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ORIGINAL ARTICLE

Exosomal *MALAT1* from macrophages treated with high levels of glucose upregulates *LC3B* expression via *miR-204-5p* downregulation

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Abstract

Background: Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) plays a critical role in the pathophysiology of diabetes-related complications. However, whether macrophage-derived *MALAT1* affects autophagic activity under hyperglycemic conditions is unclear. Therefore, we investigated the molecular regulatory mechanisms of macrophage-derived *MALAT1* and autophagy under hyperglycemic conditions.

Methods: Hyperglycemia was induced by culturing macrophages in 25 mM glucose for 1 hour. Exosomes were extracted from the culture media. A rat model of carotid artery balloon injury was established to assess the effect of *MALAT1* on vascular injury. Reverse transcription, real-time quantitative polymerase chain reaction, western blotting, immunohistochemical staining, and luciferase activity assays were performed.

Results: Stimulation with high levels of glucose significantly enhanced *MALAT1* expression in macrophage-derived exosomes. *MALAT1* inhibited *miR-204-5p* expression in macrophage-derived exosomes under hyperglycemic conditions. siRNA-induced silencing of *MALAT1* significantly reversed macrophage-derived exosome-induced *miR-204-5p* expression. Hyperglycemic treatment caused a significant, exosome-induced increase in the expression of the autophagy marker *LC3B* in macrophages. Silencing *MALAT1* and overexpression of *miR-204-5p* significantly decreased *LC3B* expression induced by macrophage-derived exosomes. Overexpression of *miR-204-5p* significantly reduced *LC3B* luciferase activity induced by macrophage-derived exosomes. Balloon injury to the carotid artery in rats significantly enhanced *MALAT1* and *LC3B* expression, and significantly reduced *miR-204-5p* expression in carotid artery tissue. Silencing *MALAT1* significantly reversed *miR-204-5p* expression in carotid artery tissue after balloon injury. *MALAT1* silencing or *miR-204-5p* overexpression significantly reduced *LC3B* expression after balloon injury.

Conclusion: This study demonstrated that hyperglycemia upregulates *MALAT1*. *MALAT1* suppresses *miR-204-5p* expression and counteracts the inhibitory effect of *miR-204-5p* on *LC3B* expression in macrophages to promote vascular disease.

Keywords: Autophagy; Exosomes; Macrophage; *MALAT1*; Rat

1. INTRODUCTION

Autophagy is a key catabolic process that promotes cell survival by protecting cells from different types of stress. It plays an important role in cellular quality control and homeostasis under both normal and pathologic conditions.[1] Autophagy activity is strongly linked to cardiovascular development and preservation of cardiac and vascular homeostasis, as well as the onset and progression of cardiovascular diseases (CVDs).[2,3] Increasing evidence has shown that macrophage autophagy plays a crucial role in inflammation, apoptosis, and cholesterol efflux.[4,5] This evidence suggests that the regulation of macrophage autophagy may be a potential strategy for CVD treatment.

Hyperglycemia is considered an independent risk factor for atherosclerosis and cardiovascular events[6] and is a major risk factor for morbidity and mortality in diabetes mellitus (DM).[7] Kanter et al[8] have demonstrated that increased glucose availability enhances glucose flux and glycolysis in macrophages, causing inflammatory activation within these cells. This increase in inflammatory activation of macrophages is a hallmark of diabetes.

Exosomes are considered natural nanocarriers and intercellular messengers that regulate cell-to-cell communication. Exosomes play an important role in the induction of autophagic flux by transporting autophagy activators and/or autophagy-related molecules to target cells.[9] The crosstalk between exosomes and autophagy may contribute to the maintenance of cellular homeostasis under external and internal stresses. Previous studies have shown that exosome-induced autophagy plays a pivotal role in cellular homeostasis, and may offer insight into novel therapeutic approaches.[10,11]

Long noncoding RNAs (lncRNAs) are non-protein-coding RNAs that are characterized by a length of at least 200 bp,

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with highly conserved sequences.[12] Yang et al[13] have reported that lncRNAs regulate autophagy via diverse mechanisms in eukaryotes. Furthermore, Wang et al[14] have demonstrated that a lncRNA called autophagy-promoting factor regulates autophagy and myocardial infarction by functioning as a CeRNA sponge for *miR-188-5p*. This indicates that the lncRNA-microRNA-mRNA-CeRNA network may play a critical role in the regulation of autophagy. Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is a highly conserved lncRNA whose expression correlates with autophagy and many human diseases. Fu et al[15] demonstrated that *MALAT1* activates autophagy and promotes cell proliferation by sponging *miR-101* and upregulating STMN1, RAB5A, and ATG4D expression in glioma. Li et al[16] also revealed that *MALAT1* interacts with the RNA-binding protein human antigen R, and silencing of *MALAT1* greatly enhances the post-transcriptional regulation of TIA-1, further inhibiting autophagy.

MALAT1 is significantly upregulated under hyperglycemic conditions and in fibrovascular membranes of the retina in patients with proliferative diabetic retinopathy.[17] Liu et al[18] demonstrated that *MALAT1* promotes neovascularization in human retinal microvascular endothelial cells treated with high levels of glucose. *MALAT1* gene polymorphisms have been shown to be associated with coronary artery disease in a Chinese population.[19] More recently, *MALAT1* has been reported to promote foot ulcer healing in diabetic rats.[20] These data indicate that *MALAT1* plays a crucial role in the relationship between hyperglycemia and vascular diseases.

MicroRNA (miRNA)-204 is a well-studied tumor suppressor. As a target of *MALAT1*, *miR-204* regulates the expression of the autophagy marker *LC3B* in patients with acute kidney injury and in various types of cancer.[21–24]

Although macrophages express *MALAT1*, it is unclear whether macrophage *MALAT1* affects autophagic activity under hyperglycemic conditions. In this study, we investigated the molecular regulatory mechanisms of macrophage-derived *MALAT1* and autophagy under hyperglycemic conditions.

2. METHODS

2.1. Cell culture

Murine macrophages (RAW264.7) were originally obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) at 37°C with 5% CO_2. Cells were grown to 80% to 90% confluence in 100 mm culture dishes and subcultured at a ratio of 1:3. For exosome collection, 10 mL of cell-free supernatant from the macrophage culture was collected after treatment with 25 mM glucose for 1 hour.

