Overexpression of miR-17-5p protects against high glucose-induced endothelial cell injury by targeting E2F1-mediated suppression of autophagy and promotion of apoptosis

YIFENG YUAN[1,2], XUE LI[1,2] and MAOQUAN LI[1,2]

[1]Department of Interventional and Vascular Surgery, Tenth People's Hospital of Tongji University; [2]Institute of Interventional and Vascular Surgery, Tongji University, Shanghai 200072, P.R. China

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Abstract. E2 promoter binding factor 1 (E2F1) has been reported to have an important regulatory role in cell survival during hyperglycemic conditions; however, the mechanisms remain to be fully elucidated. Bioinformatics analyses have suggested that microRNA (miR)-17-5p targets the 3'untranslated region (3'UTR) of E2F1. The aim of the present study was to characterize the protective effect of miR-17-5p/E2F1 on human umbilical vein endothelial cells (HUVECs) under high glucose (HG) conditions, to confirm the regulatory effect of miR-17-5p on E2F1/AMP-activated protein kinase α2 (AMPKα2)-mediated apoptosis and E2F1/mammalian target of rapamycin complex 1 (mTORC1)-mediated autophagy. Bifluorescein experiments were performed to characterize the interaction between miR-17-5p and E2F1. The Cell Counting Kit-8 assay, flow cytometry, immunofluorescence, and reverse transcription-quantitative polymerase chain reaction and western blot analyses were used to detect cell viability, apoptosis, autophagy, and relative mRNA and protein expression, respectively. The results showed that HG induced the downregulation of miR-17-5p and upregulation of E2F1 during HUVEC injury. The downregulation of E2F1 inhibited HG-induced HUVEC dysfunction by suppressing mTORC1-mediated inhibition of autophagy and AMPKα2-mediated promotion of apoptosis. The results suggested that inhibiting the expression of E2F1 protected against HG-induced HUVEC injury via the activation of autophagy. The overexpression of miR-17-5p inhibited E2F1-mediated HUVEC injury under HG conditions, which was reversed following transfection with an E2F1-overexpression vector. The bifluorescein experiments showed that miR-17-5p targeted the 3'UTR of E2F1. Taken together, the results suggested that the expression of miR-17-5p inhibited HG-induced endothelial cell injury by targeting E2F1.

Correspondence to: Dr Xue Li or Dr Maoquan Li, Department of Interventional and Vascular Surgery, Tenth People's Hospital of Tongji University, 301 Middle Yan Chang Road, Shanghai 200072, P.R. China
E-mail: lixue5303@126.com
E-mail: cjr.limaoquan@vip.163.com

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Introduction

Increasing evidence has shown that diabetes mellitus-induced hyperglycemic conditions cause vascular endothelial damage (1,2) demonstrating that hyperglycemia is associated with endothelial dysfunction and reduced blood vessel growth, with angiogenesis often compromised in patients with diabetes. Endothelial dysfunction has long been considered as the first event in the development of atherosclerosis and clinical events (3). Therefore, the maintenance of an intact endothelial layer and improvement of its functions are essential for the maintenance of healthy blood vessels and the prevention of cardiovascular complications associated with diabetes.

The E2 promoter binding factor (E2F) family of transcription factors is involved in DNA synthesis, the cell cycle, cell differentiation and apoptosis (4,5). E2 promoter binding factor 1 (E2F1), which was the first member cloned, is important in cell control, although its exact role is controversial. It has been reported that the expression of E2F1 inhibits cell apoptosis (6), whereas other have reported that its expression promotes cell apoptosis (7,8). Yang *et al* reported that the expression of E2F1 promoted cell apoptosis mediated by AMP-activated protein kinase α2 (AMPKα2) (9). AMPKα2 also mediated endothelial cell apoptosis under high glucose (HG) conditions (10), which resulted in the opposite effect of AMPKα1 (11). E2F1 is also important in the regulation of mammalian target of rapamycin complex 1 (mTORC1) during autophagy (12,13). Increasing evidence has shown that certain autophagic activations inhibit HG-induced endothelial cell apoptosis via the mTOR signaling pathway (14); however, the regulatory mechanisms of E2F1 in autophagy and HG-induced endothelial cell apoptosis remain to be fully elucidated.

MicroRNAs (miRNAs) are 20-22 nucleotide non-coding RNAs, which repress the expression of their cognate target

genes by specifically binding and cleaving mRNAs, inhibiting translation, and/or deadenylating mRNA tails (15). miRNAs have been reported to control various biological processes, including cell proliferation, apoptosis, and autophagy (16-18), and miRNA (miR)-17-5p is conserved across vertebrates and downregulated in diabetes (19-21). Bioinformatics analyses (http://www.targetscan.org/) have suggested that miR-17-5p targets the 3'untranslated region (3'UTR) of E2F1. However, the role of miR-17-5p in HG-induced endothelial cell damage and the association between miR-17-5p and E2F1 remain to be elucidated.

In the present study, it was found that HG induced the downregulation of miR-17-5p and upregulation of E2F1 during human umbilical vein endothelial cell (HUVEC) injury. The downregulation of the expression of E2F1 inhibited HG-induced HUVEC dysfunction via the suppression of mTORC1-mediated inhibition of autophagy and AMPKα2-mediated promotion of apoptosis. These results suggested that the inhibited expression of E2F1 protected against HG-induced HUVEC injury through the activation of autophagy. The overexpression of miR-17-5p inhibited E2F1-mediated HUVEC injury under HG conditions, which was reversed following transfection with an E2F1-overexpression vector. Finally, bifluorescein experiments showed that miR-17-5p targeted the 3'UTR of E2F1. Taken together, the results revealed the importance of miR-17-5p in controlling HG-induced E2F1-mediated endothelial cell damage.

Materials and methods

Cell culture. The HUVECs, purchased from the American Type Culture Collection (Manassas, VA, USA; CRL-1730™), were cultured in RPMI-1640 complete medium (Invitrogen; Thermo Fisher Scientific, Inc., USA) containing 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.). All cells were cultured at 37˚C in a humidified incubator containing 5% CO_2, and the culture medium was replaced every other day.

Flow cytometry. Flow cytometry was used to determine the percentage of apoptotic HUVECs. The apoptotic cells were differentiated from viable or necrotic cells by combined treatment with annexin V (AV)-FITC and propidium iodide (PI). The cells were washed twice and adjusted to a concentration of $1x10^6$ cells/ml with cold D-Hanks buffer. The AV-FITC (10 μl) and PI (10 μl) were then added to 100 μl of cell suspension and incubated for 15 min at room temperature in the dark. Finally, 400 μl of binding buffer was added to each sample without washing and analyzed using flow cytometry. Each experiment was performed in triplicate.

