

Analysis of potential functional significance of microRNA-3613-3p in human umbilical vein endothelial cells affected by heat stress

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Abstract. Dysregulation of microRNA-3613-3p (miR-3613-3p) was previously reported in endothelial cells (ECs) during heat stress. The aim of the present study was to investigate the precise role of miR-3613-3p in heat stress. In the present study, potential gene targets of miR-3613-3p in heat-treated ECs were assessed, and the potential effects of miR-3613-3p were determined using Gene Ontology enrichment analysis. Kyoto Encyclopedia of Genes and Genomes pathway analysis was used to identify signaling pathways that may be affected by miR-3613-3p in heat-treated cells. Reverse transcription-quantitative PCR, western blotting and annexin V-FITC/propidium iodide staining were performed to detect miRNA expression, protein expression and apoptosis, respectively. Luciferase gene reporter assay was performed to evaluate the association between miR-3613-3p and mitogen-activated protein kinase kinase 2 (MAP3K2). Bioinformatics analysis revealed 865 potential gene targets for miR-3613-3p and a series of functions and pathways in heat-treated ECs. 'Negative regulation of apoptotic process' was identified as a potential function of miR-3613-3p. In addition, functional analysis confirmed the downregulated expression levels of miR-3613-3p in ECs during heat stress, which was accompanied by an increase in apoptosis; restoration of miR-3613-3p expression inhibited apoptosis. MAP3K2 protein was demonstrated to be upregulated in heat-treated ECs, and overexpression of miR-3613-3p reduced MAP3K2 expression levels. Additionally, MAP3K2

was targeted by miR-3613-3p. These results indicated that miR-3613-3p may have complicated roles in ECs under heat stress. miR-3613-3p may serve an important role in the apoptosis of heat-treated ECs, and this effect may be partly achieved by targeting MAP3K2.

Introduction

Heat shock (HS) is a life-threatening disorder associated with excessive heat and characterized by a syndrome of multiple organ dysfunction in which central nervous system dysfunction dominates (1,2). The direct cytotoxicity of heat, inflammation and endothelial cell (EC) injury is considered to initiate multiple organ failure (3-5). ECs have a variety of biological functions, including the regulation of coagulation, cell adhesion, nutrient exchange and vascular tone; ECs also serve an important role in the balance of local pro-inflammatory and anti-inflammatory mediators (6-12). Previous studies in cell lines and animal models have shown that ECs are an early target of heat stress injury and are a prominent feature of severe HS (5,13,14). Thus, understanding the changes in ECs in response to heat stress is important for identifying novel therapeutic approaches to HS injury treatment.

MicroRNAs (miRNAs) are small RNAs that regulate gene expression at the post-transcriptional level and modulate biological responses and cell phenotypes through regulation of the expression levels of key proteins in multiple biological pathways (15). Certain miRNAs have been demonstrated to affect the function of ECs. For example, miRNA (miR)-155, miR-320, miR-222, miR-125b, miR-410 and miR-218 have been reported to regulate adherens junction disassembly responses, cell migration and cell morphology, contributing to changes in the permeability and integrity of the vasculature (16-18). As underlying regulatory mechanisms such as miRNA expression in response to HS were unknown, miRNA expression was analyzed in our previous study via miRNA microarrays; it was demonstrated that heat stress altered miRNA expression in HUVECs, with 31 miRNAs differing significantly in expression between HUVECs exposed to severe heat treatment and untreated control cells (20 miRNAs were downregulated and miRNAs were 11 upregulated) (19). Only 1% of the human miRNA assemblage (31/3,100) exhibited differential expression following heat treatment,

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indicating that the regulatory activity of heat stress may be specific toward a subset of miRNAs in HUVECs. Additionally, associations between miRNAs and genes have been previously evaluated on the basis of their differential expression levels, and according to interactions between miRNAs and genes reported in the Sanger miRNA database; these associations were used to build a miRNA-gene network that was employed to evaluate the regulatory effects of miRNAs on genes (19). Using this network model, miR-3613-3p was identified as a key miRNA that may serve a prominent role in the response to heat stress (19).

In the present study, the biological functions and underlying molecular mechanisms of miR-3613-3p in ECs during heat stress were preliminarily investigated. The results may contribute to a better understanding of the mechanisms underlying the relationships between miRNAs and biological responses of ECs and may facilitate new diagnostic and therapeutic strategies for the treatment of heat stress.

