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miR-143 is involved in endothelial cell dysfunction through suppression of glycolysis and correlated with atherosclerotic plaques formation

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Abstract. – OBJECTIVE: Atherosclerosis is recognized as a chronic inflammatory disease leading to hardening of the vessel wall and narrowing of arteries. Endothelial cells (ECs) exhibit highly active glycolysis, the dysfunction of which leads to accumulation of lipids in the arterial wall and formation of atherosclerotic plaque.

MATERIALS AND METHODS: qRT-PCR was performed to compare the deregulated miR-143 between atherosclerotic plaque and normal vessel tissues. The direct target of miR-143 was verified by Western blot and luciferase assay. The metabolic enzymes in atherosclerotic plaque and normal vessel tissues were measured. HUVECs were transfected with miR-143 precursor or control microRNAs, and glucose uptake, lactate production, intracellular ATP, and oxygen consumption were measured.

RESULTS: In this study, we report a correlation between up-regulated miR-143, EC dysfunction, and atherosclerotic plaque formation. The glycolysis rate was significantly elevated in ECs, which show relatively low levels of miR-143. Importantly, miR-143 was upregulated in clinical atherosclerotic plaque samples compared with healthy arteries, suggesting that miR-143 might play important roles in the atherosclerotic plaque formation. Moreover, mRNA levels of key enzymes of glycolysis, such as HK2, LDHA, and PKM2 are significantly down-regulated in the atherosclerotic plaque samples. Overexpression of miR-143 in HUVECs suppresses glycolysis through direct targeting of HK2, leading to EC dysfunction. Restoration of HK2 expression rescues glycolysis in miR-143-overexpressing HUVECs.

CONCLUSIONS: This study provides further insight into the metabolic mechanisms involved in atherosclerotic plaque formation due to microRNAs.

Key Words:
miR-143, Endothelial cell dysfunction, Glycolysis, Atherosclerotic plaque formation.

Introduction

Atherosclerotic vascular disease is the usual cause of heart attacks, strokes, and peripheral vascular disease, and remains the number one cause of death and morbidity in the world[1-3]. It is widely recognized as a chronic inflammatory disease that leads to vessel wall hardening and narrowing of the arteries[2,3]. In its early stages, atherosclerosis involves endothelial activation and dysfunction, which lead to accumulation of lipids in the arterial wall and formation of atherosclerotic plaque[4,5]. The vascular endothelium is the major interface of the vessel wall between blood and other tissues. For maintaining their functions, endothelial cells (ECs) must be well-adapted to environmental fluctuations since they are exposed to a variety of stimuli, such as nutrition deficiency and hypoxia under pathophysiological states[6]. Cellular metabolism is one mechanism that may contribute to rapid dynamic changes in cell phenotype. To maintain the homeostatic control of ECs, their cellular metabolism is tightly regulated at multiple levels, including transcriptional and post-translational stages[7-9]. De Bock et al[10] reported that ECs are in highly glycolytic states compared with other cells and that glycolysis is the predominant bioenergetic pathway for ECs, indicating that deregulated metabolism of ECs might be the pathological cause of other vascular diseases such as atherosclerosis.

MicroRNAs (miRNAs) are highly conserved noncoding RNA molecules of about 22 nt that exert post-transcriptional regulation of gene expression through complementary binding to the 3' untranslated region of their targets[11]. miRNAs have emerged to play critical roles in cancer[12]. In addition, miRNAs have been associated with inflammation, cardiovascular disease, oxidative

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stress, impaired adipogenesis, obesity, and angiogenesis[13,14]. Bidzhekov et al[15] identified differentially regulated miRNAs between atherosclerotic plaque and healthy arteries, suggesting that miRNAs might play essential roles in the formation of atherosclerotic plaque.

To better understand the pathophysiology of the atherosclerotic process, we compared the miRNA expression in atherosclerotic plaque and normal arteries. Further, by investigating the glucose metabolism of ECs, our study proves the correlation between atherosclerotic plaque and miRNA-regulated metabolism in ECs, which may help improve the clinical therapy of human atherosclerosis.

Materials and Methods

Primary Endothelial Cells and Atherosclerotic Plaque Specimens

Human aortic ECs (HAECs) and adult human dermal blood microvascular ECs (HMV) were purchased from Lonza (Basel, Switzerland) and cultured in the EGM-2 medium supplemented with Single Quots (Lonza, Basel, Switzerland). Other human primary cells were purchased from the American Type Culture Collection (ATCC: Manassas, VA, USA). HUVECs were cultured in M199 medium (1 mg/ml D-glucose) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 30 mg/l endothelial cell growth factor supplements (EGCS), 10 units/ml heparin, 50 IU/ml penicillin and 50 mg/ml streptomycin. Cells were cultured in the ATCC recommended medium. All cells were cultured in a humidified atmosphere with 5% CO_2 in air at 37°C.