Western blot analysis. Western blot analyses were performed using cell lysates in urea buffer (8 M urea, 1 M thiourea, 0.5% CHAPS, 50 mM dithiothreitol and 24 mM spermine). GAPDH was used as a loading control. The samples [40 μg total protein, quantified using the two-dimensional Quant kit (GE Healthcare Life Sciences, Little Chalfont, UK)] were separated using 8% SDS-PAGE and transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Following blocking in 5% nonfat milk for 1 h, the membranes were incubated with primary antibodies against E2F1 (1:1,000; cat. no. ab179445; Abcam, Cambridge, UK), AMPKα2 (1:200; cat. no. ab3760; Abcam), mTORC1 (1:200; cat. no. ab168538; Abcam), cleaved caspase-3 (1:200; cat. no. ab2302; Abcam), B-cell lymphoma 2 (Bcl-2; 1:400; cat. no. ab119506; Abcam), Bcl-2-associated X protein (Bax; 1:300; cat. no. ab32503; Abcam), P62 (1:400; cat. no. ab5641; Abcam), microtubule-associated protein 1 light chain 3 (LC3; 1:200; cat. no. ab48394; Abcam), and GAPDH (1:2,000; cat. no. ab8245; Abcam) at 4˚C overnight. Following washing with phosphate buffered saline (PBS) three times, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (1:2,000; cat. no. ab172730; Abcam) for 1 h at room temperature. The signals were detected using an ECL detection system (GE Healthcare Life Sciences) and analyzed using ImageJ 1.42q software (National Institutes of Health, Bethesda, MD, USA).

Cell viability assay. A Cell Counting Kit-8 assay (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to assess cell viability. The HUVECs ($1x10^4$) were seeded into 96-well plates and incubated overnight under the conditions described above. The cells were pretreated with 5.5, 15, 30 or 50 mM glucose for 24-72 h. The medium was then removed and the cells were washed three times with PBS. RPMI-1640 (90 μl) and CCK8 (10 μl) were subsequently added to each well and incubated for 1.5 h at 37˚C, and a microplate reader was used to measure the optical density at 450 nm.

Matrigel assay. *In vitro* neovascularization assays were performed in human fibrin matrices. Briefly, following the different treatments, the HUVECs were seeded onto Matrigel-coated plates (BD Biosciences, Franklin Lakes, NJ, USA) in RPMI-1640 medium and incubated at 37˚C for 12 h. The tubular structures of the HUVECs in the Matrigel were analyzed by phase contrast microscopy. The average numbers of tube formations from six random phase contrast photomicrographs were used for subsequent analyses.

Vector construction and transfection. For the overexpression of miR-17-5p, the miR-17-5p mimic or corresponding negative control (miR-NC) were purchased from GenePharma (Shanghai, China). The HUVECs were transfected with either the miR-17-5p mimic or miR-NC at a final concentration of 50 nM using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells were then used for miR-17-5p expression analyses or for other experiments following transfection for 48 h.

For the downregulation of E2F1, small interfering (si)RNA against E2F1 (5'-CGGACUCAGUGAUAAUAAUUU-3') was synthesized by GenePharma and transfected at a final concentration of 50 nM using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

For the overexpression of E2F1, AMPKα2, mTORC1, human E2F1 and mTORC1, cDNA with the 3'UTR was cloned into the pMSCV-hygro vector (Takara Bio, Inc., Otsu, Japan). The primers corresponded to the National Center for Biotechnology Information reference sequence, and included the following: E2F1 (NG_046988.1), forward 5'-CAAAGC TTATGGCCTTGGCCGGGGCC-3' and reverse 5'-GGCTCG AGTCAGAAATCCAGGGG-3'; AMPKα2 (BC069823.1),

forward 5'-CAAAGCTTATGGCTGAGAAGCAG-3' and reverse 5'-GGCTCGAGTCAACGGGCTAAAG-3'; and mTORC1 (AF148645.1), forward 5'-CAAAGCTTATGCCGC AGTCCAAG-3' and reverse 5'-GGCTCGAGTCACATCACC GAGCATG-3'. The E2F1 cDNA was inserted into a pMD18-T Simple vector (Takara Bio, Inc.) to form the pMD18-T-E2F1, pMD18-T-AMPKα2, and pMD18-T-mTORC1 vectors. Following sequencing, the recombinant segment of the correct clone was incised by *Hind*III and *Xho*I (Takara Bio, Inc.). The recombinant segment was inserted into the pMSCV-hygro vector, which was incised by the same two restriction endonucleases. The clones were sequenced and the correct clones were amplified and identified by restriction enzyme digestion.

Prior to transfection, ~1x10^6 HUVECs were seeded in media onto a 60-mm dish and incubated for 24 h. The following day, the cells were transfected using the Sofast gene transfection reagent kit (Sunma Biotechenology Co., Ltd., Xiamen, China) according to the manufacturer's protocol. The transfected cells were selected using hygromycin for 3-4 weeks for subsequent experiments. The monoclonal cells were then cloned and screened for the expression of E2F1, AMPKα2 and mTORC1.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis for the detection of miR-17-5p. Total RNA was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription was performed using the RT-qPCR system (Promega Corporation, Madison, WI, USA). RT-qPCR analysis was performed in a total reaction volume of 20 μl (10 μl 2xmaster mix, 4 μl cDNA, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), and 4 μl of double-distilled water) using SYBR® Green I Supermix (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The primer sequences used for PCR were as follows: E2F1 forward, 5'-ATGGCCTTGGCC GGGGCC-3' and reverse, 5'-TCAGAAATCCAGGGG-3'; AMPKα2 forward, 5'-ATGGCTGAGAAGCAG-3' and reverse, 5'-TCAACGGGCTAAAG-3'; mTORC1 forward, 5'-ATGCCG CAGTCCAAG-3' and reverse, 5'-TCACATCACCGAGCA TG-3'. All reactions were run in triplicate on an iCycler IQ Multicolor Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the following cycling parameters: 95˚C for 10 sec, followed by 40 cycles of 94˚C for 15 sec, annealing at 55˚C for 30 sec, and a final extension step at 70˚C for 30 sec. All quantifications were normalized to the level of human U6 small nuclear RNA or GAPDH in the reaction. Comparative quantification cycle (Cq) ($2^{-\Delta\Delta Cq}$) method, which compares differences in Cq values between common reference RNA and target gene RNA, was used to obtain the relative fold changes in gene expression (22), which compares differences in Cq values between the common reference RNA and target gene RNAs, was used to obtain the relative fold changes in gene expression. The miR-17-5p, E2F1, TORC1, and AMPKα2 primers for PCR were designed by GenePharma. The results are expressed as the mean ± standard deviation.

