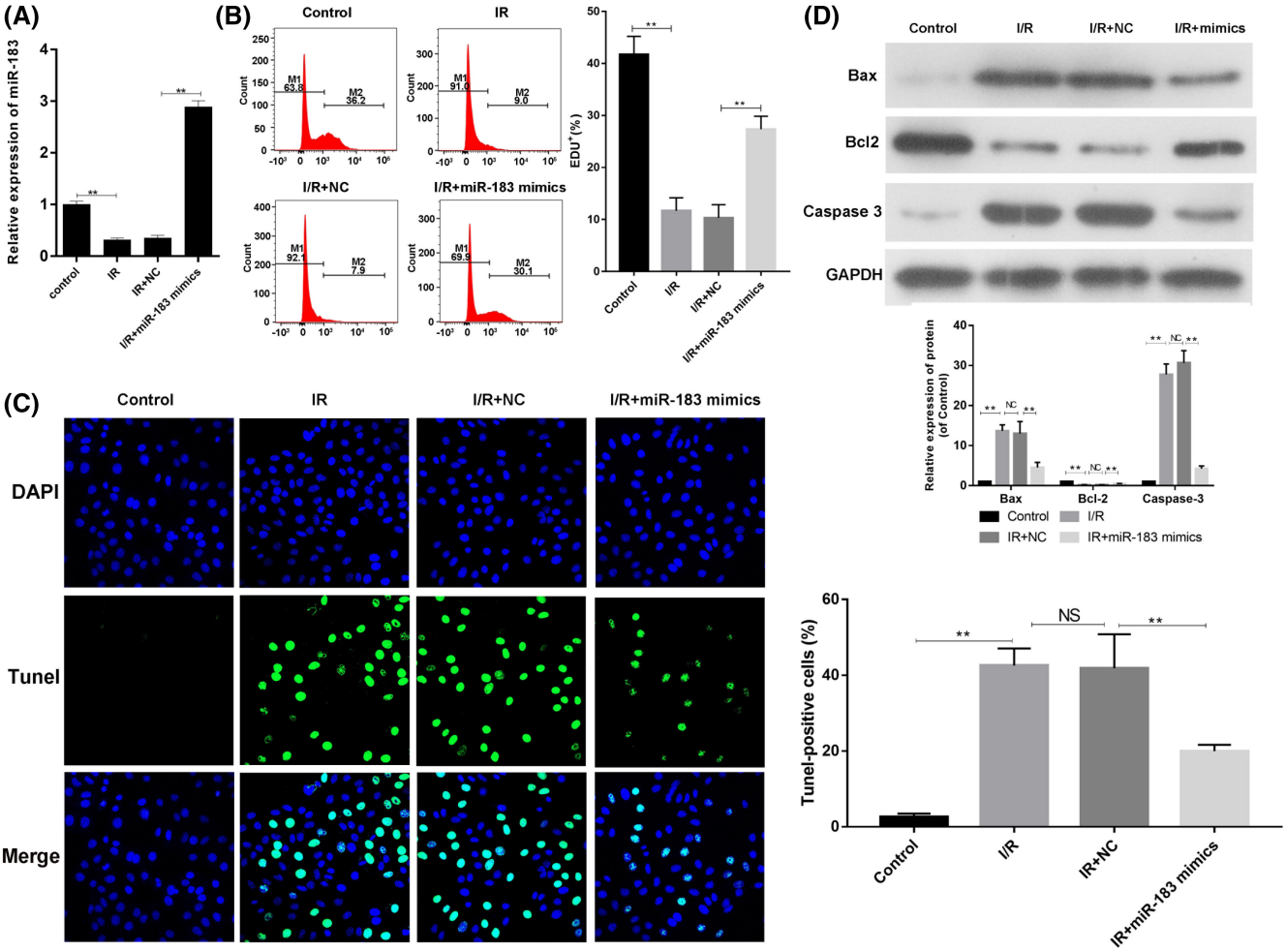

FIGURE 5 Overexpression of miR-183 markedly accelerates proliferation and prevents apoptosis in HL-7702 cells after I/R injury. (A) The effect of miR-183 overexpression was analyzed by RT-qPCR in HL-7702 cells with I/R injury transfected with NC or miR-183 mimics. (B) The impact of miR-183 on the proliferation of HL-7702 cells with I/R injury was assessed by flow cytometry. Quantitative analysis of EdU-positive cells is displayed. (C) After miR-183 overexpression, cell apoptosis was monitored using TUNEL staining. Magnification, ×200. And the number of TUNEL-positive cells was quantified. (D) western blotting was applied to test the influence of miR-183 on Bax, Bcl2, and caspase 3 expression in HL-7702 cells with I/R injury. And protein levels were quantified according to grayscale values. $^{**}p < .01$. Each experiment was performed at least three times.