All primary human atherosclerotic plaque specimens were obtained from patients undergoing surgery for carotid endarterectomy from 2012 to 2014 at Department of Cardiology, Second Hospital of Jilin University and were stored in liquid nitrogen until analysis. All patients provided written informed consent. The study was approved by the Ethics Committee of Department of Cardiology, Second Hospital of Jilin University, China.

Antibodies and Reagents

The antibodies used for this study are listed as follows: Hexokinase 2 (HK2), PKM2, and LDHA (Cell Signaling Glycolysis Antibody Sampler Kit #8337, Danvers, MA, USA); β-actin (Cell Signaling #4967, Danvers, MA, USA); 3-BrPA was purchased from Sigma-Aldrich (Hong-Kong, China).

Pre-miRNA or Plasmid DNA Transfection

miRNA precursors (pre-miR-143) and control miRNAs were purchased from Applied Biosystems (Foster City, CA, USA). Overexpression vectors containing wild-type HK2 and the control vector were obtained from Addgene (Cambridge, MA 02139, USA). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection of miRNAs or plasmid DNA. Forty-eight hours after transfection, the expression of miR-143 was detected by real-time-PCR, and HK2 expression was assessed by Western blotting.

Glucose Consumption Assay

Cells were seeded in 12-well plates at 2×10^5 cells/well. Culture media was collected at 48 hr for measurement of glucose consumption using an Amplex Red Glucose/Glucose oxidase assay kit (Molecular Probes, Eugene, OR, USA). Absorbance was measured at 563 nm using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA), and the results were normalized to the amount of total protein compared with the control cells.

Lactate Production Assay

Cells were seeded in 12-well plates at 2×10^5 cells/well. Culture media was collected at 48 hr for the measurement of lactate production using a Lactate assay kit (BioVision, Inc., Milpitas, CA, USA). Results were normalized to the quantity of total protein compared with the control cells.

Oxygen Consumption

Cells were seeded at 1×10^5 cells per well on Seahorse XF24 tissue culture plates (Seahorse Bioscience Europe, Copenhagen, Denmark). The measurement of oxygen consumption was performed at 10 min intervals for 3 hr using the Seahorse XF24 analyzer.

Measurements of Intracellular ATP

Cells were seeded in 12-well plates at 2×10^5 cells/well for measurement of ATP levels using the ATP Determination Kit (Life Technologies #A22066, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative RT-PCR (qRT-PCR)

RNA was isolated from cultured cells using the RNeasy mini-kit (Qiagen, Valencia, CA, USA) (with an on-column DNAse digestion step according to the manufacturer's instructions). Briefly, cell lysates were passed through a Qiashredder (Qiagen, Valencia, CA, USA) and the eluted lysates were mixed 1:1 with 70% ethanol. The lysates were applied to a mini-column and after washing and DNAse I digestion, the RNA samples were eluted in 30-50 µl of RNAse-free water. The quantity and quality of total RNA samples were checked by agarose-gel-electrophoresis and using the Bioanalyzer RNA 6000 Nano assay (Agilent, Waldbronn, Germany). For miRNA expression analysis, qRT-PCR was performed using the TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and the TaqMan microRNA assays kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocols. For detecting mRNAs of HK2, LDHA and PKM2, cDNA synthesis was performed using the SuperScript First-Standard Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Quantitative PCR analyses were performed using the Assay-on-Demand primers and the TaqMan Universal PCR Master Mix reagent (Applied Biosystems, Foster City, CA, USA). The primers used for q-PCR were as follows: LDHA: forward, 5'-TGGAGTGGAATGAATGTTGC-3' and reverse, 5'-ATAGCCCAGGATGTGTAGCC-3'; HK2: forward, 5'-CAAAGTGACAGTGGGTGTGG-3' and reverse 5'-GCCAGGTCCTTCACTGTCTC-3'; PKM2: forward, 5'-TCGCATGCAGCACCTGATT-3' and reverse, 5'-CCTCGAATAGCTGCAAGTGGTA-3' and β-actin: forward, 5'-CTGGCTCCTAGCACCATGAAGAT-3' and reverse 5'-GGTGGACAGTGAGGCCAGGAT-3'. The expression levels of β-actin were used to normalize the expression levels of other genes. All reactions were performed in triplicate. The relative amounts of mRNA were calculated by using the comparative $2^{-\Delta\Delta CT}$ method.