Luciferase reporter assay. To construct the luciferase reporter vectors, the 3'UTR of E2F1 cDNA fragments containing the predicted potential miR-17-5p binding sites [predicted using TargetScan (http://www.targetscan.org/) for bioinformatics analyses on September 25, 2017 using the search term 'E2F1'] were amplified by PCR and subcloned downstream of the luciferase gene in the PYr-MirTarget luciferase vector (Ambion; Thermo Fisher Scientific, Inc.). The 3'UTR of E2F1 (containing the binding sites for miR-17-5p) was amplified from a cDNA library with the following primers: Forward 5'-CTCGAGGCGCGTGGGGGGGCT CTAACTGCACTTTCGGCC-3' and reverse 5'-GCGGCC GCCAGGGACCCCTGCCCTTG-3'. The mutant 3'UTR of E2F1 (in which five nucleotides were mutated in the binding sites) was amplified using the following primer sequences: Forward 5'-CTCGAGGCGCGTGGGGGGGCTCTAACT GGTGAATCGGCC-3' and reverse 5'-GCGGCCGCTGCC ACAGTTTTGGCAGTGAAC-3'.

For the luciferase assays, 2x10^5 293T cells (American Type Culture Collection, Manassas, VA, USA) were cultured in 24-well plates and co-transfected with 50 ng of the corresponding vectors containing firefly luciferase together with 25 ng of miR-17-5p or the control. Transfection was performed using Lipofectamine® 2000 reagent. At 48 h post-transfection, the relative luciferase activity was calculated by normalizing the firefly luminescence to the Renilla luminescence using the Dual-Luciferase Reporter assay (Promega Corporation) according to the manufacturer's protocol.

Immunofluorescence. The cells were incubated with LC3 antibodies (cat. no. 3215; 1:500; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight, and were then incubated with FITC-conjugated goat anti-mouse secondary antibody (cat. no. 3654; 1:1,000; Cell Signaling Technology, Inc.) for 1 h at room temperature in the dark. Following several washes with PBS, the slides were incubated with DAPI for 3 min and then mounted in glycerol. The fluorescence was assessed using a fluorescence microscope.

Statistical analysis. Continuous variables are expressed as the mean ± standard deviation. One-way analysis of variance was performed for multiple comparisons using GraphPad Prism software, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

miR-17-5p and E2F1 are involved in HG-induced HUVEC injury. To determine the effect of HG on endothelial cells, the HUVECs were treated with different concentrations of glucose (5.5-50 mM) for different durations (0-72 h). The cell viability was then determined using a CCK8 kit assay. The results showed that HG (15-50 mM) treatment significantly suppressed the cell viability, compared with that under normal glucose levels (5.5 mM). This effect was concentration- and time-dependent. Treatment with HG (30 mM) for 48 h resulted in an almost 50% loss of viability (Fig. 1A), therefore, 30 mM glucose and an induction duration of 48 h were we selected for the subsequent experiments.

Following induction for 48 h with HG (30 mM), the HUVECs were collected for apoptosis analyses using flow cytometry. The results showed that the percentage of apoptotic HUVECs reached almost 35%, compared with the normal groups (Fig. 1B and C). The RT-qPCR analysis

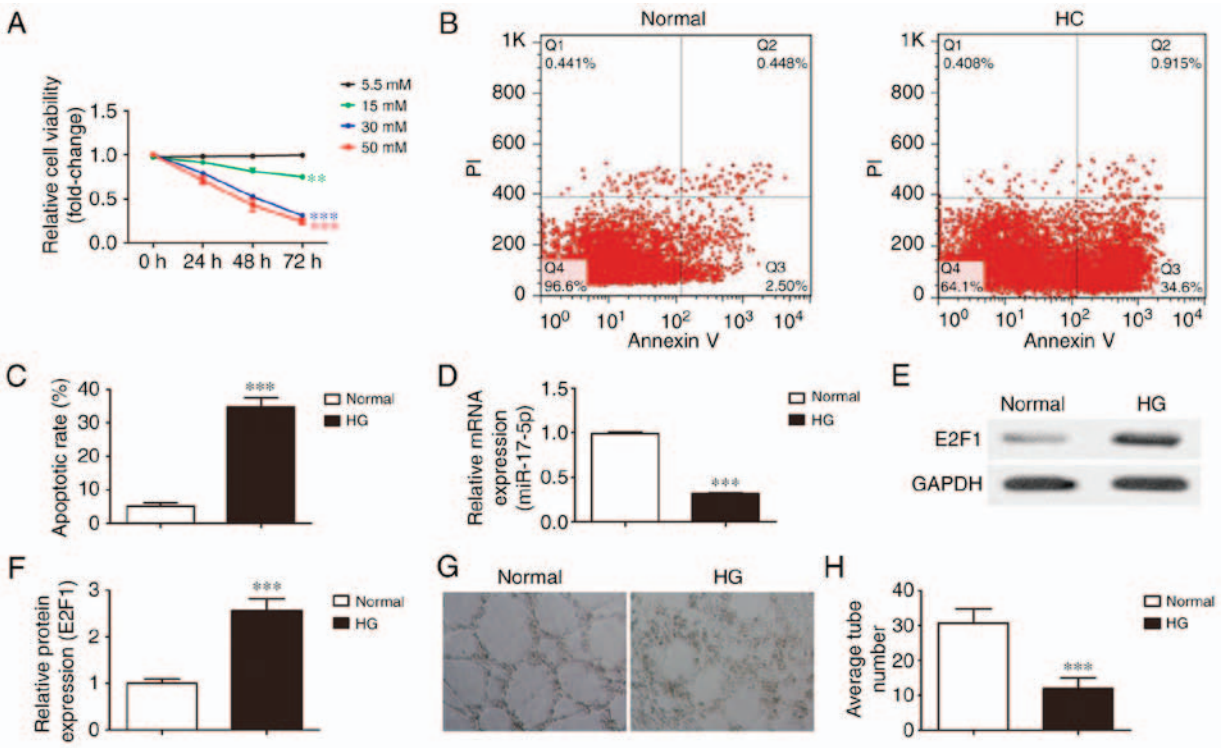

Figure 1. miR-17-5p and E2F1 are involved in HG-induced functional disruption of HUVECs. (A) HUVECs were incubated with increasing concentrations of glucose (5.5, 15, 30 and 50 mM) for 0-72 h. Cell viability was measured using a Cell Counting Kit-8 assay. The percentage of viable cells was analyzed. **P<0.01 and ***P<0.001 vs. normal group. (B) Apoptosis was determined using annexin V/propidium iodide staining following induction with NG or HG for 48 h. (C) Percentages of apoptotic cells. ***P<0.001, vs. normal group. (D) Expression of miR-17-5p in HUVECs detected by reverse transcription-quantitative polymerase chain reaction analysis following induction with normal glucose (5.5 mM) or HG for 48 h. ***P<0.001, vs. normal group. (E) Expression of E2F1 was measured by western blot analysis and (F) relative protein expression was analyzed. ***P<0.001, vs. normal group. (G) Tube formation capability of HUVECs from different treatment groups was measured (magnification, x400); tube formation capability was decreased following treatment with HG. (H) Average numbers of tube formations for each field were statistically analyzed. ***P<0.001, vs. NG group. All data are expressed as the mean ± standard deviation (n=3). HUVECs, human umbilical vein endothelial cells; HG, high glucose (30 mM); miR, microRNA; E2F1, E2 promoter binding factor 1.

