

Exosomes Derived from miR-183-Overexpressing Human Adipose-Derived Stem Cells Alleviate Hepatic Ischemia-Reperfusion (I/R) Injury through miR-183/ALOX5 axis

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Research

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Abstract

Background

Ischemia-reperfusion (I/R) injury a crucial factor causing liver injury in clinic. Recent research confirmed that human adipose-derived stem cells (ADSCs) can differentiate into functional hepatocytes. However, the mechanism of ADSCs in the treatment of liver injury remains unclear.

Methods

Characteristics of ADSCs were first identified, and exosomes from miR-183-overexpressed ADSCs were isolated and identified. And the function and mechanism of microRNA-183 (miR-183) and arachidonate 5-lipoxygenase (ALOX5) were monitored through biological experiments in HL-7702 cells with I/R injury or I/R rats.

Results

We proved that inhibition of exosomes from the identified ADSCs prevented proliferation, induced apoptosis and downregulated miR-183 in HL-7702 cells with I/R injury, while exosomes derived from ADSCs played an opposite role on the proliferation and apoptosis of HL-7702 cells with I/R injury in comparison with exosome inhibitors by upregulating miR-183. Meanwhile, the effect of miR-183 alone was similar to that of exosomes derived from ADSCs. Besides, ALOX5, as a target gene of miR-183, was involved in the related functions of miR-183. Moreover, *in vivo* experiments further confirmed miR-183 or exosomes from ADSCs also could improve liver injury of rats and downregulate MAPK and NF-κB pathways.

Conclusion

All these findings testified that exosomes derived from miR-183-overexpressing ADSCs have a significant protective effect on hepatic I/R injury by regulating miR-183/ALOX5 axis, which might provide therapeutic strategy for liver injury.

Introduction

The liver is the largest organ in the body of mammals, with multiple functions, such as protein synthesis, detoxification, glycogen storage, etc[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]. And it can reduce the capacities of liver metabolism and detoxification, cause microcirculation disorders and even liver failure, reduce the success rate of surgery, and affect 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 reduce liver I/R injury.

ADSCs are a type of pluripotent stem cells with high self-renewal ability with adipogenic, osteogenic, chondrogenic and myoblast differentiation[8]. Researches have proved 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, etc[9, 10]. Current researches have testified that ADSCs can be applied to treat liver diseases such as hepatitis, liver fibrosis and liver failure[11-13]. Among them, ADSCs transplantation is an effective method to promote the recovery of normal function and liver regeneration after large-scale injury[14]. It was reported that ADSCs can secrete cytokines and growth factors associated with liver regeneration[15, 16]. In animal models, ADSCs transplantation showed a protective effect against liver injury and stable integration into the 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, and nerve growth[19, 20]. Researches also verified that ADSCs-derived exosomes play essential roles in tissue repair and immune regulation[21]. Besides, exosomes have stable properties, convenient storage and transportation, and no risk of immune rejection and tumor formation[12]. Therefore, it is expected to be a new therapeutic strategy. However, the mechanism of ADSCs-paracrine exosomes on liver injury has not been fully elucidated. In this study, we isolated and identified exosomes from the authenticated ADSCs to elucidate the latent role and mechanism of specific ADSCs-derived exosomes in liver I/R injury treatment.

Materials And Methods

Cell culture

Human ADSCs were obtained from Cellular Engineering Technologies (Coralville, IA). HL-7702 cells were supplied by American Type Culture Collection (Manassas). ADSCs and HL-7702 cells were cultured in 10% fetal bovine serum (FBS)-containing DMEM medium at 37°C, 5% CO₂. The grown ADSCs were observed under a microscope.

Cell treatment

HL-7702 cells were grown in hypoxia condition (94% N₂, 5% CO₂, 1% O₂) at 37 °C for 1 h, follow by normal condition for 8 h to establish the I/R injury model. We obtained ALOX5-overexpressed plasmid and empty vector from Hanbio (Shanghai, China), and negative control (NC), miR-183 mimics from Ribobio (Guangzhou, China). ADSCs or HL-7702 cells were transfected with NC (100 nM), miR-183 mimics (100 nM), ALOX5-overexpressed plasmid (1 µg) or empty vector (1 µg) using Lipofectamine 3000 (Invitrogen) in line with manufacturer's instructions.

Animal

Male Sprague-Dawley rats (180-200g) were obtained from Laboratory Animal Center of Sun Yat-Sen University. The experiment was carried out after clinical examination of health under the same conditions (temperature ($20 \pm 2^{\circ}\text{C}$), 50% humidity, 12 h light/dark cycle). All animal experiments have been approved by the Committee of the Ethics Committee of Southwest Hospital, Third Military Medical University (Army Medical University).

Rat model of hepatic I/R

On account of the previous researches[22, 23], Briefly, after anesthesia, a traumatic clip was applied to occlude liver arterial/portal venous blood for 60 min, then the clip was removed for reperfusion.

Detection of ADSCs surface antigen

ADSCs were collected and counted ($1 \times 10^6/\text{mL}$). And 100 μL cell suspension was added with FITC-CD31, FITC-CD34, FITC-CD45, FITC-CD90 and FITC-CD105 at 4°C for 60 min in dark.

After washing with PBS, cells were resuspended with 500 μL PBS. Then the positive rate of cell surface antigen in each group was examined by flow cytometry.

Oil red staining

The harvested ADSCs ($1 \times 10^5/\text{mL}$) were incubated with adipogenic differentiation medium (Millipore) for 16 days. After washing using PBS, ADSCs were fixed using 4% formaldehyde for 30 min and treated with Oil red dye for 30 min. The staining effect was observed under a microscope.

Alizarin Red Staining

The harvested ADSCs ($1 \times 10^5/\text{mL}$) were grown with 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.

Exosome extraction

As described in previous study[24], ADSCs were transfected with miR-183 mimics for 48 h, and the medium was harvested and filtered using 0.22 μm filter (Merck–Millipore). ExoQuick-TCExosome Precipitation Solution (System Biosciences) was applied to extract exosomes from ADSCs.

RT-qPCR analysis

The extraction of total RNAs was completed using TRIzol reagent (Invitrogen). cDNAs were synthesized using SuperScript II (Vazyme, Nanjing, China) with total RNAs. Amplification reaction was conducted using SYBR Green Mix (Promega). Amplification data was obtained through ABI Prism 7900 (Applied Biosystems) and counted using $2^{-\Delta\Delta\text{Ct}}$ method.