2.2. Extraction of exosomes from cell media

Total Exosome Isolation Reagent (Invitrogen, Thermo Fisher Scientific, MA) was used to isolate exosomes from the cell culture media according to the manufacturer's instructions as previously described.[25] Exosomes were quantitated using ExoQuant™ quantification assay kit according to the manufacturer's instructions (BioVision, Milpitas, CA).

2.3. Reverse transcription and real-time quantitative polymerase chain reaction

To quantitate *MALAT1*-exosome RNA transcripts, 12 µL of a 14 µL RNA eluate was subjected to reverse transcription (RT) with random hexamers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, MA). For quantitative polymerase chain reaction (qPCR), 10% of each cDNA reaction was analyzed using standard SYBR chemistry and cycler conditions. The Fast SYBR® Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) was used for the further assays. Polymerase chain reaction (PCRs) was performed in a 96-well plate using an ABI StepOnePlus cycler. The cycler profile is described as follows: (1) initial denaturation: 15 minutes at 95°C; (2) annealing and extension: 94°C for 15 seconds, 55°C for 30 seconds, 70°C for 30 seconds in reactions of 40 cycles. Analysis of relative gene expression levels was performed using formula $2^{-\Delta CT}$ with ΔCT = CT (target gene) – CT (control). Individual PCR products were sequenced to verify product purity as previously described.[26]

2.4. PCR product construction and sequencing

Cloning was performed using pGEM®-T Easy Vector System (Promega, Madison, WI). Ligation was performed in a final reaction volume of 10 µL consisting of 5 µL of 2X Rapid Ligation Buffer, 1 µL of pGEM®-T or pGEM®-T Easy Vector (50 ng), 1 µL of T4 DNA Ligase, and 3 µL of PCR product as described previously.[25] Primers used for direct sequencing were identical to those used in the amplification reactions. The nucleotide sequences of both strands were determined using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) equipped with a long-read sequencing capillary and POP-7 sequencing polymer.

2.5. Partial mouse *MALAT1* DNA fragment (containing *miR-204-5p* binding site) construction

ENSMUST00000172812.2 _267–766 bp; Chromosome 19:5,795,690-5,802,672; http://www.ensembl.org/index.html was generated by artificial synthesis, digested with SacI and Xbal restriction enzymes, and ligated into the pmirNanoGLO plasmid vector (Promega). The cloned mouse *MALAT1* DNA fragment contained *miR-204-5p* potential binding sites (from 517 to 539 bp). For the mutant, the conserved site AGGCATGAGTTGGAAACAGGGAA was mutated into CTTACGTATTGTGACCCCTTTCC and constructed using the same method described above. All cloned plasmids were confirmed by DNA sequencing (Seeing Bioscience Co., Ltd., Taipei, Taiwan).

2.6. Luciferase activity assay

The test plasmid (2 µg) was transfected using ViaFect™ Transfection Reagent (Promega) according to the manufacturer's protocol as previously described.[27] Briefly, a mixture of transfection reagent and DNA was incubated for 20 minutes at room temperature. The mixture was added to the cell culture medium for 24 hours. The culture medium was then replaced with normal culture medium. Following treatment, cell extracts were prepared using the Nano-Glo dual-luciferase reporter assay system (Promega), and luciferase activity was measured using a luminometer (Glomax Multi Detection System, Promega).

2.7. Western blot analysis

Cultured macrophages were harvested by scraping and centrifuged (300×g) for 10 minutes at 4°C. The pellet was resuspended and homogenized in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Rockford, IL) and centrifuged at 14 000×g for 15 minutes. A Bio-Rad Protein Assay was used to measure protein content. Equal amounts of protein (30 µg) were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels, and subjected to electrophoresis. Western blot was performed as previously described.[25] Equal protein loading of the samples was further verified by quantifying β-actin levels using mouse monoclonal β-actin (Sigma-Aldrich, Saint Louis, MO). All western blots were quantified using densitometry.

Cells treated with rapamycin (Sigma-Aldrich), an autophagy inducer and 3-methyladenine (3-MA; Sigma-Aldrich), an autophagy inhibitor were used as positive and negative controls, respectively.

2.8. Balloon injury of the carotid artery in diabetic rats and delivery of exosomes containing macrophage-derived *MALAT1*

Male Wistar rats (300-320 g), aged 18 weeks, purchased from BioLASC (BioLASC Taiwan Co., Ltd., Taipei, Taiwan), were injected with a single dose of intraperitoneal streptozotocin (STZ, Sigma-Aldrich) at 60 to 120 mg/kg to induce diabetes. Diabetes was confirmed by the presence of hyperglycemia (blood glucose concentration of 19 mmol/L) for a minimum of 1 week. One week after STZ injection, the rats were anesthetized with isoflurane (2%) and subjected to balloon catheter injury in the right carotid artery as previously described.[28] At the end of the experiment, the rats were euthanized with carbon dioxide using a special acrylic box and high-pressure barreled carbon dioxide equipment. Rats were placed in the box and subjected to a low concentration of carbon dioxide at first. Then, the carbon dioxide levels were increased rapidly so that the rat quickly lost consciousness. We continued to perfuse the gas for at least 5 minutes after the rat was lost consciousness and confirmed death before removing the rat from the euthanasia container. Immunofluorescence staining was performed as described previously.[28] The intimal, medial, and adventitial cross-sectional areas were measured using imaging software (Nikon NIS-Elements, Tokyo, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of Shin Kong Wu Ho-Su Memorial Hospital (approval number: 1110MOST002) and were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*.

2.9. Statistical analysis

The data are expressed as mean ± SD. Statistical significance was determined by analysis of variance (ANOVA) (GraphPad Software Inc., San Diego, CA). The Tukey-Kramer comparison test was used for pairwise comparisons between multiple groups after ANOVA. Statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. High glucose stimulation induced *MALAT1* expression in cultured macrophages

To test the effect of glucose stimulation on *MALAT1* expression in macrophages, different concentrations of glucose were added to the culture medium for 1 hour. As shown in Supplementary Fig. 1A, http://links.lww.com/JCMA/A250, *MALAT1* was significantly upregulated in the presence of 12.5 to 100 mM glucose. We found that 25 mM glucose treatment induced the maximal effect on *MALAT1* expression, after which its expression gradually decreased and reached a level similar to that of the control after 6 hours (Supplementary Fig. 1B, http://links.lww.com/JCMA/A250). Therefore, 25 mM glucose treatment was used for subsequent experiments.