showed that the expression of miR-17-5p was decreased by HG induction (Fig. 1D) and the results of the western blot analysis showed that HG treatment promoted the expression of E2F1 (Fig. 1E and F). A tube formation assay was then performed. As shown in Fig. 1G and H, there was a significant decrease in endothelial tube formation following exposure to HG conditions. The number of endothelial branch points decreased by ~60%, compared with that in the control group, suggesting that HG induced HUVEC apoptosis and inhibited differentiation into blood vessels. Therefore, miR-17-5p and E2F1 may be involved in these processes.

Inhibition of the expression of E2F1 attenuates HG-induced HUVEC injury via the activation of autophagy. To further determine whether the expression of E2F1 was involved in HG-induced endothelial injury, siRNA against E2F1 was constructed and transfected into HUVECs. Following culture for 48 h, the protein (Fig. 2A) and mRNA (Fig. 2B) expression levels of E2F1 were measured using western blot and RT-qPCR analyses, respectively. The results showed that the mRNA and protein expression levels of E2F1 were significantly decreased following transfection with siRNA against E2F1. HUVECs with or without downregulated E2F1 were then treated with either normal or HG conditions for 48 h. The flow cytometry showed that the downregulation of E2F1 reversed HG-induced HUVEC apoptosis (Fig. 2C and D). The western blot analyses showed that inhibition of the expression of E2F1 reversed HG-induced apoptosis in relation to protein expression levels, including those of caspase-3 and Bax, but promoted the expression of Bcl-2 (Fig. 2E-H), suggesting that the expression of E2F1 affected HG-induced HUVEC apoptosis. Taken together, the results showed that downregulation of the expression of E2F1 significantly suppressed HG-induced endothelial cell injury.

The immunofluorescence results showed that the downregulation of E2F1 promoted the autophagy of HUVECs under HG conditions (Fig. 2I). The western blot analysis showed that the protein expression of LC3-II was increased and that of P62 was decreased following the downregulated expression of E2F1 (Fig. 2J-M), suggesting that the downregulation of E2F1 inhibited HG-induced endothelial cell injury relative to the activation of autophagy. A previous study reported that certain types of autophagy inhibited cell damage under stress conditions (14). The tube formation assay showed that the downregulation of E2F1 restored angiogenesis, even under HG conditions (Fig. 2N and O).

AMPKα2 and mTORC1 are involved in E2F-mediated regulation of autophagy and apoptosis. Increasing evidence has shown that E2F1 regulates AMPKα2 and mTORC1, which are associated with the regulation of autophagy and apoptosis, respectively (9,12,23). To further determine whether