Cell proliferation detection

The treated HL-7702 cells (3×10^4 cells) were seeded into a 24-well plate and added with 200 μ l Edu (Invitrogen, Cat. No. A10044)/medium (1:1000) for 2 h incubation at 37°C with 5% CO₂. Then the cells were harvested and resuspended with PBS. Besides, the cells were fixed with 4% formaldehyde for 30 min, neutralized with 2mg/ml glycine for 5 min, and permeated by 0.5% Triton X-100 for 10 min. Edu-positive cells were identified by flow cytometry (Applied Biosystems).

Hoechst staining

The treated HL-7702 cells (3×10^4 cells) were seeded into a 24-well plate for 48 h incubation at 37°C with 5% CO₂, and added with 1 ml diluent Hoechst 33342 (Beyotime Institute of Biotechnology) at 37°C for 30 min. The stained apoptotic cells were observed using a fluorescence microscope (Nikon).

Western blotting analysis

The total protein was extracted from the treated HL-7702 cells, rat liver tissues and exosomes using RIPA buffer (Beyotime Biotechnology, Jiangsu, China) supplementing with 1% protease inhibitors (Pierce). After quantification with bicinchoninic acid (BCA, Thermo Fisher Scientific), the protein (40 μ g) was separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore). After blocking, the membranes including the target protein were processed with primary antibodies against Bax (Abcam), Bcl2 (Abcam), caspase 3 (Abcam), TSG101 (Abcam), CD9 (Abcam), CD63 (Abcam), ALOX5 (Abcam), p-p65 (Abcam), p-JNK (Abcam), p-p38 (Abcam), p-ERK (Abcam) and GAPDH (Abcam) overnight at 4°C, and then secondary antibody (Abcam) for 1 h. Enhanced chemiluminescence (ECL, Millipore) was applied for visualization.

Transmission electron microscopy (TEM)

The extracted exosomes from ADSCs were suspended in 0.2% paraformaldehyde (Sigma-Aldrich), and 3 μ L suspension was added onto Electron Microscopy Sciences coated with Formvar-Carbon at room temperature for 5 min. Then the samples were dyed with 1.75% uranyl acetate. After drying, the exosomes were observed using TEM (HITACHI H-7500, Hitachi, Tokyo, Japan).

Dynamic light scattering (DLS)

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

Isolation of RNA and protein from exosomes

Based on the instructions, total Exosome RNA & Protein Isolation Kit (Invitrogen) was adopted to extract the RNA and protein from exosomes.

Exosome uptake

Referring to previous researches[25, 26], exosome uptake was analyzed using Vybrant® DiD (Life Technologies) at room temperature for 10 min.

ELISA analysis

In accordance with the kit instructions, AST, ALT and LDH levels were examined using respective kits. AST ELISA kit (Cat. No. C0010-2-1) and ALT ELISA kit (Cat. No. C009-2-1) were obtained from Jiancheng Bioengineering Institute (Nanjing, China); LDH ELISA kit (Cat. No. C0016) was purchased from Beyotime Biotechnology (Beijing, China).

TUNEL staining

Liver tissue section of rats in each group was dewaxed, and Apoptosis was examined using DeadEnd™ Fluorometric TUNEL System kit (Promega, Cat. no. G3250) in line with the manufacturer's instructions.

Immunohistochemistry (IHC) assay

After routine dewaxing and hydration, liver tissue sections were added with 3% H₂O₂ at room temperature for 10 min. After thermal antigen repair, the sections were sealed using 5% BSA at 37°C for 30 min, incubated with ALOX5 antibody (1:200, Abcam) overnight at 4 °C, followed by secondary antibody for 30 min. After DAB coloring for 10 min, the samples were dehydrated, transparent and then sealed with neutral gum. The results were photographed using a microscope.

Dual-luciferase reporter assay

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

Statistical analysis

Measurement data with normal distribution were expressed as mean ± SD. SPSS 21.0 statistical software was used for data processing and analysis using *t*-test or one-way analysis of variance (ANOVA) with Duncan's test. *P*<0.05 signified that the data was statistically significant.

Results

Characteristics of ADSCs

To collect exosomes from ADSCs, we purchased and identified ADSCs. Firstly, the surface markers of ADSCs were analyzed by flow cytometry, and the data signified that ADSCs were negative for CD31, CD34 and CD45, and positive for CD90 and CD105 (Figure 1A). Under the microscope, we discovered that cells were grown in a spindle-like shape (Figure 1B). Oil red staining results proved that there were small

cytoplasmic lipid droplets in ADSCs, suggesting the adipogenic potential of ADSCs (Figure 1C). And Alizarin Red Staining results manifested that calcium mineral deposits were present in large numbers in the ADSCs, indicating the osteogenic potential of ADSCs (Figure 1D). Thus, we successfully identified ADSCs with the specific characteristics.

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

Subsequently, we further confirmed whether exosomes can affect the function of liver injury, including proliferation and apoptosis. Exosome inhibitor (GW4869) was adopted to treat the co-cultured ADSCs and HL-7702 cells with I/R injury. RT-qPCR data testified that was greatly reduced in I/R injury group relative to control (blank) group, while the reduction of miR-183 level could be reversed by co-culture of ADSCs in HL-7702 cells with I/R injury; interestingly, GW4869 could also prominently downregulate miR-183 in HL-7702 cells with I/R injury after co-culture with ADSCs (Figure 2A). Next, through the detection of flow cytometry, we proved that the decrease of cell proliferation (Edu-positive cells) could be signally attenuated by co-culture of ADSCs in HL-7702 cells with I/R injury, while this increase of cell proliferation in HL-7702 cells with I/R injury after co-culture with ADSCs also could be markedly reversed by GW4869 ($P < 0.01$, Figure 2B). Additionally, we indicated that the trend of apoptosis was just opposite to the trend of cell proliferation in each group, that is, co-culture of ADSCs observably suppressed the apoptosis of HL-7702 cells with I/R injury, which also could be memorably reversed by GW4869 (Figure 2C). Besides, as displayed in Figure 2D, co-culture of ADSCs dramatically downregulated caspase 3 and Bax, and upregulated Bcl2 in HL-7702 cells with I/R injury, while these protein changes mediated by co-culture of ADSCs also could be notably reversed by GW4869 (Figure 2D). In summary, these data testified that inhibition of exosomes in ADSCs could prominently reverse the proliferation induction and apoptosis inhibition mediated by co-culture of ADSCs in HL-7702 cells with I/R injury.

Extraction and identification of exosomes in ADSCs after miR-183 overexpression

To further verify the roles of exosomes in ADSCs on HL-7702 cells after I/R injury, we extracted and identified exosomes from ADSCs. The TEM images exhibited 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). Besides, western blotting data certified that the expressions of exosome markers (TSG101, CD9 and CD63) were markedly elevated in the extracted exosomes (Figure 3C). Therefore, we proved that the exosomes were successfully extracted. Meanwhile, we also certified that after co-culture, DID-labeling exosomes have been transferred in to HL7702 cells (Figure 3D).