Thus, exosomes were isolated from macrophages treated with 25 mM glucose. These glucose-treated macrophage-derived exosomes significantly enhanced cytoplasmic *MALAT1* expression in a dose-dependent manner compared to control cells or cells treated with exosomes derived from control macrophages (data not shown). Exosomes derived from macrophages stimulated with 25 mM glucose did not induce significant cytotoxicity when compared with the control.

3.2. Glucose-treated macrophage-derived exosomes decreased *miR-204-5p* and increased *LC3B* expression in cultured macrophages

MALAT1 has been shown to sponge *miR-204-5p* in other cell types.[21–23] The exogenous addition of 50 μg of exosomes induced maximal reduction of cytoplasmic *miR-204-5p* expression (Fig. 1). The same amount of control exosomes did not reduce *miR-204-5p* expression. Therefore, 50 μg of exosomes derived from macrophages treated with 25 mM glucose were used for subsequent experiments.

Treatment with 50 μg of glucose-treated macrophage-derived exosomes significantly enhanced cytoplasmic *MALAT1* levels in macrophages. However, cytoplasmic *miR-204-5p* mRNA levels were significantly reduced after treatment with exosomes from macrophages that weren't treated with glucose. Cytoplasmic *LC3B* levels exhibited a similar pattern to that of *MALAT1* after treatment with glucose-treated macrophage-derived exosomes (Fig. 2). Macrophage-derived exosomes gradually upregulated *LC3B* protein expression from 1 to 6 hours, with *LC3B* expression peaking at 5 hours (Fig. 3A, B). Silencing *MALAT1* using *MALAT1* siRNA significantly reduced cytoplasmic *LC3B* expression induced by glucose-treated macrophage-derived exosomes. Scrambled *MALAT1* siRNA did not significantly influence *LC3B* expression following treatment with macrophage-derived exosomes. Overexpression

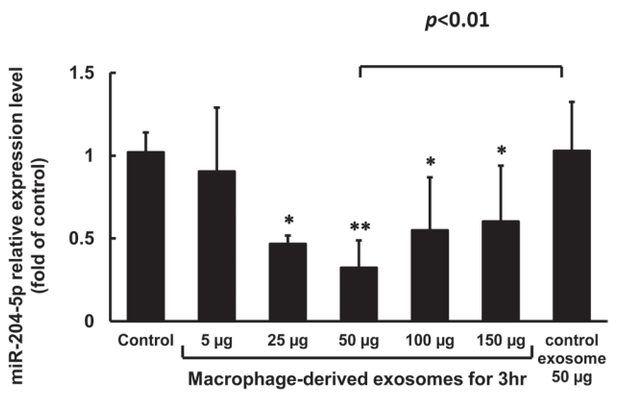

Fig. 1 Levels of *miR-204-5p* in macrophages treated with different amounts of exogenous macrophage-derived exosomes for 3 h. The macrophage-derived exosomes were extracted from macrophages treated with 25 mM glucose for 1 h. *$p < 0.05$ vs control; **$p < 0.01$ vs control. N = 6 per group.

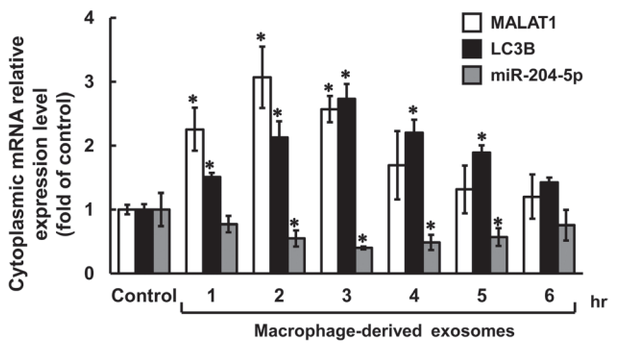

Fig. 2 Effect of macrophage-derived exosome on cytoplasmic *MALAT1*, *LC3B*, and *miR-204-5p* expression in cultured macrophages treated with exogenous macrophage-derived exosomes for different periods of time. The macrophage-derived exosomes were extracted from macrophages treated with 25 mM glucose treatment for 1 h. *$p < 0.01$ vs control. N = 4 per group.