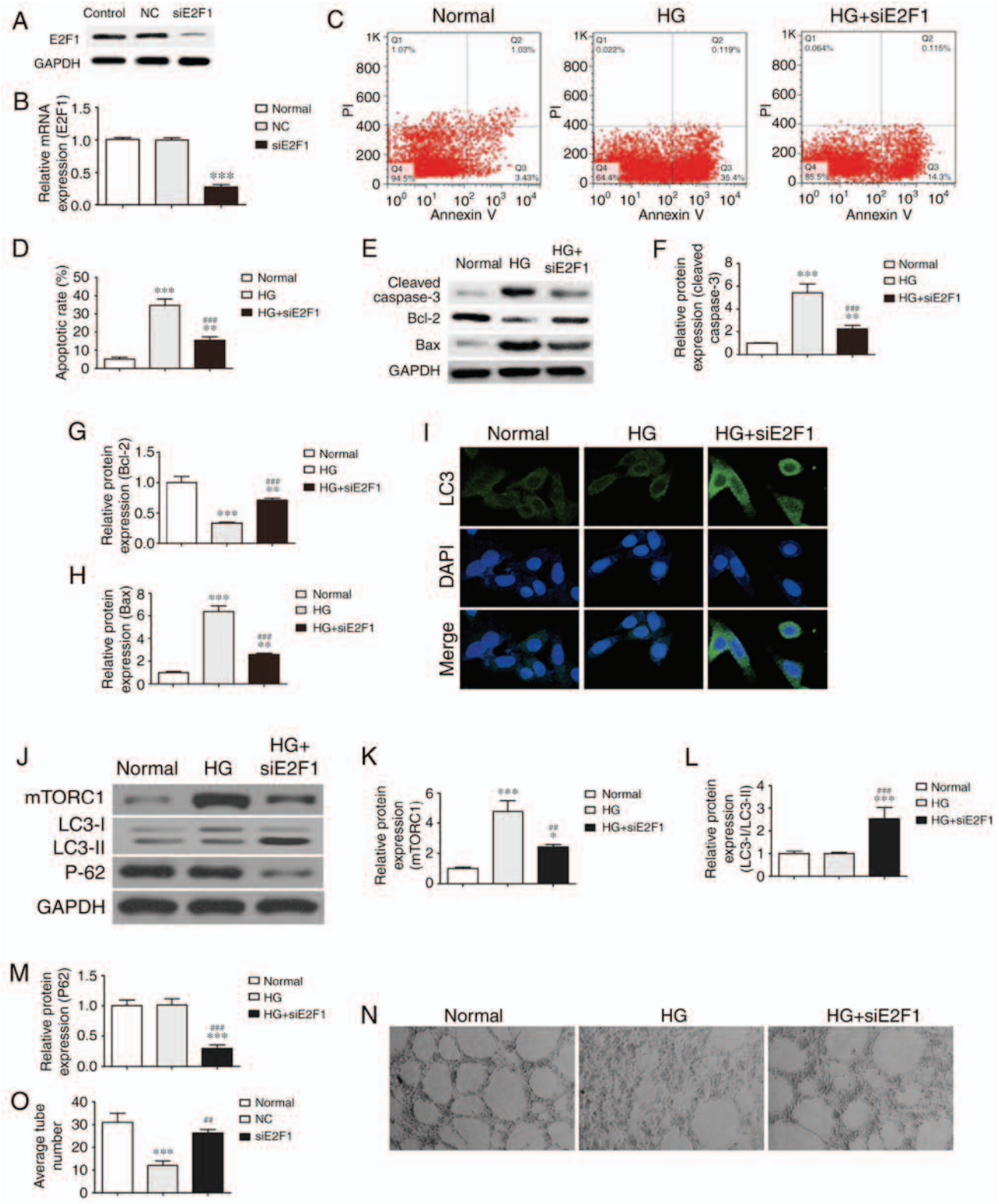

Figure 2. Downregulation of E2F1 inhibits HG-induced HUVEC dysfunction by promoting the activation of autophagy. HUVECs were transfected with siRNA against E2F1 for 48 h prior to induction with normal glucose (5.5 mM) or HG (30 mM) for 48 h. Expression of E2F1 was detection by (A) western blot analysis and (B) reverse transcription-quantitative polymerase chain reaction analysis. ***P<0.001, vs. control. (C) Apoptosis was determined by annexin V/propidium iodide staining following transfection with siRNA against E2F1 prior to induction with normal glucose (5.5 mM) or HG (30 mM) for 48 h. (D) Percentage of apoptotic cells was measured. **P<0.01 and ***P<0.001, vs. normal group; $^{###}$P<0.001, vs. HG group. (E) Western blot analyses showed the expression of apoptosis-related proteins AMPKα2, (F) cleaved caspase-3, (G) Bcl-2 and (H) Bax. Relative protein levels were analyzed. *P<0.05, **P<0.01 and ***P<0.001, vs. normal group; $^{#}$P<0.05, $^{##}$P<0.01 and $^{###}$P<0.001, vs. HG group. (I) Autophagy plaques were analyzed by immunofluorescence (magnification, x400). (J) Western blot analyses showed the expression of autophagy-related proteins, (K) mTORC1, (L) LC3 and (M) P62. The relative protein levels were analyzed. *P<0.05 and ***P<0.001, vs. normal group; $^{##}$P<0.01 and $^{###}$P<0.001, vs. HG group. (N) Tube formation capability of HUVECs from different treatment groups was measured (magnification, x400). (O) Average number of tube formations for each field was statistically analyzed. ***P<0.001, vs. normal group; $^{##}$P<0.01, vs. HG group. All data are expressed as the mean ± standard deviation (n=3). HUVECs, human umbilical vein endothelial cells; HG, HG, high glucose; E2F1, E2 promoter binding factor 1; AMPKα2, AMP-activated protein kinase α2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; mTORC1, mammalian target of rapamycin complex 1; LC3, microtubule-associated protein 1 light chain 3; NC, negative control; si, small interfering RNA.