Exosomes derived from miR-183-overexpressed ADSCs dramatically enhances proliferation and inhibits apoptosis of HL-7702 cells after I/R injury by upregulating miR-183

Next, we explored the impacts of exosomes and miR-183 on the proliferation and apoptosis of HL-7702 cells after I/R injury. Exosomes or/and miR-183 mimics were applied to treat HL-7702 cells after I/R injury. RT-qPCR data indicated that miR-183 level was observably decreased in I/R injury group relative to

blank group, while the decrease of miR-183 level could also be signally reversed by exosomes in HL-7702 cells with I/R injury; meanwhile, we discovered that miR-183 overexpression then prominently upregulated miR-183, which was mediated by exosomes, in HL-7702 cells with I/R injury (Figure 4A). Functionally, just like co-culture of ADSCs, exosomes alone could also significantly promote the proliferation of HL-7702 cells with I/R injury, which also could be memorably energized by miR-183 overexpression ($P < 0.01$, Figure 4B). Hoechst staining results also verified that the apoptosis of HL-7702 cells with I/R injury could be dramatically inhibited by exosomes, and exosome-mediated inhibition of apoptosis also could be further enhanced by miR-183 overexpression (Figure 4C). Besides, the data from western blotting analysis uncovered that exosomes markedly downregulated caspase 3 and Bax, and upregulated Bcl2 in HL-7702 cells with I/R injury, and changes in the expression of these proteins also could be further strengthened by miR-183 overexpression (Figure 4D). Overall, we disclosed that exosomes derived from ADSCs could alleviate liver I/R injury by upregulating miR-183 *in vitro*.

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

In addition, we further tested whether miR-183 could independently affect proliferation and apoptosis of HL-7702 cells after I/R injury. Firstly, miR-183 was notably overexpressed in HL-7702 cells after I/R injury through the transfection of miR-183 mimics (Figure 5A). Secondly, flow cytometry data also manifested that overexpression of miR-183 dramatically accelerated the proliferation of HL-7702 cells after I/R injury (Figure 5B). Hoechst staining data certified that overexpression of miR-183 observably restrained the apoptosis of HL-7702 cells after I/R injury (Figure 5C). And we also found that overexpression of miR-183 memorably reduced caspase 3 and Bax expressions, and elevated Bcl2 expression in HL-7702 cells with I/R injury (Figure 5D). Consequently, these data proved that overexpression of miR-183 also could markedly alleviate liver I/R injury *in vitro*.

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

Moreover, we further investigated the latent target gene of miR-183. As displayed in Figure 6A, we found that overexpression of miR-183 could signally downregulate ALOX5 in HL-7702 cells with I/R injury. Bioinformatics analysis results also signified the presence of binding sites for miR-183 and ALOX5 (Figure 6B). And dual-luciferase reporter results disclosed that the elevated miR-183 prominently attenuated the luciferase activity of WT-ALOX5, but not that of Mut-ALOX5 (Figure 6C). More importantly, HL-7702 cells with I/R injury were transfected with ALOX5 or/and miR-183 overexpression, and the data showed that ALOX5 overexpression markedly upregulated ALOX5, which then downregulated by miR-183 in HL-7702 cells with I/R injury (Figure 6D). Then we discovered that overexpression of ALOX5 could dramatically inhibited proliferation and facilitated apoptosis of HL-7702 cells with I/R injury, which also could be partially reversed by miR-183 ($P < 0.01$, Figure 6E-6F). And we also found that of ALOX5 overexpression memorably increased caspase 3 and Bax expressions, and decreased Bcl2 expression in

HL-7702 cells with I/R injury (Figure 6H). Thus, we revealed that miR-183 could attenuate liver I/R injury by targeting ALOX5 *in vitro*.

MiR-183 or exosomes from ADSCs ameliorates the liver function of I/R rats and inhibits MAPK and NF-κB pathways

Furthermore, we also verified the potential of exosomes from ADSCs 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 memorably elevated in the I/R rats relative to that in the control rats, while exosomes introduction or miR-183 overexpression observably reduced AST, ALT and LDH levels in I/R rats ($P < 0.01$, Figure 7A). Additionally, TUNEL staining results revealed that I/R injury could lead to significant enhancement of apoptosis in rats, while this enhancement of apoptosis also could be signally weakened (Figure 7B). IHC results then uncovered that ALOX5 expression was prominently increased in the I/R rats with respect to that in the control rats, while this increase also could be partially reversed by exosomes or miR-183 overexpression in the liver tissues of I/R rats (Figure 7C). Moreover, our data certified that ALOX5, p-p65, p-JNK, p-p38 and p-ERK were memorably upregulated in the liver tissues of the I/R rats compared to that the control rats, while exosomes or miR-183 overexpression also could markedly attenuate the upregulation of these proteins in the liver tissues of the I/R rats (Figure 7D). On the whole, we demonstrated that exosomes introduction or miR-183 overexpression also could signally improve the liver function of I/R rats.

Discussion

Liver I/R injury is a complex and dynamic pathophysiological process, which mainly includes ischemia injury of local tissue cells and inflammation-mediated reperfusion injury[3, 27]. Researchers discovered that ADSCs transplantation can play a therapeutic role in liver injury by inhibiting inflammation, apoptosis, oxidative stress and autophagy, and promoting liver cell regeneration[28-30]. In order to further investigate the therapeutic mechanism of ADSCs for liver I/R injury, we obtained human ADSCs. In our study, we found that the obtained ADSCs were negative for CD31, CD34 and CD45, and positive for CD90 and CD105. Meanwhile, we proved that the obtained ADSCs have the adipogenic and osteogenic potentials. It was reported that ADSCs are spindle-shaped *in vitro*; ADSCs can express stem cell specific surface markers (CD29, CD44, CD73, CD90, CD105 and CD166), but lack hematopoietic markers (CD45 and CD34) and surface marker of neovascular endothelial cells (CD31); meanwhile, ADSCs have a strong collagen-forming ability[31, 32]. So, we successfully identified the obtained ADSCs.