Luciferase Reporter Assay

The pMIR-reporter luciferase vector containing the wild-type 3'-UTR, the 3'-UTR with binding site mutations for HK2, and the empty vector were constructed. For the luciferase assay, cells at a density of 2×10^5 per well in 24-well plates were co-transfected with MIR-REPORT luciferase reporters with 3'-UTR of wild-type HK2 or binding site mutant of HK2, pre-miR-143, or control miRNAs using Lipofectamine 2000 reagent. Forty-eight hour later, the cells were harvested and lysed with passive lysis buffer (Promega, Madison, WI, USA). Luciferase activity was then measured using a dual luciferase reporter assay (Promega, Madison, WI, USA). The pRL-TK vector (Promega, Madison, WI, USA) was used as an internal control. The results were expressed as relative luciferase activity (firefly Luc/Renilla Luc).

Cell Viability Assay

A total of 1×10^5 cells per well were seeded in 6-well plates and incubated overnight. The medium was replaced with fresh medium with or without 3-BrPA at 0, 10 µM, 20 µM and 40 µM and then incubated for 24 hr. Cell viability was measured using the 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Absorbance was measured spectrophotometrically at 570 nm by the Universal Microplate Reader EL800 (Bio-Tek Instruments, Inc., Vermont, MA, USA).

Western Blot Analysis

Whole cells were lysed in 1 × SDS sample buffer and resolved by electrophoresis using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose membranes. The membranes were probed with primary antibodies overnight and, then, incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 2 hr at room temperature, followed by the detection with a Super Signal Enhanced Chemiluminescence kit (Pierce, Rockford, IL, USA). For sequential blotting, the membranes were stripped with Stripping Buffer (Pierce, Rockford, IL, USA) and re-probed with appropriate antibodies.

Statistical Analysis

Data represent mean ± SEM of representative experiments unless otherwise stated. Statistical significance was calculated by standard *t*-test (Prism v5.0). $p < 0.05$ was considered statistically significant.

Results

Glucose Metabolism is Higher in Endothelial Cells Than in Other Cells

Since deregulation of EC function is responsible for the formation of atherosclerotic plaque[4,5],

we first explored the metabolic pathways that are critical for EC dysfunction. By measuring the flux of metabolic pathways in multiple cells lines from different human tissues, we observed that glucose metabolism is highly active in venous (HUVEC), arterial (HA), and microvascular (HMV) ECs than in other normal cells (Figure 1). Notably, glucose uptake (Figure 1A), as well as lactate production (Figure 1B), both of which are glycolytic flux indicators, are higher in ECs. We measured the oxygen consumption rate (OCR), which showed lower respiration in ECs than in other cell types, indicating that mitochondrial respiration was lower in ECs than in other oxidative cell types (Figure 1C). Our data suggests that glycolysis is the predominant bioenergetics pathway for ECs and that deregulation of EC glycolysis might compromise the regular functions of ECs.

Expression of miR-143 is Lower in Healthy ECs than in Atherosclerotic Plaque

To investigate the mechanisms accounting for upregulated glycolytic rates in ECs, we surveyed miRNA expression in HUVECs, arterial (HA), and microvascular (HMV) ECs and in other normal cells. Interestingly, we observed several differentially expressed microRNAs in ECs compared with other cells. Among these, miR-143 was significantly down-regulated in ECs (Figure 2A), suggesting that miR-143 might act as a suppressor of glycolysis in ECs. To further explore the correlation between miR-143 and atherosclerosis, we assessed the expression of miR-143 in atherosclerotic plaque obtained from endarterectomy specimens of human carotid arteries in comparison to healthy arteries. As expected, the expression of miR-143 was upregulated in atherosclerotic plaque compared with healthy arteries (Figure 2B). Taken together, these findings indicate a correlation between miR-143 expression in ECs glycolysis and atherosclerotic plaque formation.

Atherosclerotic Plaque Exhibits Attenuated Glycolytic Flux

Based on the above results, we assessed the glycolysis rates in atherosclerotic plaque. HK2 catalyzes the first committed step of glucose metabolism by catalyzing the phosphorylation of glucose to glucose-6-phosphate[16]. LDHA and PKM2 are also key enzymes that catalyze committed steps in glucose metabolism[17, 18]. We measured the mRNA levels of HK2, LDHA, and PKM2 in atherosclerotic plaque compared with those in healthy arteries. The three key glycolytic enzymes were consistently and significantly downregulated in atherosclerotic plaque (Figure 3A-C), suggesting that glycolysis is impaired in atherosclerotic plaque and might be one of the mechanisms underlying atherosclerotic plaque formation.