repair of damaged tissues by delivering mRNA and miRNA to nearby target cells.[49,50] In addition, activated proteins can be transferred by exosomes to target cells and produce corresponding biological effects.[51] Exosomes have been reported to play a major role in a variety of diseases, such as cancer,[52,53] Parkinson's disease,[54] cardiovascular diseases,[55,56] ocular diseases,[57] rheumatoid arthritis,[58] and atherosclerosis.[59] In addition, exosomes are closely associated with drug-induced liver injury,[60] acute liver failure,[61] and even liver cancer.[62] In the current study, we further revealed that an exosome inhibitor (GW4869) significantly suppressed the proliferation and induced the apoptosis of HL-7702 cells with I/R injury. Moreover, we discovered that GW4869 strongly downregulated miR-183 expression, suggesting that miR-183 is related to exosomes derived from ADSCs. The exosomes derived from miR-183-overexpressing ADSCs were mainly 80 to 120 nm in size and highly expressed TSG101, CD9 and CD63; these results suggested that we successfully isolated exosomes from miR-183-overexpressing ADSCs. Additionally, we verified that exosomes could be transferred to HL-7702 cells through coculture. At present, ADSC-derived exosomes have been proven to play significant roles in alleviating acute renal tubular injury, inducing functional recovery after stroke, relieving acute lung injury, reducing the size of myocardial infarction, and improving skin wound healing.[63–66] In our study, we further discovered that the presence of ADSC-derived exosomes could strongly enhance the proliferation and inhibit the apoptosis of HL-7702 cells with I/R injury in a mechanism related to miR-183. Furthermore, we demonstrated that exosomes derived from ADSCs have significant beneficial effects on the liver function of I/R rats.