As a vital component of cells, exosomes are composed of mRNA, small RNA and various proteins[33]. Previous literature has reported that ADSCs-derived exosomes can promote the repair of damaged tissues by delivering mRNA and miRNA to nearby target cells[34, 35]. Besides, the activated proteins can also be transferred by exosomes to target cells and produce corresponding biological effects[36]. Exosomes have been reported to play a major role in a variety of diseases, such as cancer[37, 38], Parkinson's disease[39], cardiovascular diseases[40, 41], ocular diseases[42], rheumatoid arthritis[43], atherosclerosis[44] and so

on. Besides, exosomes have also been proven to be closely associated with drug-induced liver injury [45], acute liver failure[46], even liver cancer[47]. In the current study, we further revealed that exosome inhibitor (GW4869) could significantly suppress proliferation and induce apoptosis of HL-7702 cells with I/R injury. Moreover, we discovered that GW4869 also could prominently downregulate miR-183, suggesting miR-183 is related to exosomes derived from ADSCs. Subsequently, our results proved that the isolated exosomes derived from miR-183-overexpressed ADSCs were mainly ranged from 80 to 120 nm in size with highly expressed TSG101, CD9 and CD63, suggesting that we have successfully isolated exosomes derived from miR-183-overexpressed ADSCs. Additionally, we verified that exosomes could be transferred in to HL7702 cells through co-culture. At present, ADSCs-derived exosomes have been proven to play significant roles in alleviating acute renal tubular injury, inducing functional recovery after stroke, relieving acute lung injury, and reducing the size of myocardial infarction, improving skin wound healing[48-51]. In our study, we further discovered that the presence of ADSCs-derived exosomes could prominently enhance proliferation and inhibit apoptosis of HL-7702 cells with I/R injury, which also is relevant to miR-183. Furthermore, we testified that exosomes from ADSCs also have significant relieving effects on the liver function of I/R rats.

MiRNAs belong to a class of small non-coding RNAs, which have significant effects in a variety of processes by regulating genes closely related to cell functions[52, 53]. According to the literatures, we discovered that miR-183 is closely associated with a variety of diseases including cancer[54, 55], lupus nephritis[56], retinal dysfunction[57], etc. In our study, we further uncovered that miR-183 overexpression also could accelerate proliferation and prevent 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 liver I/R injury. Meanwhile, we proved that and ADSCs-derived exosomes and miR-183 also could prevent MAPK and NF- κ B pathways. More importantly, we first found that ALOX5 is as a target gene of miR-183 through bioinformatics analysis and experimental verification.

ALOX5 gene, which is located on chromosome 10q11.2, can encode 5-lipoxygenase (5-LO) containing 673 amino acids[58]. After activation, ALOX5 can be transferred to the nuclear membrane and interact with ALOX5 activated protein (FLAP), then converts AA to leukotriene and 5-hydroperoxy-eicostetraeic acid (5-HpETE), which is further metabolized into leukotriene A4 (LTA4), LTB4 and LTC4[59]. Research manifested that LTA4, as an active lipid, 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 allergic diseases, etc[59-63]. Our study further demonstrated that ALOX5 could dramatically reverse miR-183-mediated proliferation and apoptosis of HL-7702 cells with I/R injury, indicating that miR-183 could notably attenuate liver I/R injury by targeting ALOX5.

We disclosed that exosomal miR-183 derived from ADSCs functions as an effective regulator by downregulating ALOX5 in liver I/R injury. Therefore, we indicated that control of exosomal miR-183 expression might be as a novel approach for the treatment of liver I/R injury.

Declarations

Availability of data and materials

The datasets supporting the conclusions of this article had been included within the manuscript.

Ethics approval and consent to participate

All the animal experiments were approved by the Ethics Committee for Animal Experimentation of Southwest Hospital.

Consent for publication

All the co-authors agreed to publish the final version of the present manuscript.

Competing interests

All the co-authors declared that there existed no conflicts of interest in this study.

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Figures

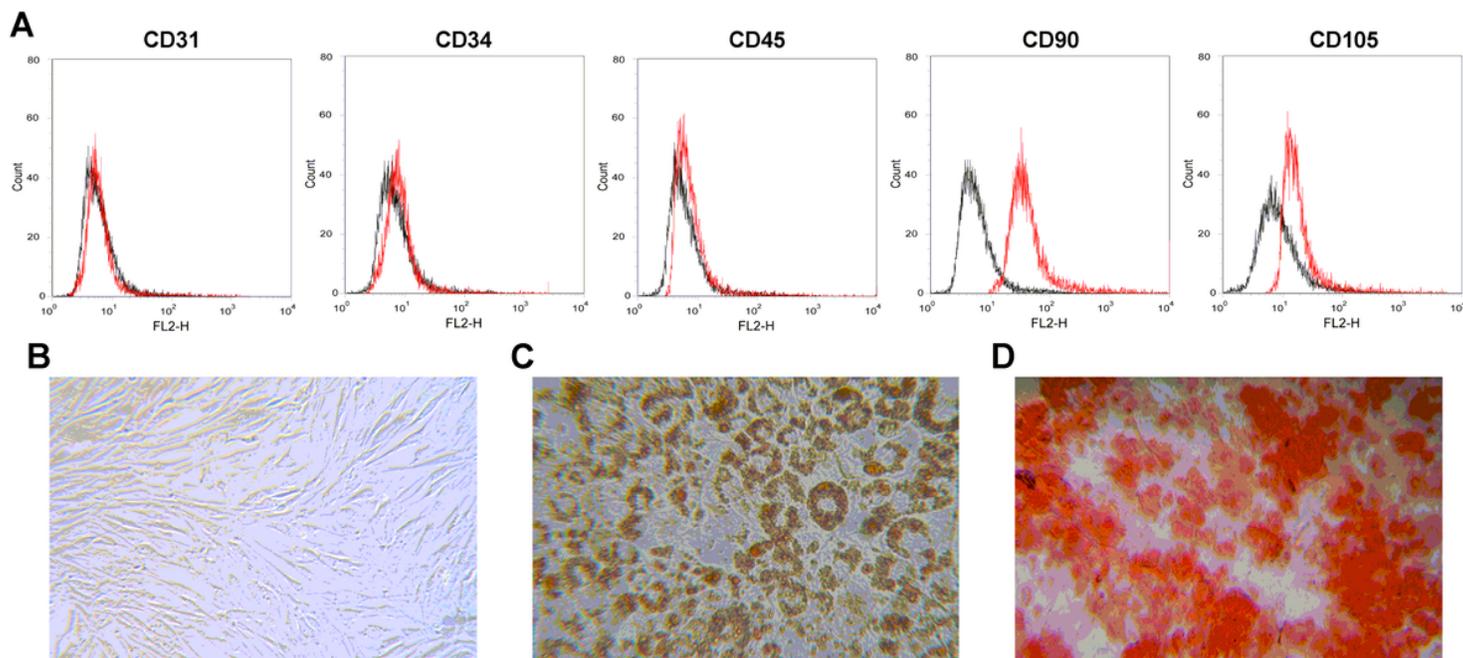


Figure 1

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 and CD105. (B) The morphology of ADSCs was identified under the microscope. Magnification, $\times 200$. (C) Formation of intracellular lipid droplets was observed by Oil red staining in ADSCs. Magnification, $\times 200$. (D) Formation of mineralized nodules was evaluated by Alizarin Red Staining in ADSCs. Magnification, $\times 200$.