miR-143 Directly Targets HK2 in ECs

We searched the miRNA databases for potential miR-143 targets that may be involved in glycolysis to further explore functions of deregulat-

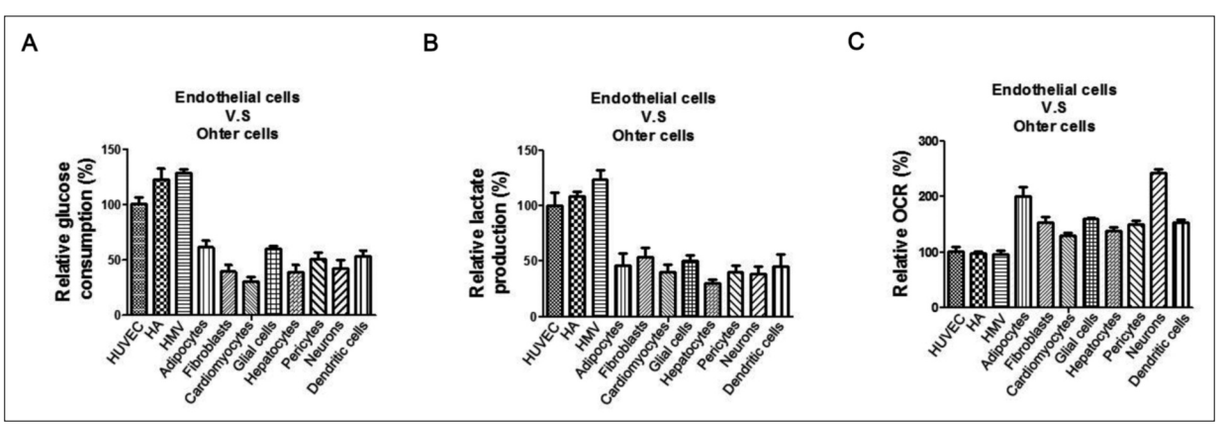

Figure 1. Human endothelial cells exhibit elevated glucose metabolism than other healthy cells ($p < 0.05$). ***A,*** Relative glucose consumption was compared between ECs–HUVECs (HUV), arterial ECs (HA), and microvascular ECs (HMV) –and other human primary cells. ***B,*** The relative lactate production was compared between ECs–HUVECs (HUV), arterial ECs (HA), microvascular ECs (HMV) –and other human primary cells. ***C,*** The relative oxygen consumption was compared between ECs–HUVECs (HUV), arterial ECs (HA), and microvascular ECs (HMV) –and other human primary cells. Columns, mean of three independent experiments; bars, SE. *, $p < 0.05$; **, $p < 0.01$.

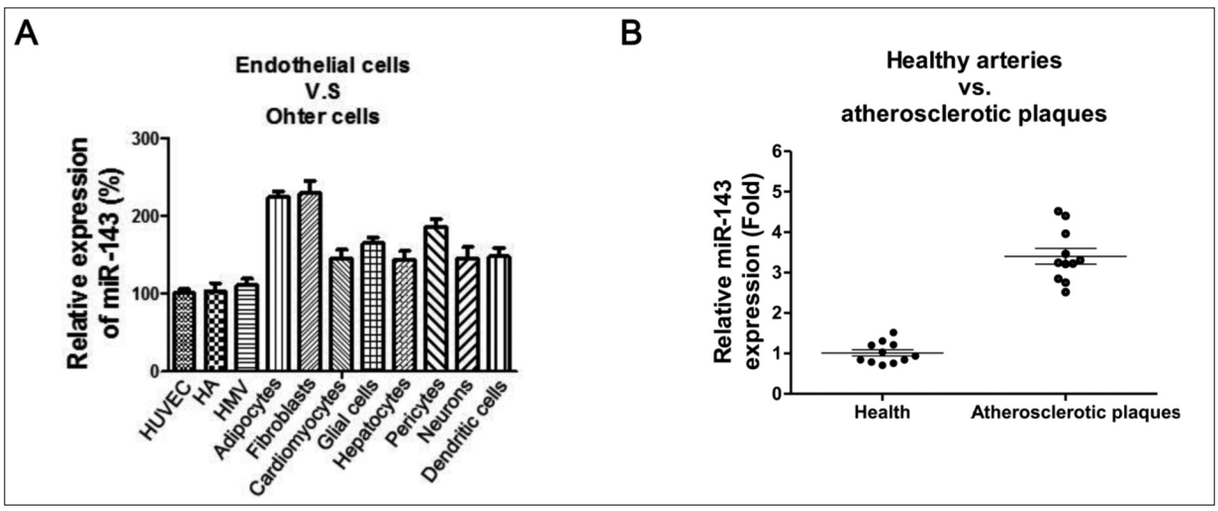

Figure 2. miR-143 is down-regulated in ECs and is positively correlated with human atherosclerosis. ***A,*** miR-143 expression in ECs and other cells ($p < 0.05$). ***B,*** miR-143 expression in healthy human arteries and atherosclerotic plaque. Columns, mean of three independent experiments; bars, SE.