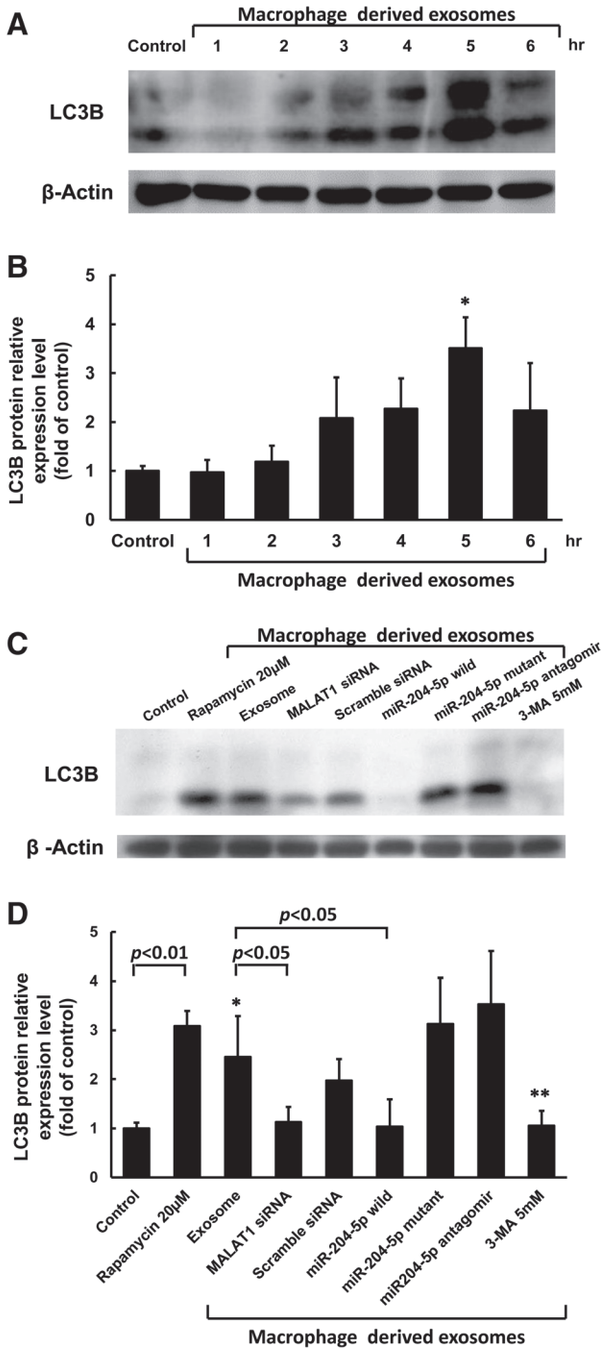

Fig. 3 Effect of macrophage-derived exosomes on *LC3B* protein expression in cultured macrophages. A, Representative western blots for *LC3B* and β-actin from macrophages subjected to macrophage-derived exosome treatment for different periods of time. B, Quantitative analysis of *LC3B* protein levels. The values for stimulated macrophages have been normalized to the control cell values. *$p < 0.01$ vs control; **$p < 0.05$ vs control. N = 3 per group. C, Representative western blots for *LC3B* and β-actin levels in macrophages treated with different amounts of macrophage-derived exosomes. D, Quantitative analysis of *LC3B* protein levels. The values for the stimulated macrophages have been normalized to the control cell values. Scramble siRNA was the control siRNA. *$p < 0.01$ vs control; **$p < 0.05$ vs control. N = 3 per group. E, Representative image of autophagosomes and/or autolysosomes by DALGreen fluorescent dye stain in macrophages treated with different agents. Treatment with macrophage-derived exosomes, wild-type *miR-204-5p*, mutant *miR-204-5p*, and rapamycin increased DALGreen fluorescent dye labeling. Similar results were observed in another two experiments.

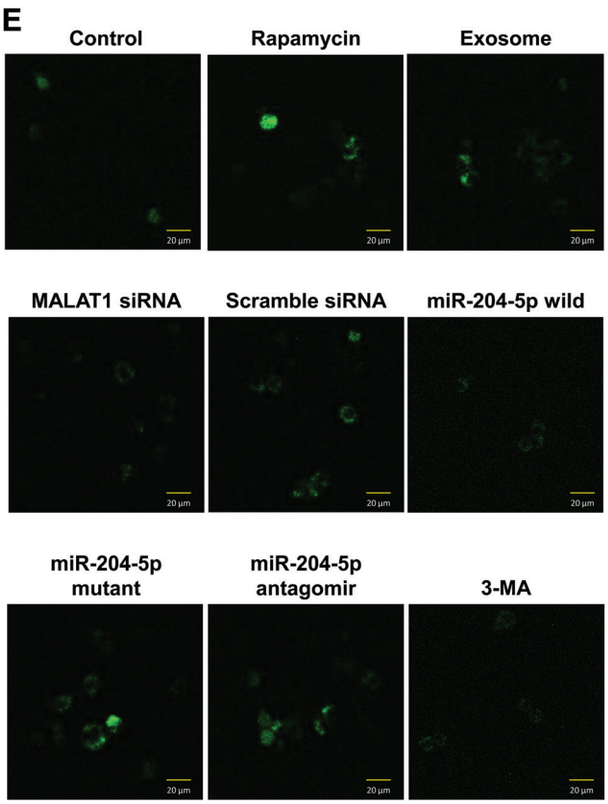

Fig. 3 Continued

of wild-type *miR-204-5p* was significantly inhibited by macrophage-derived exosome-induced *LC3B* expression, whereas overexpression of mutant *miR-204-5p* or its antagomir did not affect macrophage-derived exosome-induced *LC3B* expression. Treatment with rapamycin (an autophagy inducer) and 3-MA (an autophagy inhibitor) significantly increased and decreased *LC3B* expression compared to that in the control, respectively (Fig. 3C, D). Treatment with macrophage-derived exosomes, wild-type *miR-204-5p*, mutant *miR-204-5p*, or rapamycin increased the development of autophagosomes and/or autolysosomes in macrophages, as shown by DALGreen fluorescent dye staining (Fig. 3E). Silencing *MALATA1* using *MALAT1* siRNA significantly reversed cytoplasmic *miR-204-5p* expression in macrophages (Fig. 4A). Treatment with scrambled *MALAT1* siRNA did not significantly influence cytoplasmic *miR-204-5p* expression following treatment with macrophage-derived exosomes. Exosomes derived from macrophages stimulated with 25 mM glucose significantly enhanced the *LC3B* expression (Fig. 4B). *MALAT1* siRNA significantly inhibits *LC3B* mRNA expression in macrophages treated with macrophage-derived exosomes. Scrambled *MALAT1* siRNA did not significantly affect *LC3B* mRNA expression following macrophage-derived exosome treatment. Overexpression of wild-type *miR-204-5p* significantly inhibited *LC3B* expression induced by macrophage-derived exosomes, while overexpression of the mutant *miR-204-5p* or the antagomir of *miR-204-5p* did not inhibit *LC3B* expression induced by macrophage-derived exosomes. Rapamycin, and 3-MA treatment significantly increased and decreased *LC3B* expression compared to that in the control, respectively (Fig. 4A, B). These results indicate that macrophage-derived exosome stimulation enhanced *MALAT1* expression but reduced *miR-204-5p* expression to