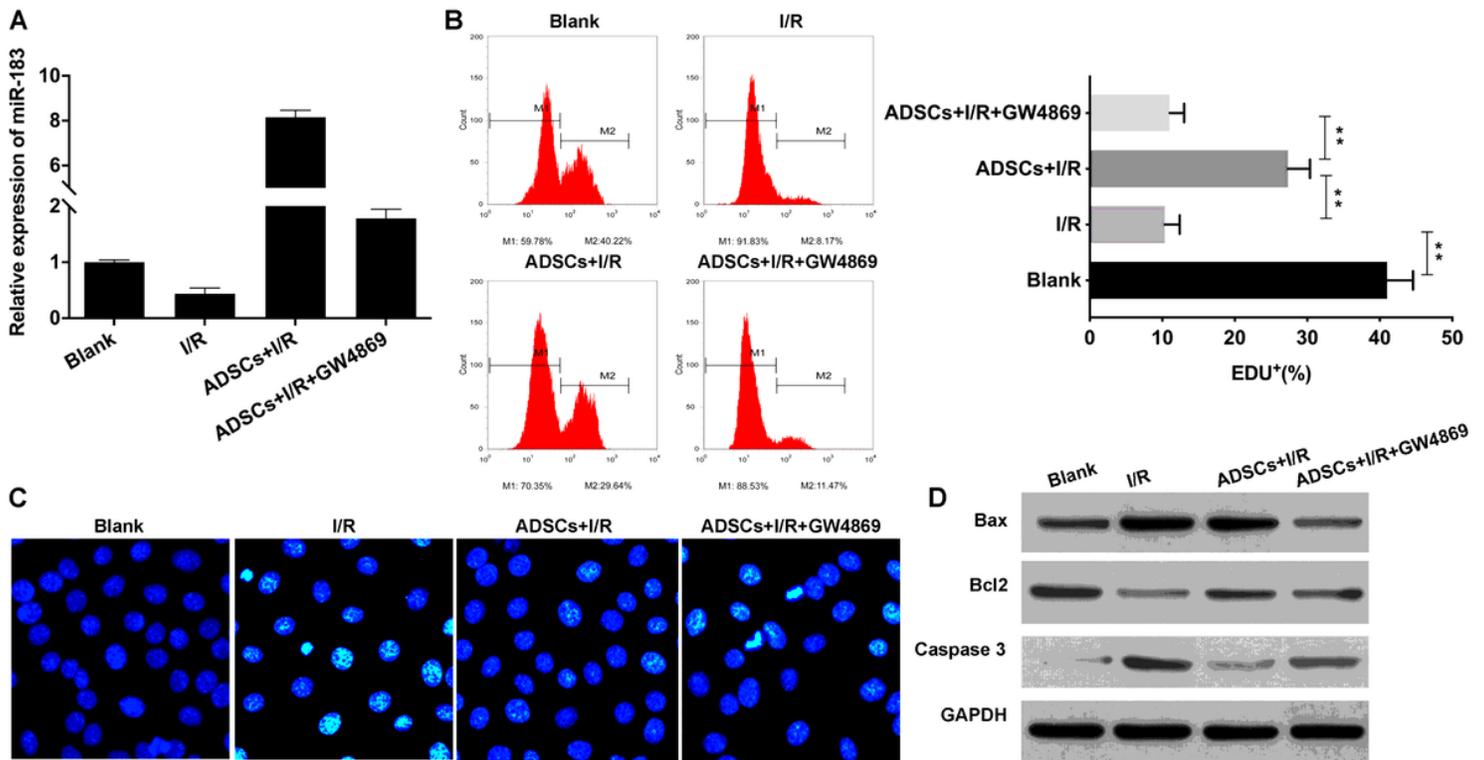


Figure 2

Inhibition of exosomes prominently downregulates miR-183, suppresses proliferation and induces apoptosis in HL-7702 cells after I/R injury. HL-7702 cells after I/R injury were co-cultured with ADSCs and then addressed with 10M GW4869 for 48h. (A) The level of miR-183 was certified using RT-qPCR assay in each group. (B) Cell proliferation was appraised by flow cytometry, and the rate of Edu-positive cells was calculated. (C) Hoechst staining was conducted for cell apoptosis in each group. Magnification, $\times 200$. (D) Bax, Bcl2 and caspase 3 expressions were tested using western blotting analysis.