ed miR-143 in ECs and in atherosclerotic plaque formation. The public miRNA database, TargetScan predicted that HK2 might be a target for miR-143 and that the 3'-UTR of HK2 contains a highly conserved binding site for miR-143 (Figure 4A). So far, no publication has reported that HK2 is a direct target of miR-143 in ECs. To determine whether miR-143 could directly target HK2 in ECs, we performed the luciferase reporter analysis by co-transfecting a vector containing pMIR reporter-luciferase fused with the original sequence or with the predicted binding site mutant of the 3'-UTR of HK2 mRNA and pre-miR-143 or control microRNA. Overexpression of miR-143 decreased the luciferase activity of the reporter with wild-type 3'-UTR of HK2 by about 60% in HUVECs (Figure 4B). However, no inhibitory effect of miR-143 was detected on reporter activity with the binding site mutant of 3'-UTR of HK2 (Figure 4B), Taken together, these results demonstrate that HK2 is a direct target of miR-143 in human ECs.

Overexpression of miR-143 Inhibits Glycolysis of ECs Through Direct Targeting of HK2

Based on our data, we hypothesized that the abnormally overexpressed miR-143 in ECs might be responsible for atherosclerotic plaque formation through suppression of glycolysis. To verify this, we transfected pre-miR-143 into HUVECs (Figure 5A) to detect whether miR-143 could

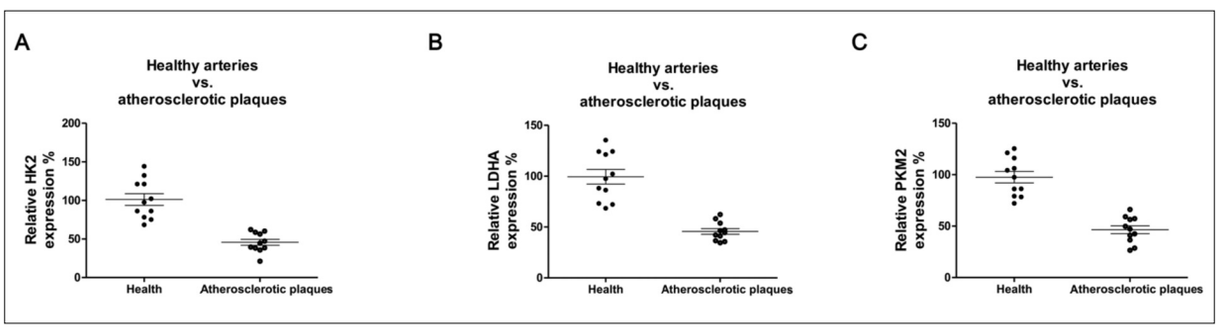

Figure 3. Key glycolysis enzymes were downregulated in atherosclerotic plaque. ***A,*** The mRNA levels of HK2 were measured in healthy human arteries and atherosclerotic plaque. ***B,*** The mRNA levels of LDHA were measured in healthy human arteries and atherosclerotic plaque. ***C,*** The mRNA levels of PKM2 were measured in healthy human arteries and atherosclerotic plaque.

regulate the rate of glycolysis. As expected, the intracellular ATP, glucose consumption, and lactate production were significantly decreased by overexpression of miR-143 (Figure 5B-D). Consistently, the protein levels of HK2, PKM2, and LDHA were downregulated by the overexpression of miR-143 in HUVECs, indicating that miR-143 suppresses glycolysis of ECs. To further support our above results, we treated normal or miR-143-overexpressing HUVECs with the glycolysis inhibitor, 3-BrPA, which is a specific inhibitor of HK2[16]. HUVECs with higher expression of miR-143 showed relative insensitivity to the glycolysis inhibitor (Figure 5E), suggesting that glycolysis is suppressed in miR-143 overexpressing cells.

Restoration of HK2 in miR-143 Overexpressing Rescues Glycolysis Level of HUVECs

To examine whether glycolysis suppression by overexpression of miR-143 was through HK2 inhibition, we transfected an overexpression vector containing wild-type HK2 into HUVECs pretransfected with miR-143. Exogenous overexpression of HK2 restored the expression of HK2 to the original level (Figure 6A), indicating that the activity of the glycolysis pathway was recovered by overexpression of HK2 in miR-143 overexpressing cells. The restoration of the activity of glycolysis pathway in miR-143 overexpressing cells resulted in the recovery of intracellular ATP levels, glucose consumption, and lactate production (Figure 6B-D), suggesting that miR-143 suppresses the glycolytic flux in ECs through inhibition of HK2.