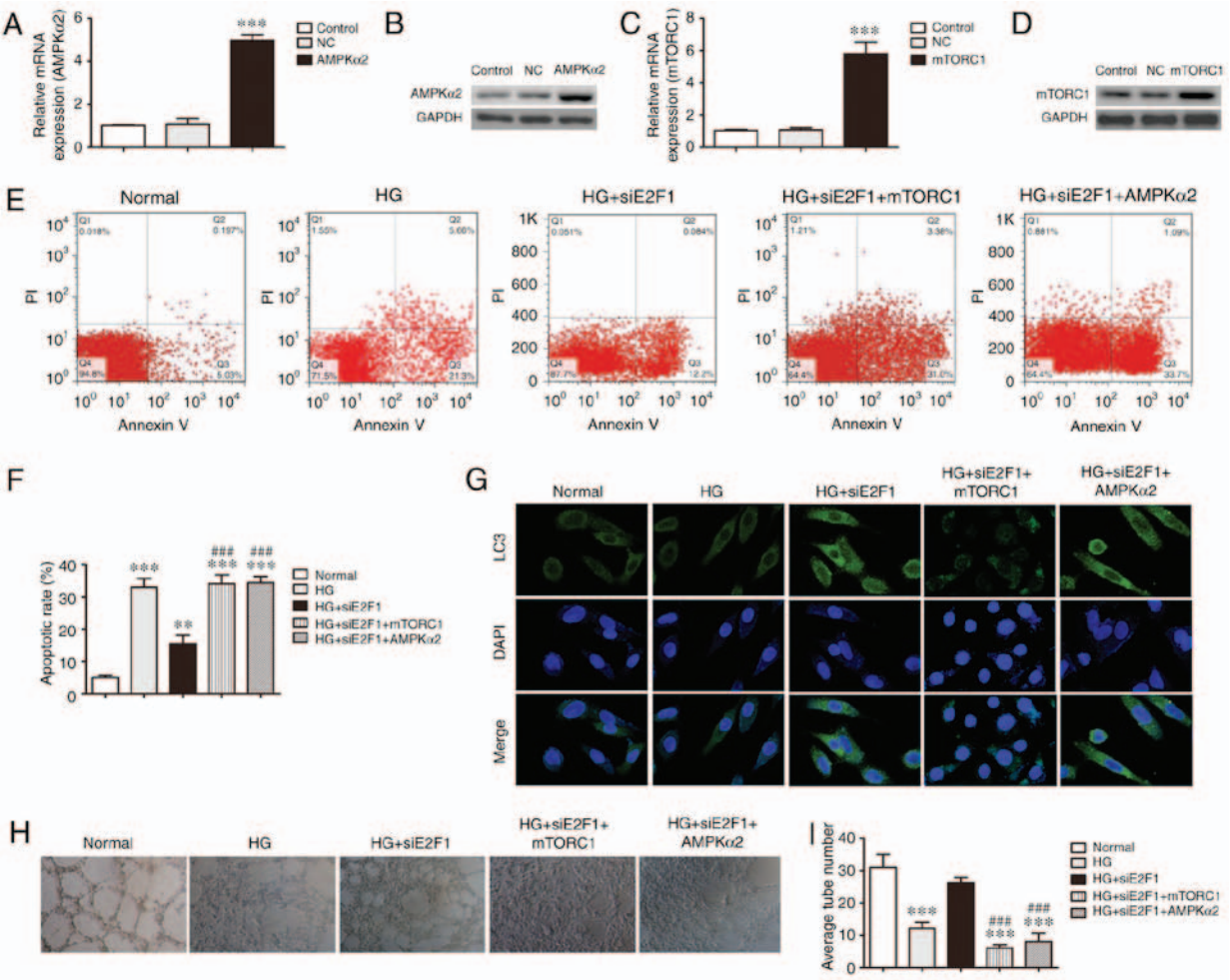

Figure 3. AMPKα2 and mTORC1 are involved in E2F1-mediated autophagy and apoptosis regulation. (A) RT-qPCR and (B) western blot analyses showing the expression of AMPKα2 in HUVECs transfected with the AMPKα2-overexpression vector for 48 h. ***P<0.0011, vs. control group. (C) RT-qPCR and (D) western blot analyses showing the expression of mTORC1 in HUVECs transfected with the mTORC1-overexpression vector for 48 h. ***P<0.001, vs. control group. (E) Apoptosis was determined by annexin V/propidium iodide staining following transfection with siRNA against E2F1 and the AMPKα2- or mTORC1-overexpression vector alone or in combination prior to induction with normal glucose (5.5 mM) or HG (30 mM) for 48 h. (F) Percentage of apoptotic cells was measured. **P<0.01 and ***P<0.001, vs. normal group; ###P<0.001, vs. HG + siE2F1 group. (G) Autophagy plaques were analyzed by immunofluorescence (magnification, x400). (H) Tube formation capability of HUVECs from different treatment groups was measured (magnification, x400). (I) Average numbers of tube formations for each field were statistically analyzed. ***P<0.001, vs. normal group; ###P<0.001, vs. HG + siE2F1 group. All data are expressed as the mean ± standard deviation (n=3). HUVECs, human umbilical vein endothelial cells; HG, HG, high glucose; E2F1, E2 promoter binding factor 1; AMPKα2, AMP-activated protein kinase α2; mTORC1, mammalian target of rapamycin complex 1; LC3, microtubule-associated protein 1 light chain 3; NC, negative control; si, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction analysis.

the regulation of autophagy and apoptosis by E2F1 involves AMPKα2 and mTORC1, AMPKα2- and mTORC1-overexpression vectors were constructed and transfection into HUVECs for 48 h. The mRNA and protein expression levels of AMPKα2 and mTORC1 were measured by RT-qPCR and western blot analyses, respectively (Fig. 3A-D). The results of the flow cytometry showed that the overexpression of AMPKα2 or mTORC1 recovered HG-induced HUVEC apoptosis, even following the downregulation of E2F1, suggesting the involvement of AMPKα2 and mTORC1 in the regulation of apoptosis. The immunofluorescence showed that the overexpression of mTORC1 suppressed autophagy under HG conditions, even following the downregulation of E2F1, however, the overexpression of AMPKα2 had no effect under exposure to HG following the downregulation of E2F1 (Fig. 3G), suggesting that the downregulation of E2F1 promoted autophagy by suppressing the expression of mTORC1, but not AMPKα2. The tube formation assay showed that the overexpression of AMPKα2 and mTORC1 suppressed angiogenesis under exposure to HG conditions, even following the downregulation of E2F1 (Fig. 3H and I).

Overexpression of miR-17-5p protects against HG-induced endothelial cell injury by targeting E2F1-mediated autophagy suppression and apoptosis promotion. Boinformatics analyses (http://www.targetscan.org/) suggested that miR-17-5p targets the 3'UTR of E2F1. To determine whether miR-17-5p-regulated HG-induced endothelial injury was associated with E2F1, an E2F1-overexpression vector was constructed and transfected into HUVECs. Following 48-h transfection, the mRNA and protein expression levels were detected by RT-qPCR (Fig. 4A) and western blot (Fig. 4B) analyses, respectively. The results showed that the expression of E2F1 was significantly increased. The RT-qPCR analysis