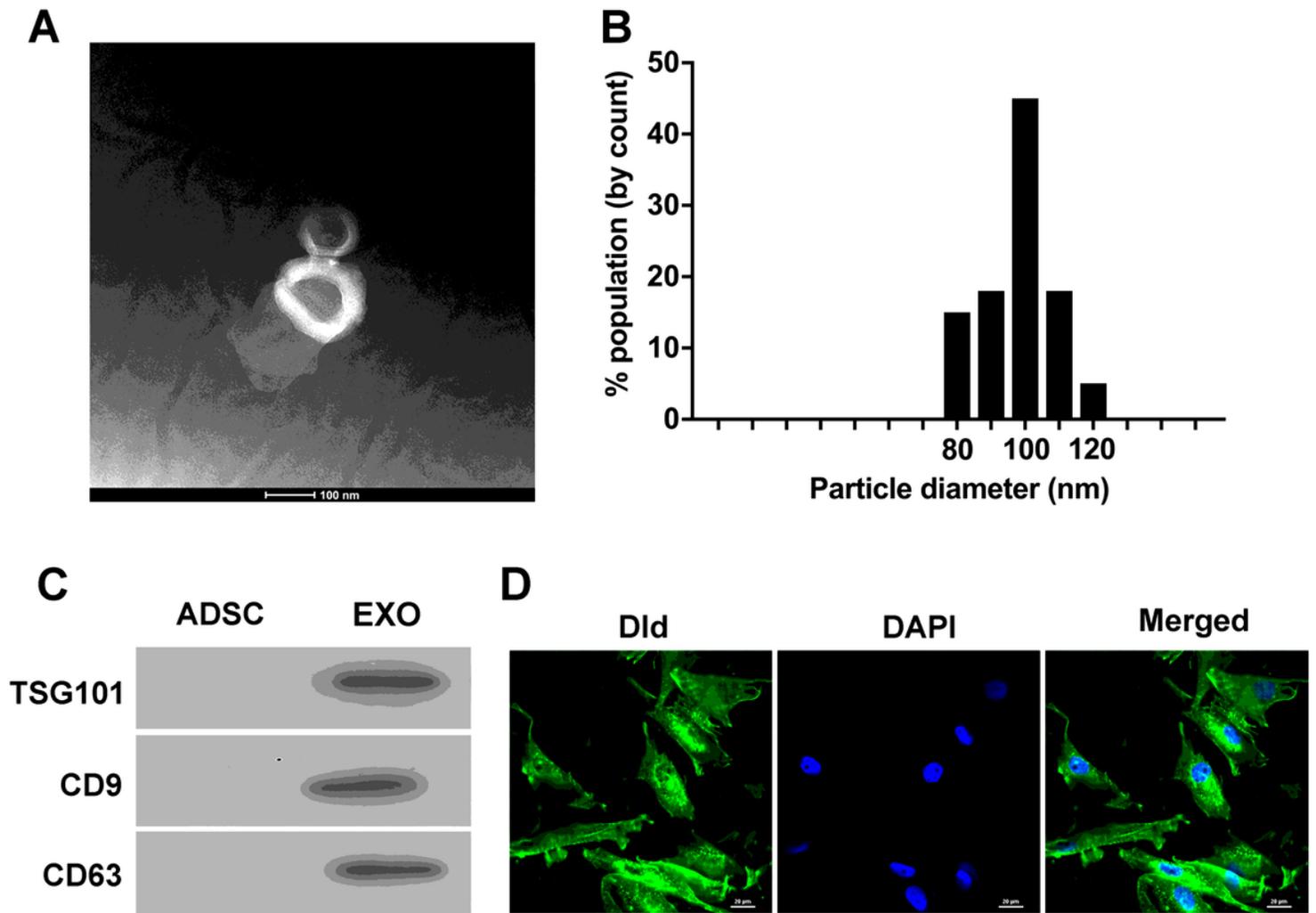


Figure 3

Extraction and identification of exosomes in ADSCs after miR-183 overexpression. Exosomes were extracted from ADSCs transfected with miR-183 mimics. (A) TEM was adopted to observe the morphology of exosomes. Scale bar=100 nm. (B) DLC was applied to confirm the particle diameter of exosomes. (C) The levels of exosome surface markers (TSG101, CD9 and CD63) were certified with western blot in ADSCs and exosomes. (D) The transfer of exosomes was evaluated by IF staining with DID-labeling in HL7702 cells. Magnification, $\times 400$; Scale bar=20 μ m.

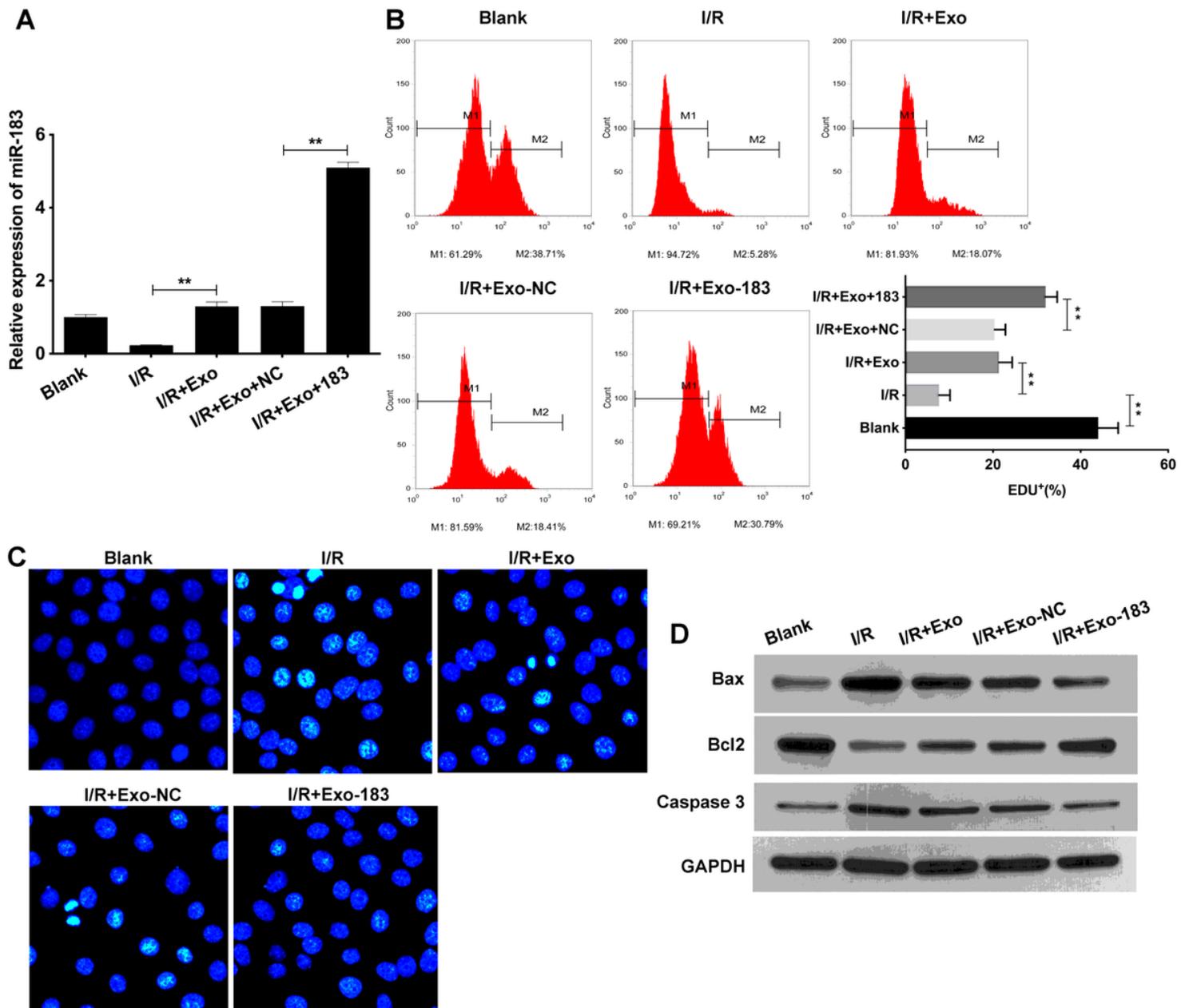


Figure 4

Exosomes derived from miR-183-overexpressed ADSCs dramatically enhances proliferation and inhibits apoptosis of HL-7702 cells after I/R injury by upregulating miR-183. HL-7702 cells after I/R injury were processed with exosomes or/and miR-183 mimics, respectively. (A) RT-qPCR analysis for miR-183 expression in each group. (B) Edu-positive cells in each group were monitored and counted by flow cytometry. (C) Representative images of Hoechst staining were presented for cell apoptosis. Magnification, $\times 200$. (D) Western blotting analysis was utilized for the detection of Bax, Bcl2 and caspase 3 expressions. $**P < 0.01$.