Materials and methods

Bioinformatics analysis. Potential target genes of miR-3613-3p were predicted using three databases: TargetScan 7.2 (<http://www.targetscan.org/>), miRanda-mirSVR 5.0 (<http://www.microrna.org/microrna/home.do>) and miRDB 5.0 (<http://www.mirdb.org>). Functional annotation using Gene Ontology (GO) (20,21) enrichment analysis was performed to determine the main functions of putative target genes of miR-3613-3p. Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) (22-24) database analysis was performed to identify the enriched pathways associated with these target genes. Fisher's exact and χ^2 tests were applied to assess the significance of GO terms and pathways, and the false discovery rate (FDR) was calculated to correct the P-value. Only GO terms and pathways with an adjusted $P < 0.05$ and an $FDR < 0.05$ were selected. Data containing two groups were analyzed using Fisher's exact test, and multiple comparisons between groups were assessed using the χ^2 test.

Cell culture and treatment. Human umbilical vein endothelial cells (HUVECs; American Type Culture Collection) were cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 5% FBS (Invitrogen; Thermo Fisher Scientific, Inc.), 1% EC growth supplement (PromoCell GmbH), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO₂ at 37°C. All experiments were performed using HUVECs at passage 3-6. To induce heat stress, culture dishes containing cells in media were sealed with Parafilm and immersed in a circulating water bath at 43±0.5°C for 1 h; cells in culture dishes placed in a circulating water bath at 37±0.5°C for 1 h were used as a control. After treatment, the culture medium was replaced with fresh medium, and the cells were incubated at 37°C for an additional 24 h.

RNA extraction. Total RNA from HUVECs (~5×10⁶ cells) was individually isolated using TRIzol® (Thermo Fisher Scientific, Inc.) and an miRNeasy mini kit (Qiagen, Inc.) according to the manufacturers' protocols. RNA quantity and quality were

measured using a NanoDrop™ spectrophotometer (ND-1000; NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and RNA integrity was detected by gel electrophoresis. In general, RNA concentrations of 8-15 ng/ μ l, and 10 ng/sample of total RNA were used for subsequent reverse transcription-quantitative PCR (RT-qPCR) analysis.

RT-qPCR. The miRNA expression levels were determined using RT-qPCR using primers which were synthesized and purchased from Shanghai GenePharma Co., Ltd. The following primers were used: miR-3613-3p, sense 5'-CGTCCCTTC CCAACCCGAAAAAAA-3', antisense 5'-CGCAGGGTC CGAGGTATTC-3'; and U6, sense 5'-CTCGCTTCGGCAG CACA-3' and antisense 5'-AACGCTTCACGAATTTGCGT-3'. Briefly, sample total RNA (10 ng) was reverse transcribed into cDNA using specific stem-loop primers and a TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Mixtures were incubated for 30 min at 16°C, 30 min at 42°C and 5 min at 85°C, and held at 4°C in a 15- μ l reaction volume. Following the reverse transcription reaction, qPCR was performed using SYBR Green (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and an ABI 7300 Real-Time PCR system (Bio-Rad Laboratories, Inc.). qPCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec in a 20 μ l reaction volume (25,26). Signals were normalized to U6 small nuclear RNA, which was analyzed simultaneously. The relative expression of each miRNA was calculated using the 2^{- $\Delta\Delta$ C_q} method (27). The experiments were performed at least three times.

Transient transfection. The miR-3613-3p mimic (cat. no. miR10017991-1-5) and negative control (miR-NC; cat. no. miR1N0000001-1-5) were purchased from Guangzhou RiboBio Co., Ltd. Cells in the logarithmic growth phase (5×10⁵ cells/well) were inoculated into 24-well plates and cultured to 80% confluence on the day prior to transfection. Subsequently, miR-3613-3p mimic or miR-NC were transfected into HUVECs at a final concentration of 50 nM using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 24 h according to the manufacturer's protocol. At 24 h post-transfection, the transfected cells were harvested for miRNA and protein assays.

Flow cytometric analysis of apoptosis. Apoptosis was analyzed with an Annexin V-FITC Apoptosis kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. HUVECs (~1×10⁶ cells) were collected, washed with ice-cold PBS and resuspended in binding buffer containing 5 μ l annexin V-FITC for 10 min in the dark at room temperature. Subsequently, the cells were pelleted by centrifugation at ~157 × g and 4°C for 10 min, the buffer was removed and the cells were resuspended in reaction buffer containing 10 μ l propidium iodide (PI). Following incubation in the dark at room temperature for 15 min, the stained cells were analyzed for apoptosis using a FACSCanto™ II flow cytometer (BD Biosciences). The early apoptotic rates were examined. FlowJo version 7.6.1 software (FlowJo LLC) was used to analyze the data. The experiment was performed at least three times.