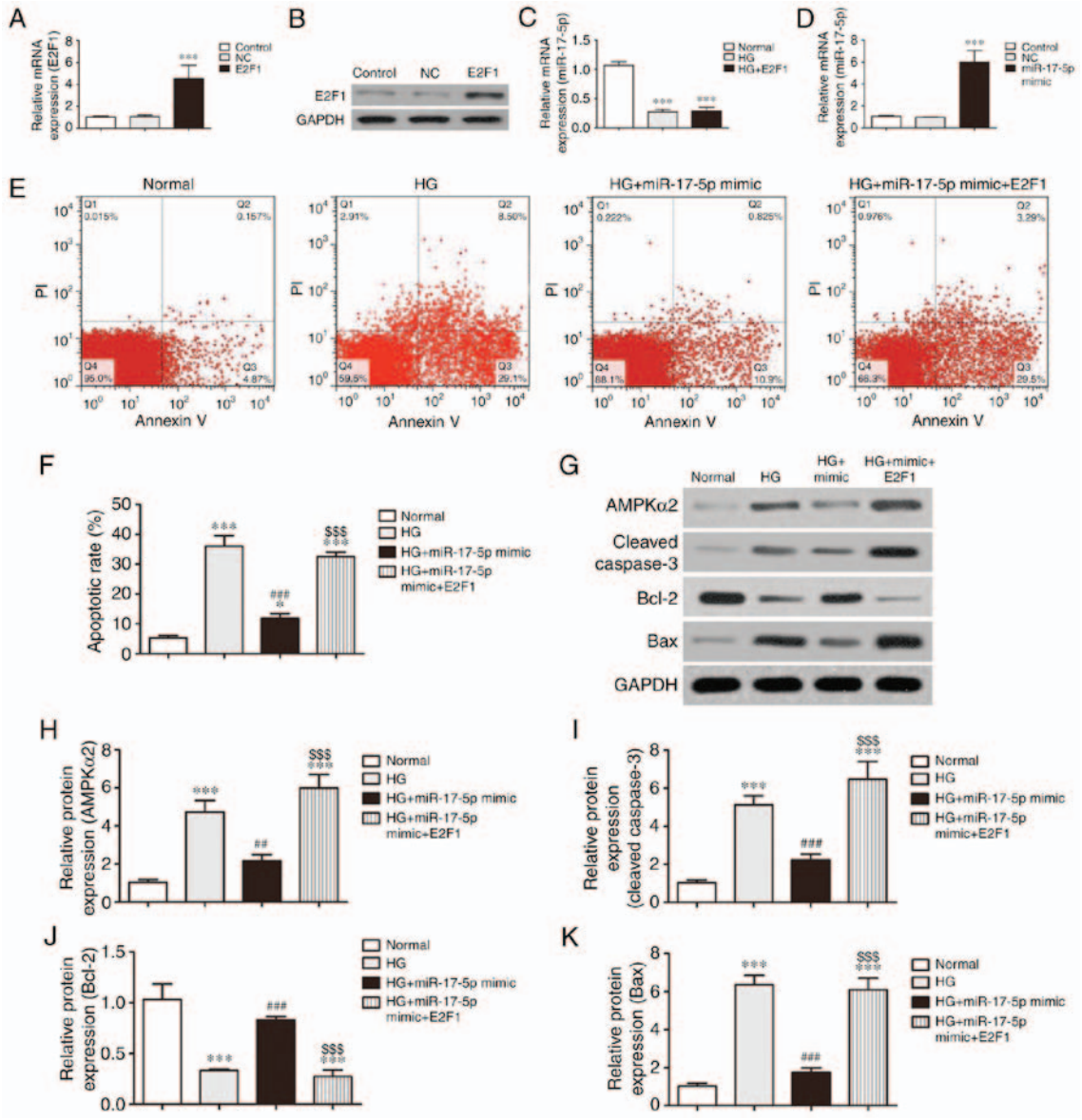

Figure 4. Overexpression of miR-17-5p protects against HG-induced endothelial cell injury by targeting E2F1-mediated suppression of autophagy and promotion of apoptosis. HUVECs were transfection with an E2F1-overexpression vector or an miR-17-5p mimic for 48 h prior to induction with normal glucose levels (5.5 mM) or HG (30 mM) for 48 h. The expression of E2F1 was detected by (A) RT-qPCR and (B) western blot analyses following transfection with an E2F1-overexpression vector or the NC vector. ***P<0.001, vs. control. (C) Effects of E2F1 and HG on the expression of miR-17-5p were determined using RT-qPCR analysis. ***P<0.001, vs. normal group. (D) Expression of miR-17-5p was detected using RT-qPCR following transfection with the miR-17-5p mimic or NC. ***P<0.001, vs. control. (E) Apoptosis was determined using annexin V/PI staining. (F) Percentage of apoptotic cells was measured. *P<0.05 and ***P<0.001, vs. normal group. $^{\#\#\#}$P<0.001, vs. HG group; $^{\$\$\$}$P<0.001, vs. the HG + miR-17-5p mimic group. (G) Western blot analysis showing the expression of apoptosis-related proteins, (H) AMPKα2, (I) cleaved caspase-3, (J) Bcl-2 and (K) Bax. The relative protein levels were analyzed. ***P<0.001, vs. normal group; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001, vs. HG group; $^{\$\$\$}$P<0.001, vs. HG + miR-17-5p mimic group.

also showed that the expression of E2F1 had no effect on miR-17-5p under exposure to HG conditions (Fig. 4C). To determine whether the overexpression of miR-17-5p reversed HG-induced endothelial injury, the HUVECs were transfected with a miR-17-5p mimic and the expression of miR-17-5p was detected by RT-qPCR analysis following transfection for 48 h. The results showed that the expression of miR-17-5p was significantly increased, compared with that in the negative control (Fig. 4D). The flow cytometry showed that the overexpression of miR-17-5p significantly suppressed HG-induced HUVEC apoptosis, however, the overexpression of E2F1 recovered the HG-induced HUVEC apoptosis, even when miR-17-5p was overexpressed (Fig. 4E and F). As miR-17-5p was unable to silence the transfected E2F1 without the 3'UTR region, it was possible that miR-17-5p suppressed HG-induced HUVEC apoptosis by targeting E2F1. The immunofluorescence showed that the overexpression of mTORC1 suppressed autophagy under HG conditions, even when E2F1 was downregulated. The western blot analysis showed that the overexpression of miR-17-5p inhibited HG-induced HUVEC apoptosis by repression of the E2F1/AMPKα2 signaling pathway, but restoration of the expression of E2F1 reversed the miR-17-5p-induced inhibition of apoptosis under HG conditions (Fig. 4G-K).

The results of the immunofluorescence showed that the overexpression of miR-17-5p promoted autophagy under HG

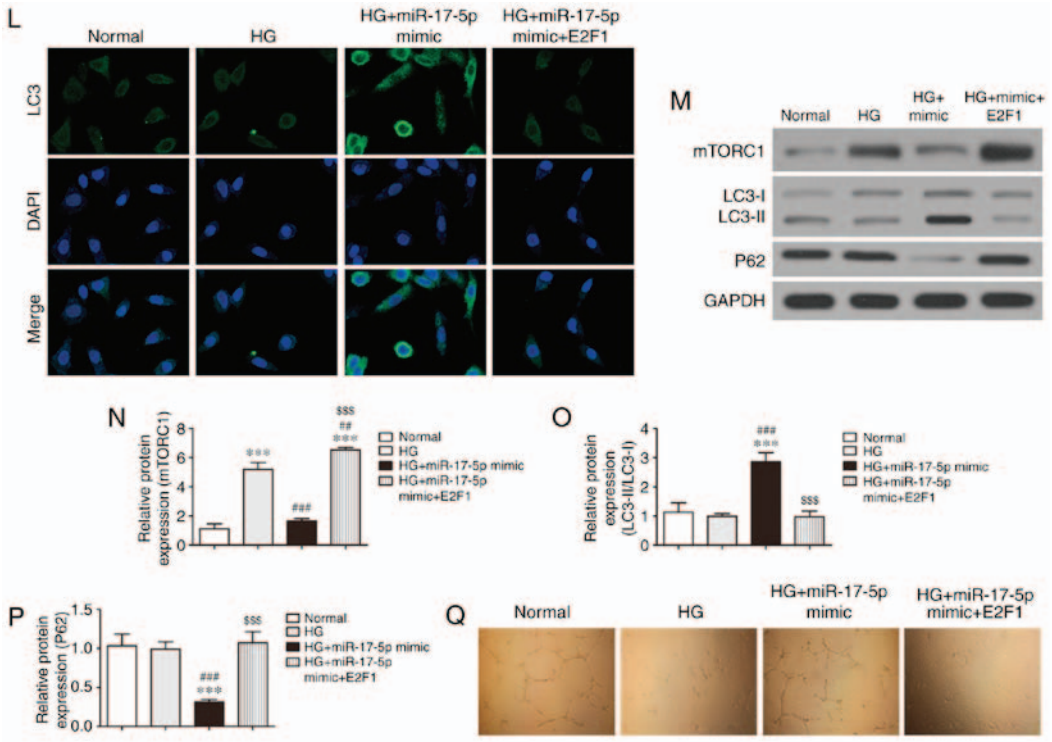

Figure 4. Continued. (L) Autophagy plaques were analyzed by immunofluorescence (magnification, x400). (M) Western blot analyses showing the expression of autophagy-related proteins, (N) mTORC1, (O) LC3 and (P) p62. The relative protein levels were analyzed. ***P<0.001, vs. normal group; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001, vs. HG group; $^{\$\$\$}$P<0.001, vs. HG + miR-17-5p mimic group. (Q) Tube formation capability of HUVECs from different treatment groups was measured (magnification, x400). The results showed that the tube formation capability of HUVECs decreased following treatment with HG. All data are expressed as the mean ± standard deviation (n=3). HUVECs, human umbilical vein endothelial cells; miR, microRNA; HG, HG, high glucose; E2F1, E2 promoter binding factor 1; AMPKα2, AMP-activated protein kinase α2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; mTORC1, mammalian target of rapamycin complex 1; LC3, microtubule-associated protein 1 light chain 3; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction analysis.