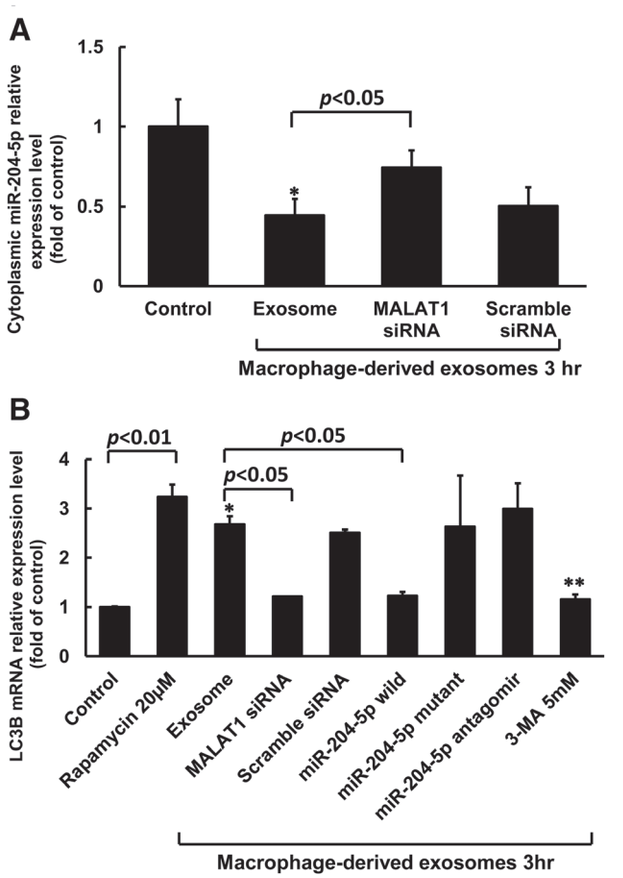

Fig. 4 Effect of macrophage-derived exosomes on *miR-204-5p* and *LC3B* expression in cultured macrophages. A, Quantitative real-time PCR analysis of *miR-204-5p* levels. Values for the treated macrophages are expressed as a ratio of the values normalized with *miR-204-5p* in the control cells. *$p < 0.01$ vs control. N = 4 per group. B, Quantitative real-time PCR analysis of *LC3B* mRNA levels. Values for the treated macrophages are expressed as a ratio of the values normalized values with *LC3B* mRNA in the control cells. Scrambled siRNA was the control siRNA. Macrophage-derived exosomes were extracted from macrophages treated with 25 mM glucose treatment for 1 h. *$p < 0.01$ vs control; **$p < 0.01$ vs Exosome. N = 4 per group.

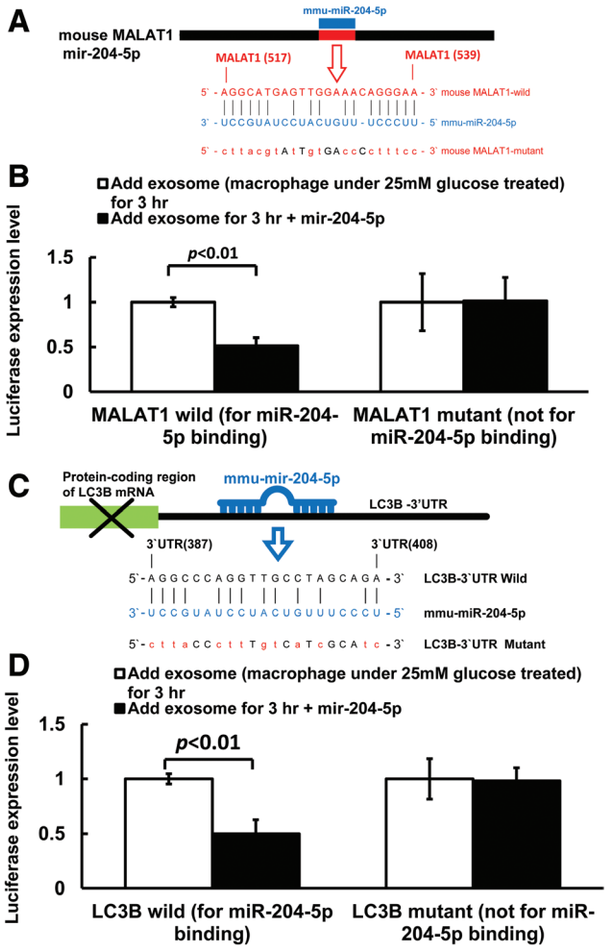

Fig. 5 Effect of *miR-204-5p* on *MALAT1* and *LC3B* luciferase activity in cultured macrophages treated with macrophage-derived exosomes. A, Sequence of the mouse *miR-204-5p* binding site in the *MALAT1* 3'UTR (nucleotides 517-539 bp). B, *MALAT1* 3'UTR luciferase activity after treatment with macrophage-derived exosomes treatment for 3 h with wild-type or mutant MALAT1. C, Sequence of the *miR-204-5p* binding site in the mouse *LC3B* 3'UTR, which is located on the *miR-204-5p* 3'UTR (nucleotides 387-408 bp). D, *LC3B* 3'UTR luciferase activity after treatment with macrophage-derived exosomes treatment for 3 h with wild-type or mutant *LC3B*. N = 3 per group.

increase *LC3B* protein levels in macrophages under hyperglycemic conditions.

3.3. *MiR-204-5p* decreased *MALAT1* and *LC3B* luciferase activity in macrophages treated with macrophage-derived exosomes under high glucose stimulation

Fig. 5A shows the sequence of the *MALAT1* 3'UTR target site for *miR-204-5p* binding (nucleotides 517-539). *MiR-204-5p* overexpression significantly reduced *MALAT1* luciferase activity in macrophages under 25 mM high glucose stimulation, when *miR-204-5p* was bound to the normal *MALAT1* 3'UTR (Fig. 5B). Overexpression of the mutant *miR-204-5p* did not significantly influence *MALAT1* luciferase activity, indicating that *miR-204-5p* was a target of *MALAT1*. We discovered that the *LC3B* 3'UTR (nucleotides 387-408) had a binding site for *miR-204-5p*, as indicated in Fig. 5C. *MiR-204-5p* overexpression significantly reduced *LC3B* luciferase activity in macrophages under 25 mM high glucose stimulation when *miR-204-5p* was bound to the normal *LC3B* 3'UTR (Fig. 5D). Overexpression of mutant *miR-204-5p* did not significantly influence *LC3B* luciferase activity, indicating that *LC3B* is a *miR-204-5p* target gene.