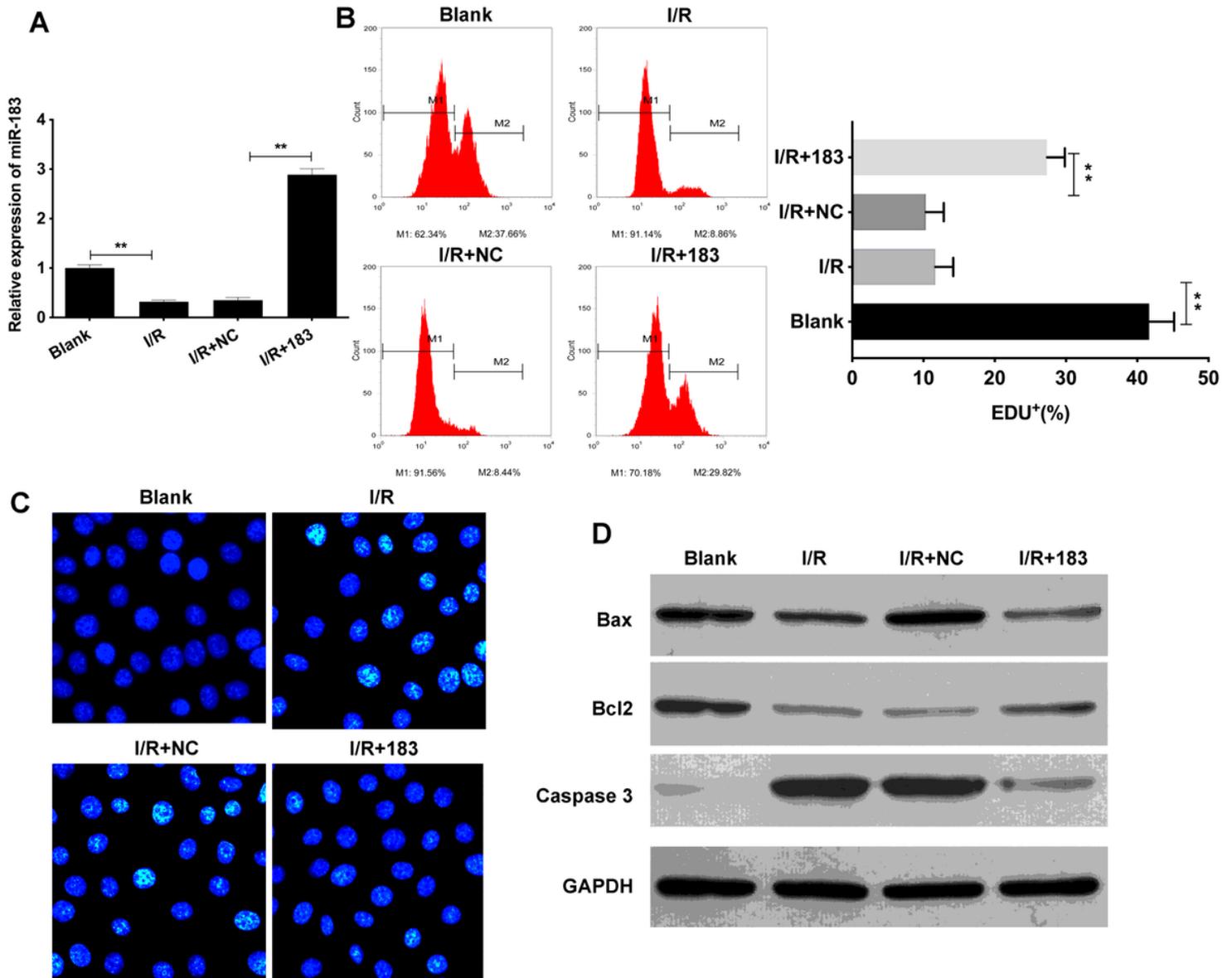


Figure 5

Overexpression of miR-183 markedly accelerates proliferation and prevents apoptosis of HL-7702 cells after I/R injury. (A) The overexpression effect of miR-183 was analyzed via RT-qPCR in HL-7702 cells with I/R injury, which were transfected with NC or miR-183 mimics, respectively. (B) The impact of miR-183 on the proliferation of HL-7702 cells with I/R injury was assessed by flow cytometry. And the quantitative analysis of Edu-positive cells was displayed. (C) After miR-183 overexpression, cell apoptosis was monitored using Hoechst staining. Magnification, $\times 200$. (D) Western blot was devoted to testify the influence of miR-183 on Bax, Bcl2 and caspase 3 expressions in HL-7702 cells with I/R injury. $**P < 0.01$.