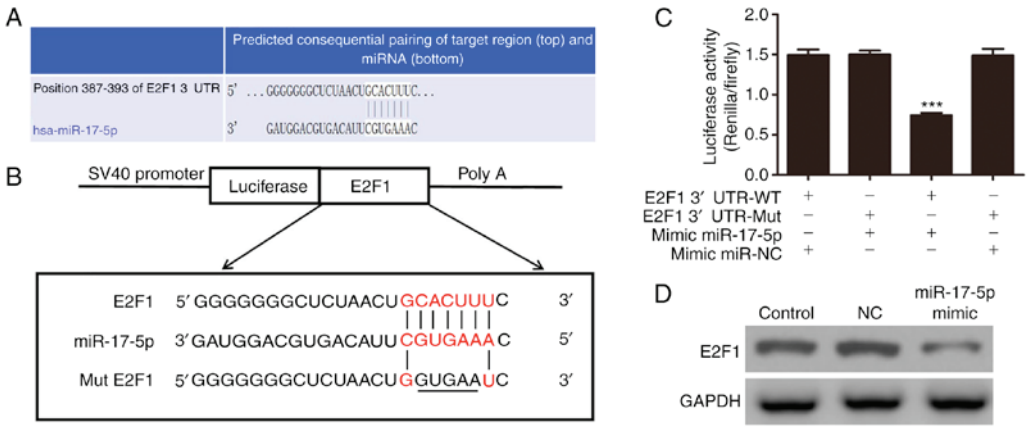

Figure 5. E2F1 is a potential target of miR-17-5p. (A) Complementary sequences between miR-17-5p and the 3'UTR of E2F1 mRNA were obtained using publicly available algorithms. (B) Mutated version of the E2F1 3'UTR is shown. (C) 3'UTR of E2F1 was fused to the luciferase coding region (PYr-E2F1 3'UTR) and co-transfected into HUVECs with the miR-17-5p mimic to confirm that E2F1 was the target of miR-17-5p. The PYr-E2F1 3'UTR and miR-17-5p mimic constructs were co-transfected into 293T cells with a control vector, and the relative luciferase activity was determined at 48 h post-transfection. The data are expressed as the mean ± standard deviation. ***P<0.001, vs. control. (D) Western blot analysis of the effect of the expression of E2F1 in HUVECs transfected with the miR-17-5p mimic (n=5). Expression levels of GAPDH were detected as an endogenous control. The data are expressed as the mean ± standard deviation. ***P<0.001, vs. control. HUVECs, human umbilical vein endothelial cells; E2F1, E2 promoter binding factor 1; 3'UTR, 3'untranslated region; miR, microRNA; WT, wild-type; Mut, mutant; NC, negative control.

conditions, however, the overexpression of E2F1 inhibited the miR-17-5p-mediated promotion of autophagy (Fig. 4L). The western blot analysis showed that the overexpression of miR-17-5p promoted HUVEC autophagy under HG conditions by repression of the E2F1/mTORC1 signaling pathway, but the restored expression of E2F1 reversed the miR-17-5p-induced promotion of autophagy under HG conditions (Fig. 4M-P). The tube formation assay showed that miR-17-5p overexpression promoted angiogenesis even under exposure to HG conditions, which was reversed when E2F1 was overexpressed (Fig. 4Q).

E2F1 is the direct target of miR-17-5p. To determine the possible interaction between miR-17-5p and E2F1, bioinformatics screening for its possible target genes was performed using an online 3'UTR binding site prediction database (http://www.targetscan.org/). The overlap analyses showed that miR-17-5p had a broadly conserved binding site (Fig. 5A). A mutated version of the E2F1 3'UTR was constructed in which five complementary nucleotides in the binding site were altered (Fig. 5B). This mutated construct was fused to the luciferase coding region (PYr-E3F1 3'UTR) and co-transfected into 293T cells with the miR-17-5p mimic (Fig. 5C). The relative luciferase activity showed that when the wild-type E2F1 3'UTR was co-transfected with the miR-17-5p mimic, the expression of E2F1 was significantly decreased ($P<0.001$), compared with the cells co-transfected with the control miRNA. However, this effect was not observed following treatment with the mutant 3'UTR of E2F1, indicating that miR-17-5p can specifically target and suppress the 3'UTR of E2F1. Western blot analyses further confirmed that the expression of miR-17-5p significantly inhibited the protein expression of E2F1 *in vitro* (Fig. 5D).

Discussion

Increasing evidence has shown that exposing cultured HUVECs to high concentrations of glucose induces oxidative/nitrosative stresses, including the generation of intracellular reactive oxygen species that cause cellular death. These injuries are associated with impaired endothelial cell tube formation (24). Several studies have reported that the dysregulation of miRNA in diabetic endothelial cells influences their functions (25,26). Therefore, targeting and regulating miRNA in an advanced pathway is a strategy for overcoming HG-induced endothelial injury.

In 1986, Kovesdi *et al* identified the cell cycle-related factor, E2F1 (27). Since its discovery, increasing evidence has shown that E2F is not only involved in cell cycle regulation and apoptotic signal transduction, but is also closely associated with growth, apoptosis, metastasis and autophagy (28-30). The results of the present study showed that the expression of E2F1 was upregulated following exposure to HG conditions, and showed that the downregulation of E2F1 improved HG-induced endothelial injury. The present study also demonstrated that HG-induced endothelial injury was associated with E2F1/AMPKα2 and E2F1/mTORC1. Fox *et al* reported that the expression of AMPKα2 mediated cell apoptosis (31), whereas the expression of mTORC1 inhibited autophagy (32,33). The present study also showed that downregulation of the expression of E2F1 significantly suppressed the expression of AMPKα2 and mTORC1, suggesting that the downregulation of E2F1 inhibited HG-induced apoptosis, and reversed the inhibition of mTORC1-mediated autophagy. Taken together, these results confirmed that increased autophagy within certain limits suppressed HG-induced cellular damage (34,35).

The present study investigated miRNAs as molecular targets of E2F1. The *in vitro* results showed that the overexpression of miR-17-5p was important for enhanced HUVEC survival under HG conditions. miR-17 has been shown to control cellular proliferation and apoptosis by targeting the E2F family of transcription factors (36,37), indicating that the overexpression of miR-17-5p significantly reverses HG-induced cellular damage by targeting the E2F1/AMPKα2- and E2F1/mTORC1-l-mediated promotion of apoptosis and inhibition of autophagy, respectively. Bifluorescein experiments confirmed that miR-17-5p bound predominantly to the 3'UTR of E2F1, resulting in downregulation of its expression.

In conclusion, the present study showed that E2F1 was expressed at high levels in HUVECs under HG conditions, leading to endothelial cell damage. The study also showed that the overexpression of miR-17-5p increased cell survival and functional recovery by targeting the E2F1/AMPKα2- and E2F1/mTORC1-mediated promotion of apoptosis and inhibition of autophagy, respectively. These findings characterized a unique mechanism involved in the HG-induced activation of E2F1 and cell apoptosis, in which the overexpression of miR-17-5p induced the activation of autophagy.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YY and ML generated and analyzed the data. XL designed the experiments and drafted the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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