3.4. Carotid artery balloon injury enhances *MALAT1* to inhibit *miR-204-5p* expression in diabetic rats

Single doses of STZ (60-120 mg/kg) were administered to induce diabetes. Streptozotocin (90 mg/kg) induced the maximal effect on increased fasting glucose levels in rats (Supplementary Figure II, http://links.lww.com/JCMA/A251) for up to 4 weeks. Thus, this dosage (90 mg/kg) was administered in the subsequent animal studies. The results showed that *MALAT1* expression in the carotid arterial tissue gradually increased from 3 to 28 days in diabetic rats compared with that in control rats, and in diabetic rats after carotid artery balloon injury compared with that in the sham group (Fig. 6A). *MALAT1* mRNA expression was significantly enhanced

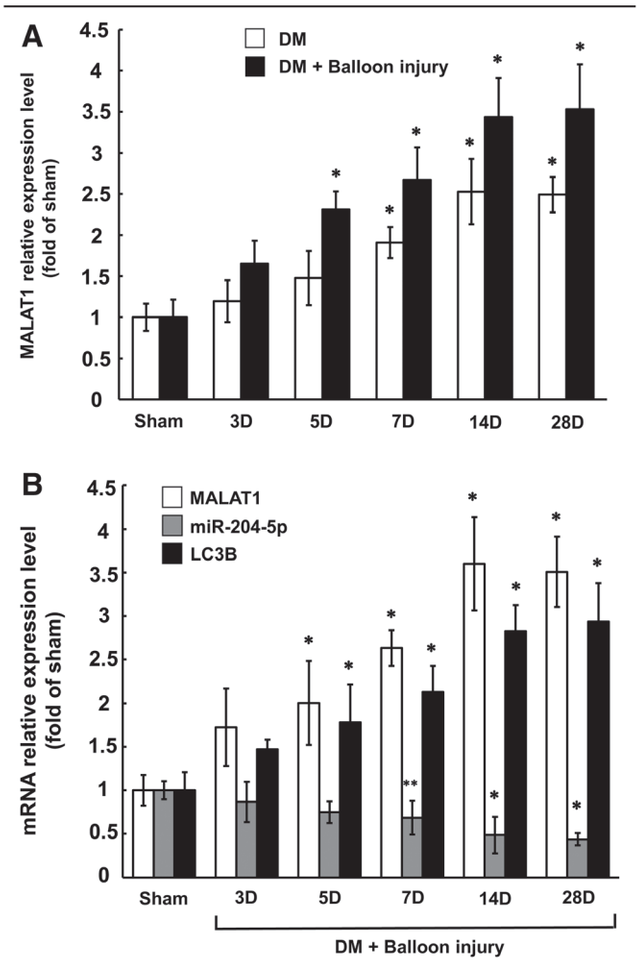

Fig. 6 Effect of carotid artery balloon injury on *MALAT1* expression in diabetic rats. A, Quantitative real-time PCR analysis of *MALAT1* levels after different time-periods in arterial tissue with or without balloon injury in diabetic rats. B, Quantitative real-time PCR analysis of *MALAT1*, *miR-204-5p*, and *LC3B* mRNA levels after different time-periods in arterial tissue after carotid artery balloon injury. *$p < 0.01$ vs control; **$p < 0.05$ vs control. N = 5 per group.

and *miR-204-5p* expression was significantly reduced between days 7 and 28 post balloon injury of the carotid artery in diabetic rats (Fig. 6B). The expression pattern of *LC3B* mRNA in the carotid artery after balloon injury was similar to that of *MALAT1* mRNA. Fourteen days after balloon injury of the carotid artery, *miR-204-5p* expression was significantly inhibited in diabetic rats treated with glucose-treated macrophage-derived exosomes. At 14 days after balloon injury to the carotid artery, *miR-204-5p* expression was significantly reversed by silencing *MALAT1* using a *MALAT1* siRNA (Fig. 7A). Scrambled *MALAT1* siRNA did not significantly influence *miR-204-5p* expression compared to diabetic rats with balloon injury. Fourteen days after balloon injury of the carotid artery, *LC3B* mRNA and protein expression was significantly enhanced by treatment with macrophage-derived exosomes and significantly; however it was reduced by silencing *MALAT1* using MALAT1 siRNA and *miR-204-5p* wild-type overexpression compared to that in injured rats treated with glucose-treated macrophage-derived exosome (Fig. 7B–D). Overexpression of scrambled siRNA or mutant *miR-204-5p* did not significantly influence *LC3B* mRNA and protein expression compared to that in the injured, glucose-treated macrophage-derived exosome group.

3.5. Macrophage-derived exosomes enhanced *LC3B* protein expression in the arterial tissue of diabetic rats after carotid artery balloon injury

Fourteen days after carotid artery balloon injury, *LC3B* fluorescent signals were enhanced in the arterial tissues of diabetic rats. *LC3B* fluorescence signals were also enhanced upon treatment with macrophage-derived exosomes (Supplementary Figure III, http://links.lww.com/JCMA/A252). *LC3B* fluorescent signals were reduced by silencing *MALAT1* using *MALAT1* siRNA at 14 days after balloon injury of the carotid artery compared to