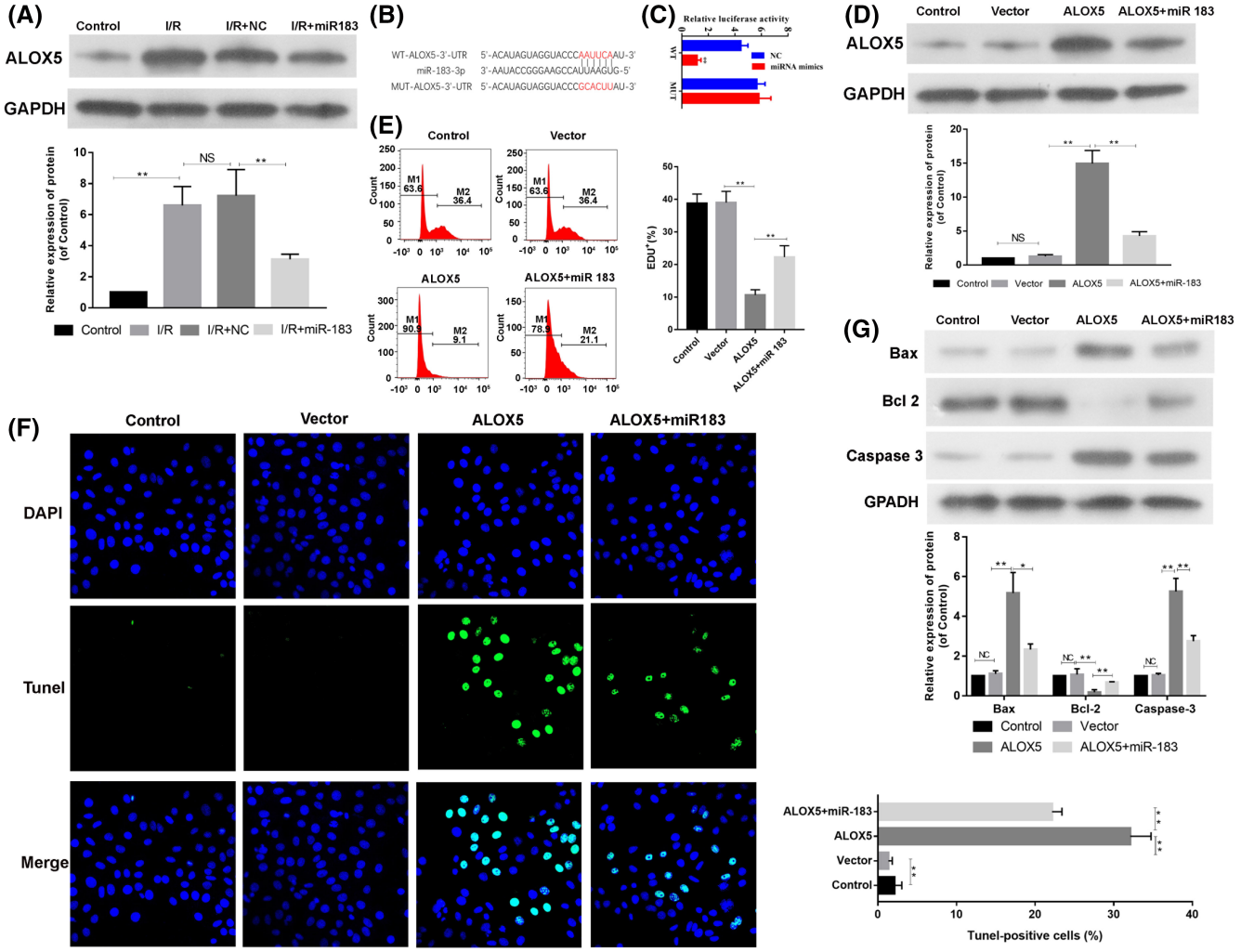

FIGURE 6 ALOX5, as a target gene of miR-183, is related to the miR-183-mediated effect on the proliferation and apoptosis of HL-7702 cells with I/R injury. (A) ALOX5 levels were tested by western blotting after miR-183 overexpression. (B) The diagram displays the homologous sequences of the binding sites of miR-183 and ALOX5. (C) The relationship between miR-183 and ALOX5 was verified via a dual-luciferase reporter assay. (D) ALOX5 levels were tested by western blotting after ALOX5 and/or miR-183 overexpression. (E) Flow cytometry was used to verify the proliferation of HL-7702 cells with I/R injury after ALOX5 and/or miR-183 overexpression. (F) After the co-transfection of ALOX5 and miR-183, apoptosis was evaluated using TUNEL staining in HL-7702 cells with I/R injury. Magnification, ×200. And TUNEL-positive cells were counted. (G) western blotting analysis of Bax, Bcl2, and caspase 3 expression. *$p < .05$, **$p < .01$. Each experiment was performed at least three times.

miRNAs belong to a class of small noncoding RNAs that have significant effects in a variety of processes by regulating genes closely related to cell functions.[67,68] According to the literature, miR-183 is closely associated with a variety of diseases, including cancer,[69,70] lupus nephritis,[71] and retinal dysfunction.[72] In our study, we further revealed that miR-183 overexpression could accelerate the proliferation and prevent the apoptosis of HL-7702 cells with I/R injury and improve the liver function of I/R rats, indicating the therapeutic effect of miR-183 on hepatic I/R injury. Moreover, our data showed that ADSC-derived exosomes and miR-183 could inhibit the MAPK and NF-κB pathways. More importantly, we are the first to report that ALOX5 is a target gene of miR-183 through bioinformatics analysis and experimental verification.

The ALOX5 gene, which is located on chromosome 10q11.2, encodes 5-lipoxygenase (5-LO) containing 673 amino acids.[73] After activation, ALOX5 is transferred to the nuclear membrane and interacts with ALOX5 activated protein (FLAP) to then convert AA to leukotriene and 5-hydroperoxy-eicostetraeoic acid (5-HpETE), which is further metabolized into leukotriene A4 (LTA4), LTB4, and LTC4.[74] Research has shown that LTA4, as an active lipid, is critically related to inflammation. Currently, ALOX5 has been reported to be involved in the pathogenesis of multiple diseases, such as asthma, cardiovascular diseases, multiple sclerosis, tumors, and