Discussion

It is well-established that the endothelial cell layer is the main regulator of vascular wall homeostasis and regulates multiple aspects of vascular physiology[4]. Therefore, EC dysfunction is regarded as an early step in atherosclerotic plaque formation due to defects in EC function. In this study, we observed that glycolysis is suppressed in atherosclerotic plaque compared with healthy arteries. Also, a highly active glycolysis rate is a significant feature of ECs, suggesting that downregulation of glycolysis, which contributes to ECs deregulation might correlate with the formation of atherosclerotic plaque. Consistently, our results demonstrated that key glucose metabolism enzymes such as HK2, LDHA, and PKM2 were significantly downregulated in clinical atherosclerotic plaque samples.

We found that ECs generated most of their ATP from glycolysis and were more glycolytic than other healthy cell types. Moreover, ECs have a smaller mitochondrial volume fraction (5%) compared to oxidative hepatocytes (30%)[19]. Our studies show that glucose metabolism in ECs is upregulated and results in increased glucose

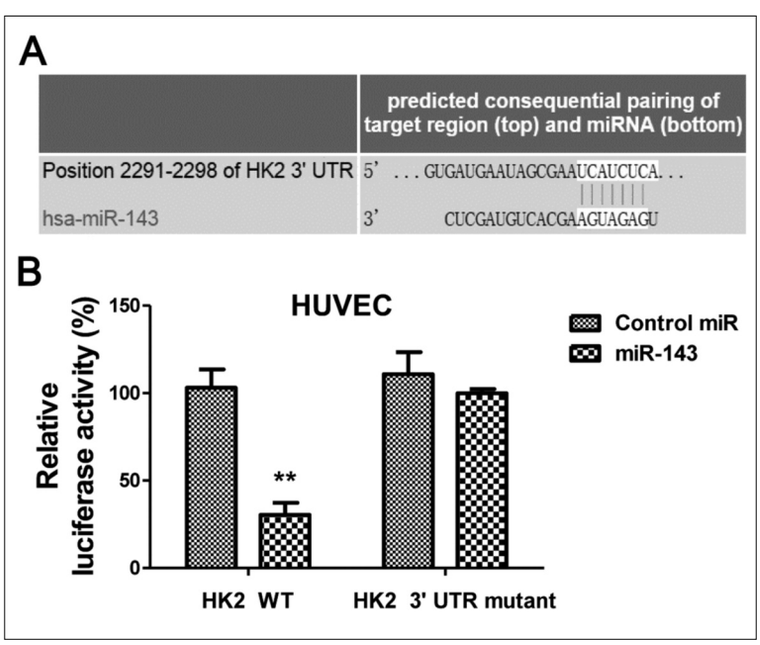

Figure 4. HK2 is a direct target of miR-143 in endothelial cells. ***A,*** Target prediction from Targetscan.org: the position 2291-2298 of the HK2 3' UTR contains putative binding sites for miR-143. ***B,*** HUVECs were co-transfected with luciferase reporter plasmids containing wild-type 3'-UTR of HK2 or mutant 3'-UTR of HK2 and pre-miR-143, or Control miR using Lipofectamine 2000 reagent. Forty-eight hours post-transfection, cells were harvested and lysed using a passive lysis buffer. Luciferase activities were measured by a dual luciferase reporter assay. The pRL-TK vector was used as an internal control. The results were expressed as relative luciferase activity (firefly LUC/Renilla LUC). Columns, mean of three independent experiments; bars, SE. **, $p < 0.01$