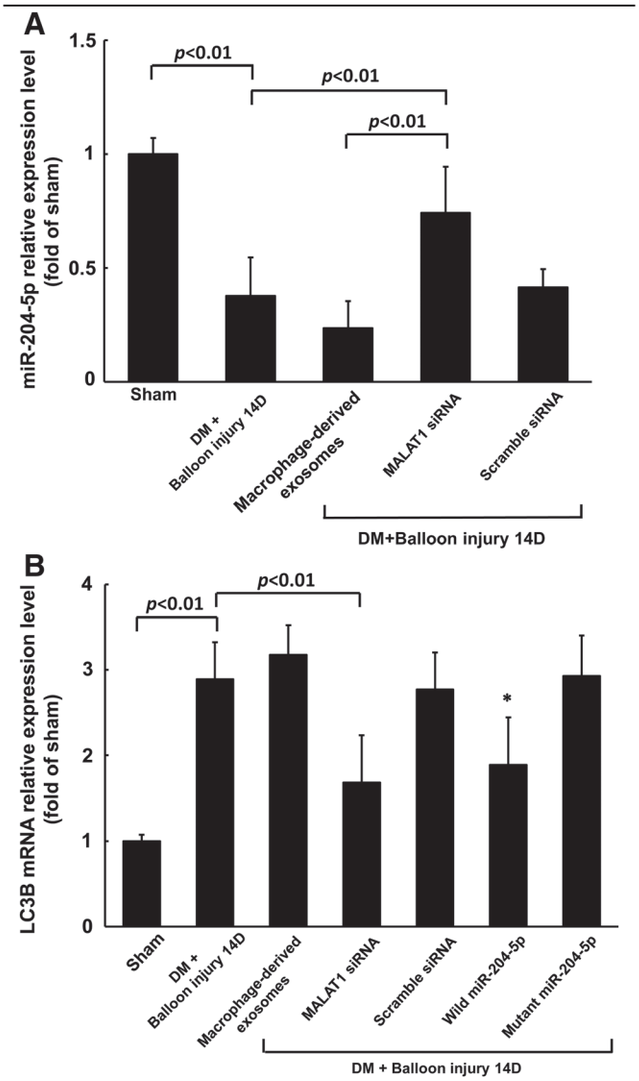

Fig. 7 Macrophage-derived *MALAT1*-mediates the reduction of *miR-204-5p* expression after balloon injury of the carotid artery in diabetic rats. A, Quantitative real-time PCR analysis of *miR-204-5p* levels in arterial tissue 14 d after balloon injury of the carotid artery in diabetic rats. The macrophage-derived exosomes were extracted from macrophages treated with 25 mM glucose for 1 h. Scrambled siRNA was the control siRNA. B, Quantitative real-time PCR of *LC3B* mRNA levels in arterial tissue 14 d after balloon injury of the carotid artery in diabetic rats. Macrophage-derived exosomes were extracted from macrophages treated with 25 mM glucose for 1 h. *$p < 0.01$ vs exosome group. C, Representative western blots for *LC3B* and β-actin levels in arterial tissues 14 d after balloon injury of the carotid artery in diabetic rats. D, Quantitative analysis of *LC3B* levels. The values for the balloon injury group have been normalized to those of the sham group. *$p < 0.05$ vs balloon injury in diabetic rats. N = 5 per group.

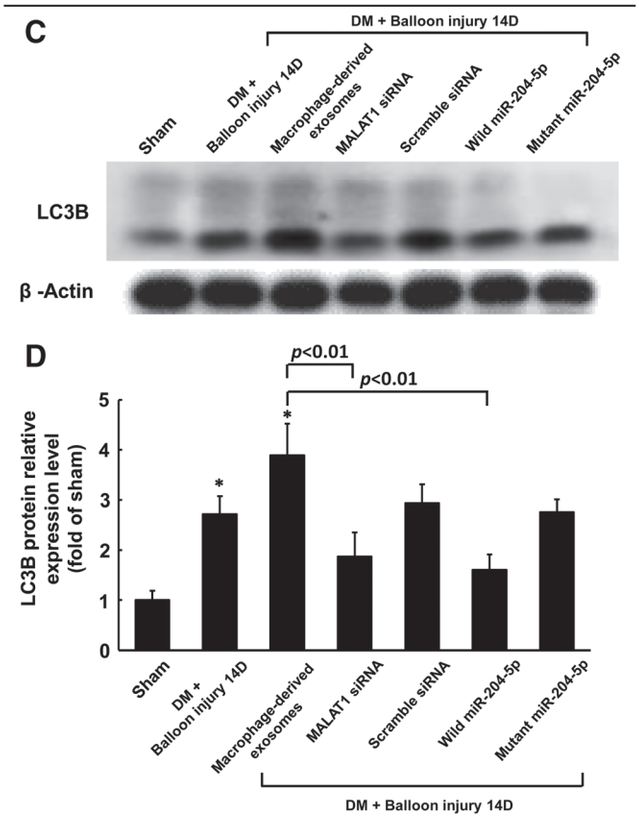

Fig. 7 Continued

the group treated with macrophage-derived exosome after balloon injury of the carotid artery. Balloon injury to the carotid artery significantly reduced lumen size and intimal area gain of the carotid artery. Treatment with macrophage-derived exosomes significantly reduced lumen size and intimal area gain compared to that in the group administered balloon injury of the carotid artery. Lumen size was significantly increased, and intimal area was significantly reduced by siRNA-mediated silencing of *MALAT1* compared with diabetic rats administered balloon injury of the carotid artery (Supplementary Fig. IV, http://links.lww.com/JCMA/A253).

4. DISCUSSION

During circulation, exosomes are the richest reservoirs of almost all lncRNAs, which are thus protected against RNases. These protected circulating lncRNAs contain valuable genetic information, making them more useful than body fluids in clinical applications.[29–31] Increasing evidences suggest that manipulating lncRNAs may serve as novel therapeutic tools and innovative biomarkers for the diagnosis and treatment of related diseases.

A clinical study has demonstrated that patients with diabetes exhibit a higher risk of atherosclerotic CVD and its associated morbidity and mortality.[32] Macrophages play an important role in the pathogenesis of atherosclerosis and vascular disease.[33] LncRNAs have been reported to exacerbate atherosclerosis and vascular disease[34] and *MALAT1* plays a causal role in the pathophysiological process associated with complications caused by DM.[35]

Previous studies have demonstrated that *MALAT1* acts as a sponge for *miR-204-5p* in lung adenocarcinoma, gastric cancer, thyroid cancer cells, and acute kidney injury.[21–24] In this study, we showed that *MALAT1* reduces *miR-204-5p* expression in macrophages under high glucose stimulation. We also identified an *miR-204-5p* binding site in the *MALAT1* promoter. Exosomes from macrophage stimulated with 25 mM glucose for 1 hour expressed *MALAT1*. Moreover, the exogenous addition of glucose-treated macrophage-derived exosomes inhibited *miR-204-5p* expression. Moreover, siRNA-mediated silencing of *MALAT1* reversed *miR-204-5p* expression.

LC3B, a ubiquitin-like molecule, is an autophagy marker[36] and an RNA-binding protein that induces rapid mRNA degradation during autophagy.[37] Recently, Miao et al[38] reported that treatment with high levels of glucose increased *LC3B* expression and the quantity of autophagic vacuoles in rat aortic endothelial cells. *LC3B* was found to be a target of *miR-204* in osteoblastic MC3T3-E1 cells.[39] However, it is not known whether treatment with high levels of glucose increases autophagy in macrophages and in an animal model of carotid artery vascular injury. In this study, we revealed that macrophage-derived exosomes containing *MALAT1* could bind to *miR-204-5p* to enhance *LC3B* expression, and that silencing *MALAT1* could significantly attenuate the effect of exosome treatment. Overexpression of *miR-204-5p* significantly altered the effect of macrophage-derived exosomes via silencing of *MALAT1*. We identified an *miR-204-5p* binding site in the 3'UTR of *LC3B*, and the overexpression of *miR-204-5p* significantly reduced *LC3B* luciferase activity in macrophages following exosome treatment. These results indicate that *LC3B* is a target gene of *miR-204-5p*. Shao et al[22] have reported that *MALAT1* activates autophagy by downregulating *miR-204* expression in gastric cancer. Zhang et al[40] have reported that *miR-204* silencing could reduce mitochondrial autophagy in a murine Alzheimer's model. In this study, we compared effect of *MALAT1* and *miR-204-5p* on autophagy with that induced by rapamycin and 5-MA, which act as an autophagy inducer and inhibitor, respectively.