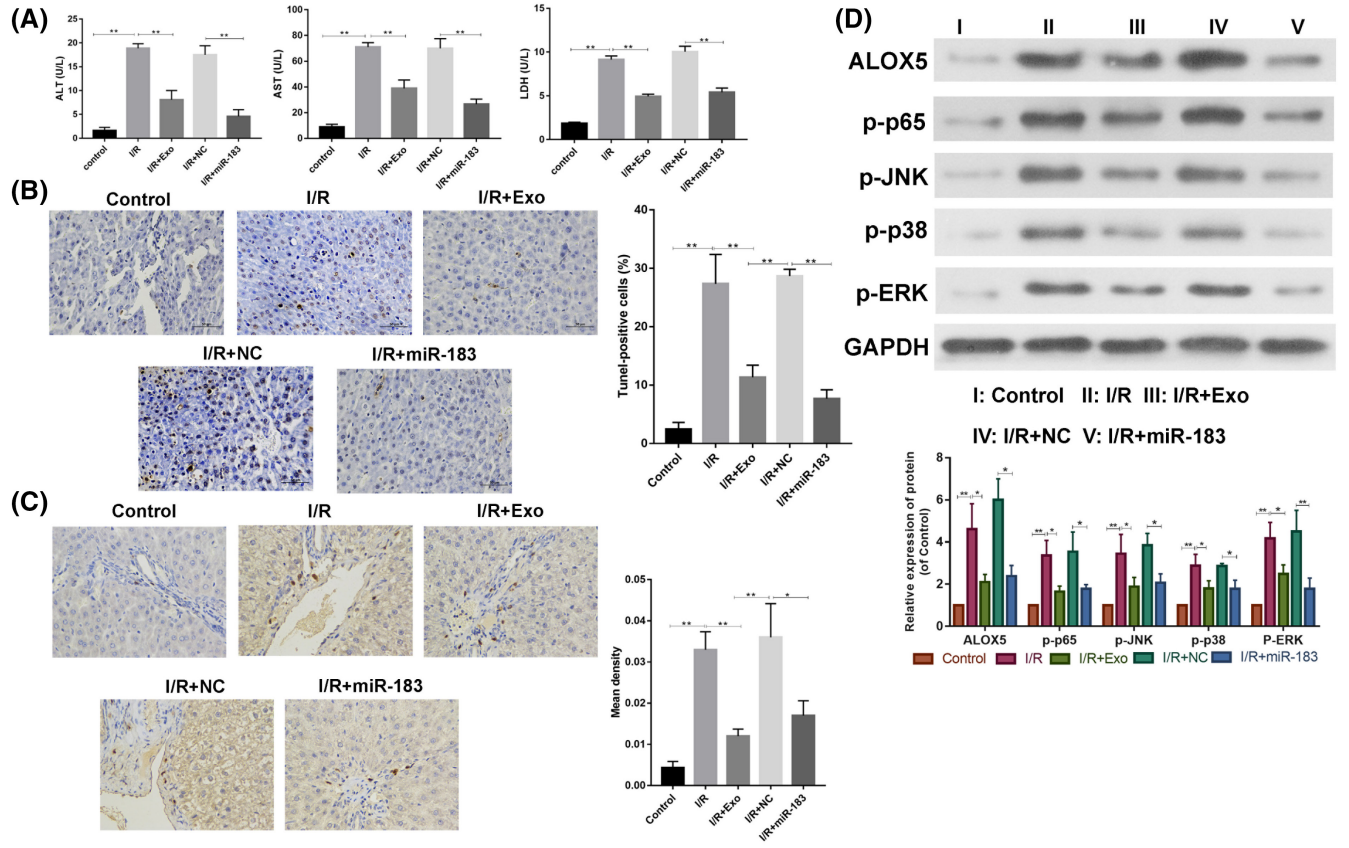

FIGURE 7 miR-183 or exosomes from ADSCs ameliorate the liver function of I/R rats and inhibit the MAPK and NF-κB pathways. I/R rats were treated with exosomes derived from miR-183-overexpressing ADSCs or miR-183. (A) The levels of AST, ALT, and LDH in the treated I/R rats were verified by ELISA. (B) Changes in apoptosis were evaluated by TUNEL staining. Magnification, ×200. Scale bar = 50 μm. And the percentage of TUNEL-positive cells was quantified (C) ALOX5 expression was analyzed by IHC in each group of liver tissues. Magnification, ×200. And the mean density of ALOX5 was quantified. (D) Western blotting analysis of ALOX5, p-p65, p-JNK, p-p38, and p-ERK expression in each group of liver tissues, which also was quantified in line with gray scale value. There were six rats in each group. $^{**}p < .01$. Each experiment was performed at least three times.

allergic diseases.[74–78] Our study further demonstrated that ALOX5 could dramatically reverse the miR-183-mediated effect on the proliferation and apoptosis of HL-7702 cells with I/R injury, indicating that miR-183 could notably attenuate hepatic I/R injury by targeting ALOX5.

5 | CONCLUSIONS

We revealed that exosomal miR-183 derived from ADSCs functions as an effective regulator by downregulating ALOX5 expression in hepatic I/R injury. Therefore, our results indicate that controlling exosomal miR-183 expression might be a novel approach for the treatment of hepatic I/R injury. However, there are several limitations to our current research. For instance, our current study did not explore the effect of exosomes without miR-183 overexpression on HL-7702 cells with I/R injury. The impact of exosomes and miR-183 overexpression on ALOX5 expression also need to be verified. It is also unclear for the ability of exosomes to enter hepatocytes through the LSEC fenestrae and the uptake of the ADSCs-derived exosomes by Kupffer cells, which also needs to be verified by extensive experiments.

AUTHOR CONTRIBUTIONS

Yi Gong, Haisu Dai, Rui Liao, Xiaojun Wang, and Zhiyu Chen were involved in study conception, performing the experiments, data analysis, and interpretation and manuscript writing; Wei Liu, Rui Liao, and Hailei Chen were involved in data collection and contributed to experiments.

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DISCLOSURES

All coauthors declare that there are no conflicts of interest in this study.

DATA AVAILABILITY STATEMENT

All relevant data are within this paper.

CONSENT FOR PUBLICATION

Not applicable.

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