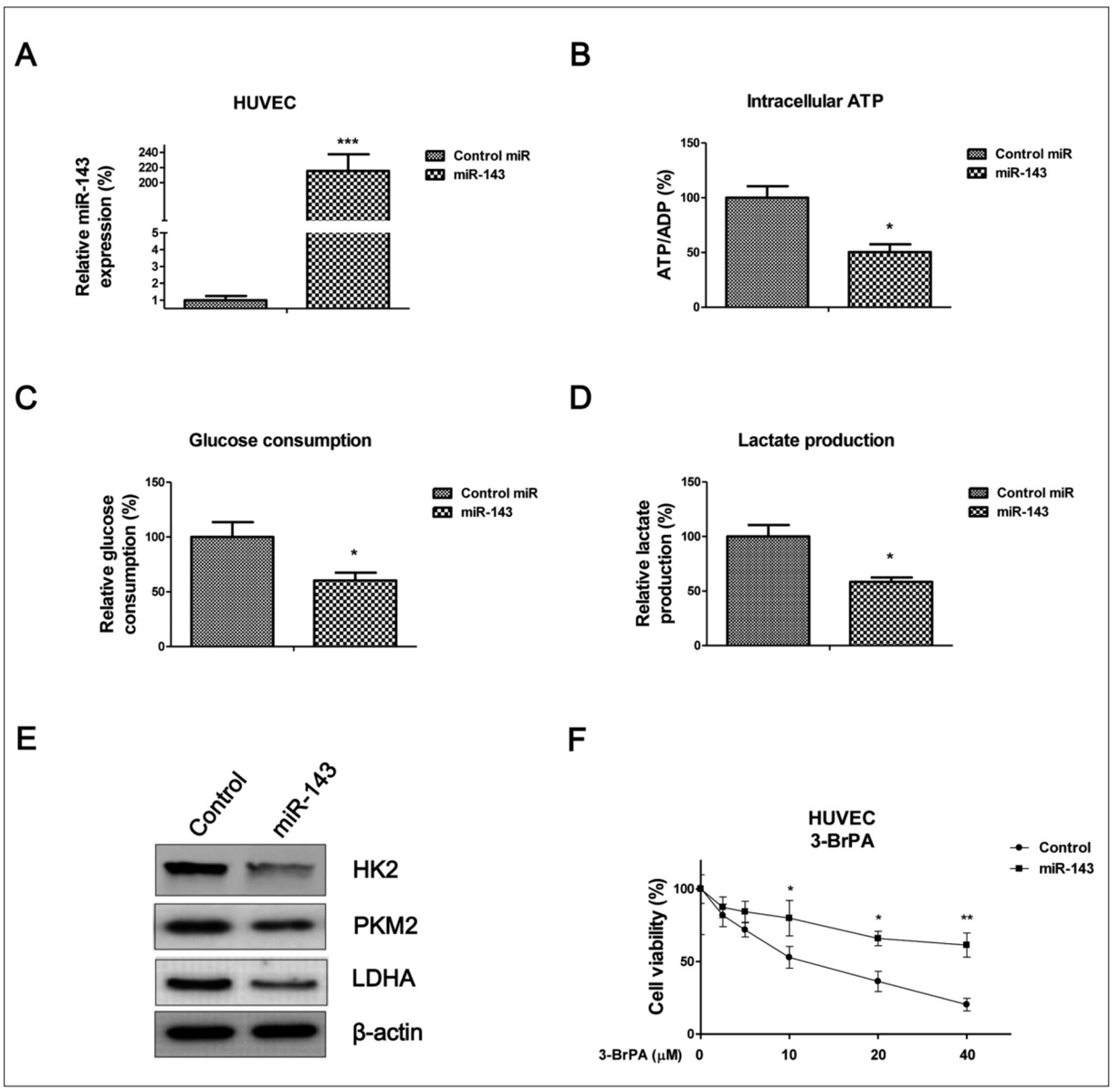

Figure 5. Overexpression of miR-143 contributes to ECs dysfunction through suppression of the glycolysis. ***A,*** HUVECs were transfected with 100 nM pre-miR-negative (Control miR) or pre-miR-143 for 48 hr, followed by measurement of miR-143 expression using qPCR. ***A,-D,*** HUVECs were transfected with 100 nM pre-miR-negative (Control miR) or pre-miR-143 for 48 hr, followed by the measurement of intracellular ATP, glucose consumption, and lactate production. ***E,*** HUVECs were transfected with 100 nM pre-miR-negative (Control miR) or pre-miR-143 for 48 hr, followed by Western blotting analysis. β-actin was used as a loading control. (F) HUVECs were transfected with 100 nM pre-miR-negative (Control miR) or pre-miR-143 for 48 hr; the cells were then treated with 3-BrPA at 0, 10 μM, 20 μM and 40 μM for 24 hr, followed by cell viability assays. Columns, mean of three independent experiments; bars, SE. *, $p < 0.05$; **, $p < 0.01$.

consumption, lactate production, and decreased oxygen consumption. ECs have lower oxygen demand since glycolysis generates ATP more rapidly than oxidative metabolism. In addition, the glycolysis side pathways are necessary for biosynthesis of macromolecules needed for cell proliferation during cell division.