Our in vivo balloon injury carotid artery model also demonstrated that macrophage-derived *MALAT1* enhanced balloon injury-induced plaque formation in carotid arterial tissue of diabetic rats. Our results demonstrated that *miR-204-5p* expression was reduced and *LC3B* expression was enhanced by balloon injury of the carotid artery in diabetic rats. We also found that *miR-204-5p* expression was reduced and *LC3B* expression was enhanced by treatment with macrophage-derived exosomes in diabetic rats that were administered balloon injury to the carotid artery. siRNA-mediated silencing of *MALAT1* reversed *miR-204-5p* expression and inhibited *LC3B* expression in the carotid artery after balloon injury. Balloon injury to the carotid artery results causes lumen size to decrease and intimal area to increase. However, *MALAT1* silencing or *miR-204-5p* overexpression increased lumen size and reduced intimal area of the carotid artery after balloon injury. Although we did not analyze human samples, Lu et al[24] reported that *MALAT1* was strongly elevated, and *miR-204* was significantly reduced in serum samples from patients with acute kidney injury.

The effect of *MALAT1* on autophagy in different diseases or cell types is an issue of debate. *MALAT1* has been shown to activate autophagy to promote tumor progression in glioma,[15] pancreatic cancer,[16] gastric cancer,[22] and multiple myeloma.[41] However, Wang et al[42] have reported that *MALAT1* promotes gastric cancer progression via inhibition of autophagy. Miao et al[38] have reported that treatment with high levels of glucose increases *LC3B* expression and enhances autophagy in rat aortic endothelial cells.[34] *MALAT1* was also shown to enhance ox-LDL-induced autophagy in macrophages.[43] Our study results revealed that hyperglycemia upregulated *MALAT1* expression and *LC3B*, an autophagy marker, in both macrophages and

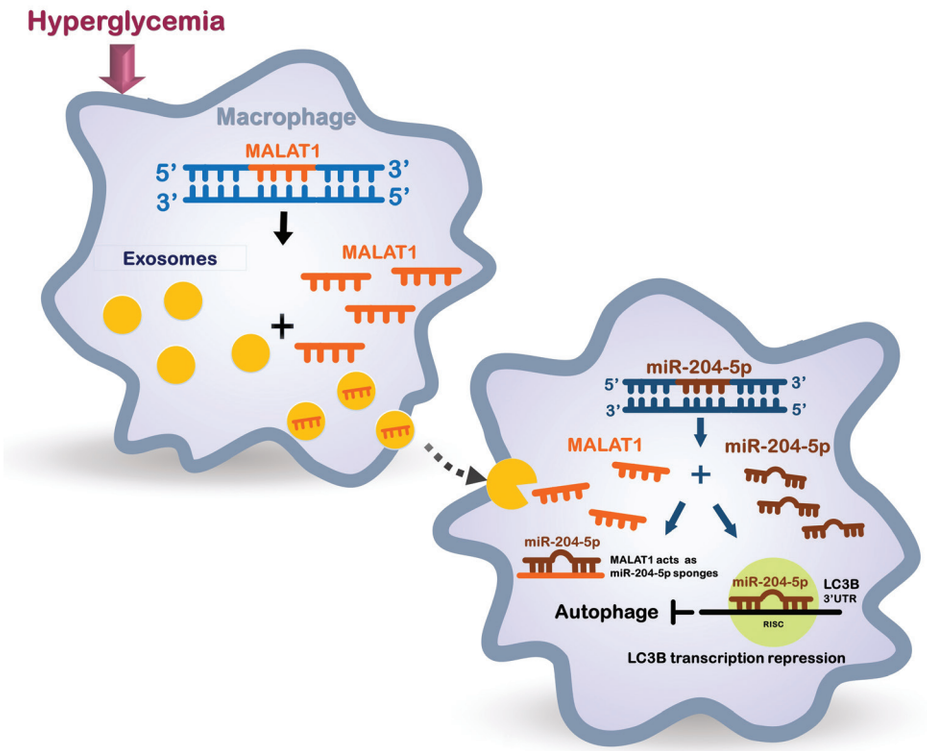

Fig. 8 Proposed pathway for autophagy mediated by hyperglycemia-induced macrophage-derived exosomes through the enhancement of *MALAT1*, inhibition of *miR-204-5p* expression and enhancement of *LC3B* expression in macrophages. This pathway illustrates the critical role of macrophage-derived exosomal *MALAT1* in vascular disease in DM. Therefore, macrophage-derived exosomal *MALAT1* can serve as a valuable therapeutic target for vascular disease management in patients with DM.

diabetic rats after balloon injury of the carotid artery. However, Yang et al[44] reported that impaired autophagy was observed in high glucose-stimulated H9C2 cells and heart tissue from diabetic patients. Analysis of the effect of *MALAT1* on different cell types and disease models may help us understand these seemingly contradictory reports on MALAT-mediated activation and inhibition of autophagy.

Macrophages exhibit different phenotypes in response to different stimuli with M1 and M2 macrophages exhibiting pro- and anti-inflammatory functions, respectively.[45] Recently, Ahmad et al[46] have shown that *MALAT1* favors the M1 macrophage because *MALAT1* knockdown enhanced the expression of M2 macrophage markers without affecting M1 macrophage markers. Based on the findings of this study, we speculate that macrophage in this study exhibited the M1 phenotype because macrophage *MALAT1* promotes vascular disease in the carotid artery balloon injury model.

To conclude, in the present study, we found that hyperglycemia upregulated *MALAT1* to inhibit *miR-204-5p* expression, thereby increasing *LC3B* expression in macrophages and promoting vascular disease. The effects of hyperglycemia on *MALAT1*, *miR-204-5p*, and *LC3B* expression in macrophages are summarized in Fig. 8. Thus, we hypothesized that macrophage-derived exosomal *MALAT1* stimulated cellular autophagy response and that targeting the MALAT/*miR-204-5p* axis may have applications in treating hyperglycemia-induced vascular diseases, including diabetic retinopathy, diabetic foot ulcers, and diabetes-associated vascular diseases. Macrophage-derived exosomal *MALAT1* may serve as a novel cell-free approach for the management of vascular diseases frequently encountered in patients with DM.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://links.lww.com/JCMA/A250.

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