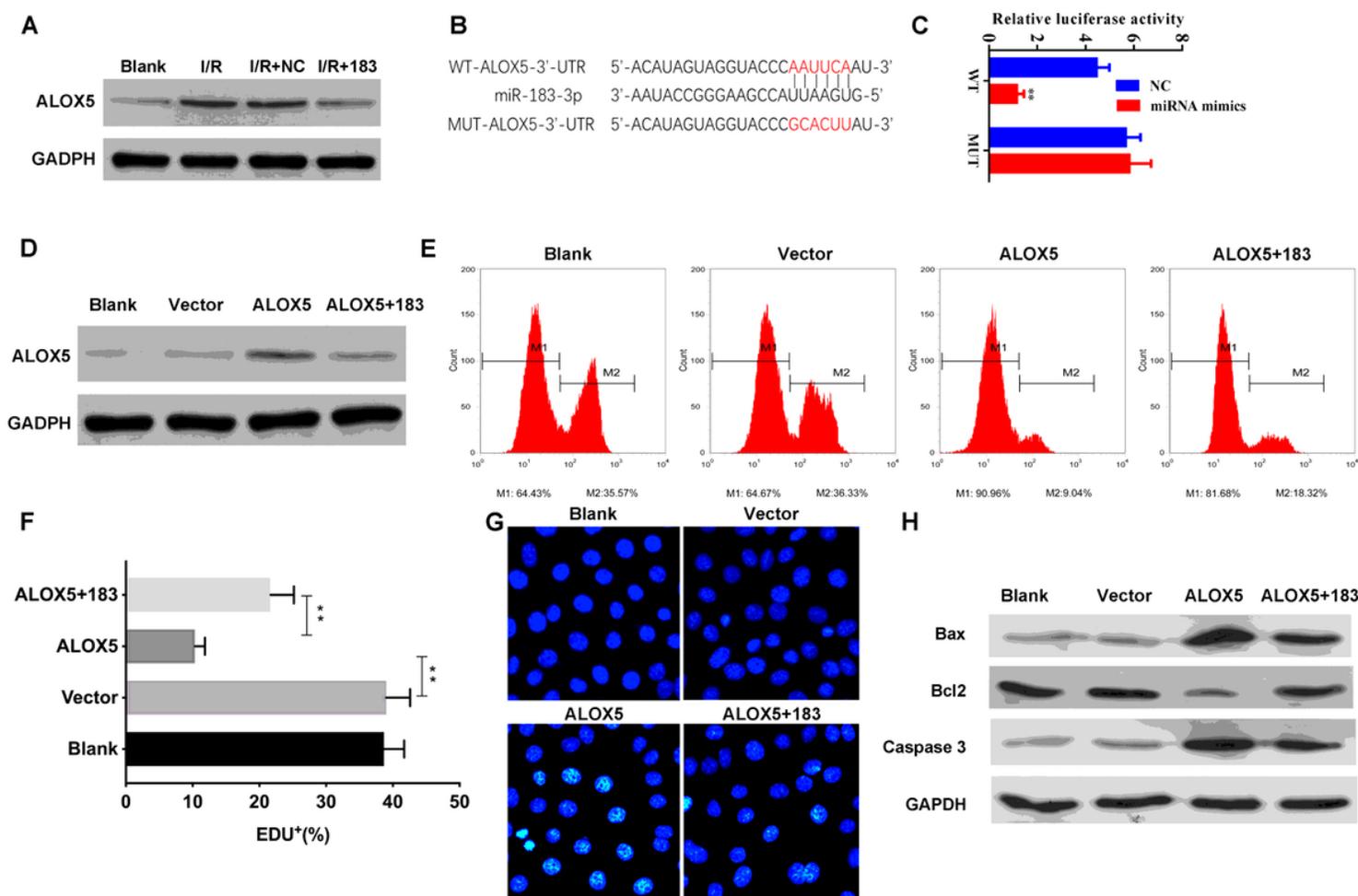


Figure 6

ALOX5, as a target gene of miR-183, is related to miR-183-mediated proliferation and apoptosis of HL-7702 cells with I/R injury. (A) ALOX5 level was tested by Western blot in after miR-183 overexpression. (B) The diagram displayed the homologous sequences of the binding sites in miR-183 and ALOX5. (C) The relationship between miR-183 and ALOX5 was verified via dual-luciferase reporter assay. (D) Flow cytometry was used for the verification of cell proliferation in HL-7702 cells with I/R injury after ALOX5 or/and miR-183 overexpression. (E) After co-transfection with ALOX5 and miR-183, cell apoptosis was tested with Hoechst staining in HL-7702 cells with I/R injury. Magnification, $\times 200$. (F) Western blotting analysis of Bax, Bcl2 and caspase 3 expressions. (G) The effect of ALOX5 and miR-183 overexpression on ALOX5 expression in HL-7702 cells with I/R injury. $**P < 0.01$.

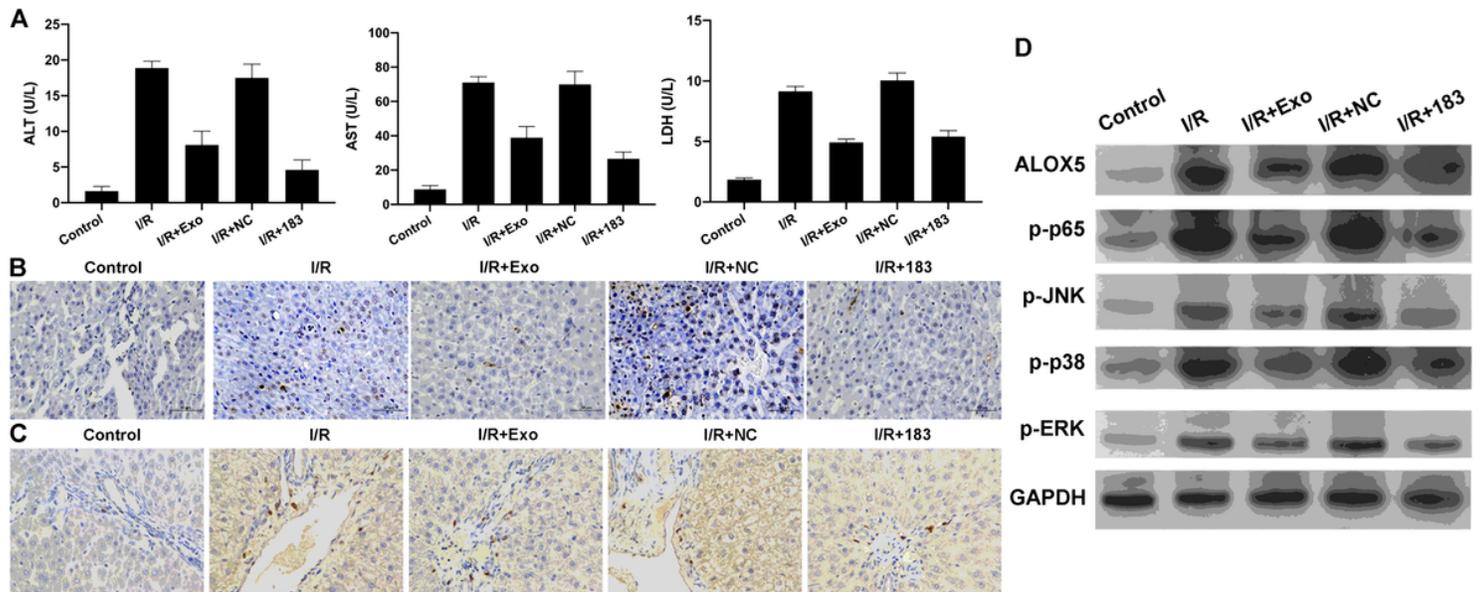


Figure 7

MiR-183 or exosomes from ADSCs ameliorates the liver function of I/R rats and inhibits MAPK and NF- κ B pathways. I/R rats were processed with exosomes derived from miR-183-overexpressed ADSCs and miR-183, respectively. (A) The levels of AST, ALT and LDH were certified by ELISA in the treated I/R rats. (B) Changes in apoptosis were identified by TUNEL staining. Magnification, $\times 200$. Scale bar=50 μ m. (C) ALOX5 expression was analyzed IHC in each group of liver tissues. Magnification, $\times 200$. (D) Western blotting analysis of ALOX5, p-p65, p-JNK, p-p38 and p-ERK expressions in each group of liver tissues.