The regulation of glycolysis by miR-143 has been implicated in cancer cells[20-22]. miR-143 expression was inversely associated with HK2 protein levels but not with mRNA levels in human lung cancer samples[20]. However, our work first described that HK2 is a direct target of miR-143 in human ECs. Overexpression of miR-143 sup-

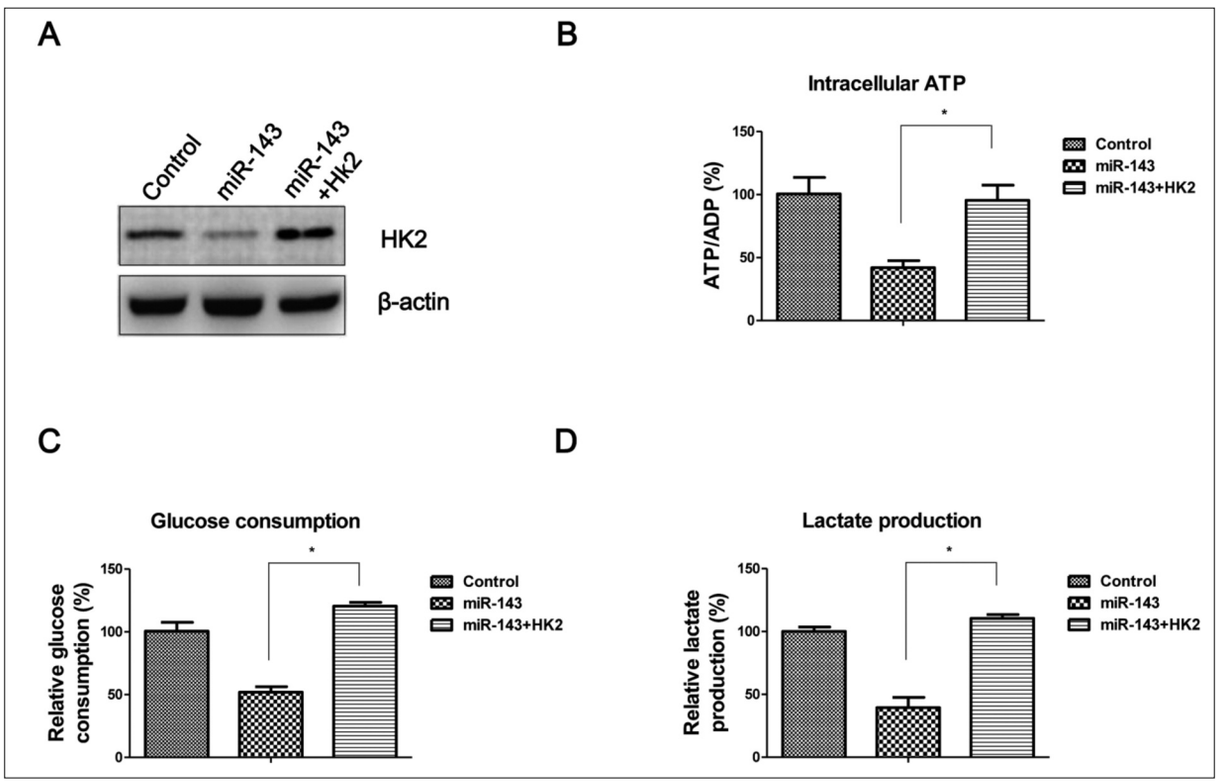

Figure 6. Restoration of HK2 expression in HUVECs rescues glycolysis. ***A,*** Western blotting experiments showed that overexpression of HK2 in miR-143-overexpressing cells restored HK2 expression to the original level. ***B-D,*** Detection of intracellular ATP, glucose consumption, and lactate production in control cells, miR-143 overexpressing cells, and cells co-transfected with miR-143 and HK2. Columns, mean of three independent experiments; bars, SE. *, $p < 0.05$.

presses the glycolysis flux by targeting HK2. Interestingly, miR-143 is significantly upregulated in clinical atherosclerotic plaque samples compared with healthy arteries, suggesting that inhibition of miR-143 might contribute to the prevention of atherosclerotic plaque formation. However, the detailed mechanisms of the process are still under investigation. Our future project will focus on the mechanisms of miR-143 and the formation of atherosclerotic plaque. Animal models will be established to evaluate the roles of miR-143 in atherosclerosis. In summary, our study reports a correlation between upregulated miR-143, ECs dysfunction, and atherosclerotic plaque formation. miR-143 is upregulated in clinical atherosclerotic plaque samples and suppresses glycolysis in ECs, which exhibit high glycolysis rates. Overexpression of miR-143 in HUVECs suppresses glycolysis through direct targeting of HK2. This research provides further insight into the metabolic mechanisms involved in microRNAs and regulation of atherosclerotic plaque formation.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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