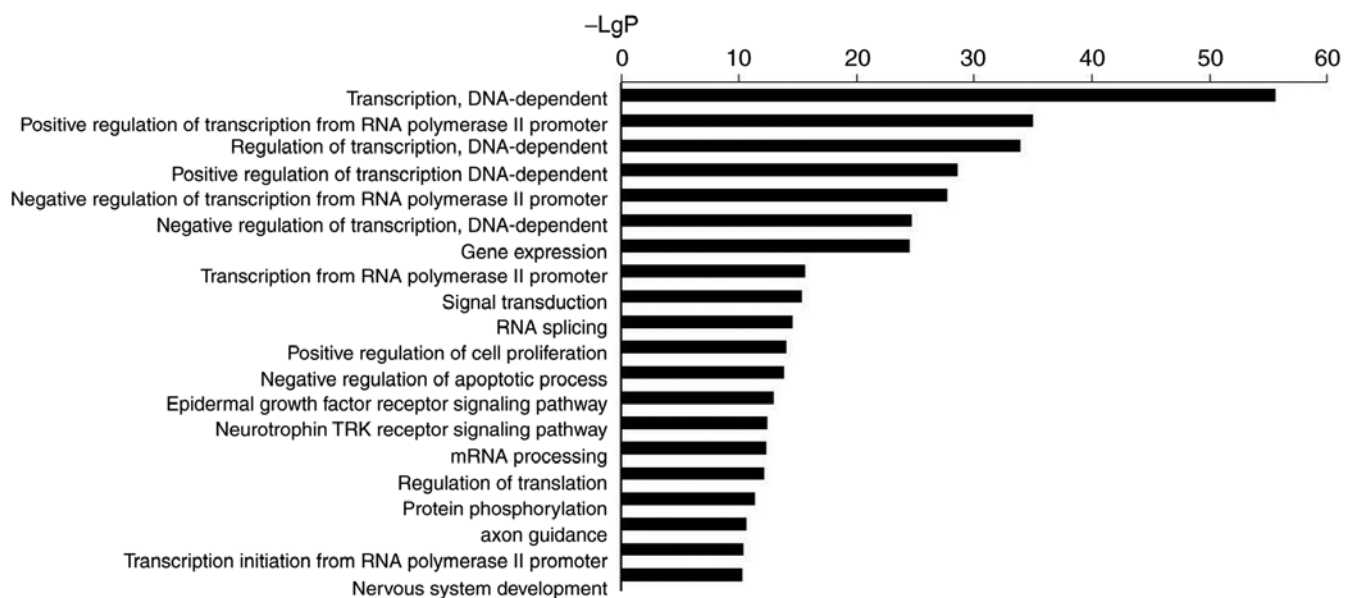


Figure 1. GO term analysis of miR-3613-3p. Top 20 significantly enriched GO biological process functions are presented in descending order. The vertical axis denotes the functional category, and the horizontal axis denotes the degree of enrichment of the function. GO, Gene Ontology; -LgP, negative logarithm of P-value; TRK, tropomyosin-related kinase.

Western blot analysis. HUVECs (5×10^5 cells/well) were seeded in 24-well plates and incubated for 24 h prior to lysis. Cells were lysed in 1X sample buffer (62.5 mM Tris, pH 6.8; 10% glycerol; 2% SDS) and homogenized. The protein concentration was measured using a Bicinchoninic Acid Protein assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Equal amounts of protein (20 μ g/well) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked with 5% non-fat milk powder in PBS + 0.1% Tween-20 at room temperature for 2 h. The membranes were subsequently incubated overnight at 4°C with mouse anti-human mitogen-activated protein kinase kinase 2 (MAP3K2; 1:1,000, cat. no. SAB5300054, Sigma Aldrich; Merck KGaA) or mouse anti-human actin (1:500, cat. no. A4700, Sigma Aldrich; Merck KGaA) primary antibodies. A goat anti-mouse horseradish peroxidase-conjugated immunoglobulin G secondary antibody (1:10,000; cat. no. SAB3701029; Sigma Aldrich; Merck KGaA) was used, with incubation at room temperature for 2 h. Protein bands were visualized with Enhanced Chemiluminescence Western Blot Detection reagent (Thermo Fisher Scientific, Inc.). The membranes were exposed to light-sensitive film and densitometric analysis was conducted using ImageJ v1.50 software (National Institutes of Health). The expression levels of protein were normalized to the actin endogenous control. The western blotting experiments were performed at least three times.

Luciferase gene reporter assay. HUVECs (1×10^5 cells/well) were seeded in 24-well plates and incubated for 24 h prior to transfection. Cells were co-transfected with 100 nM luciferase vectors (pGL3-MAP3K2 3'UTR wild-type or mutant reporter plasmid; Guangzhou RiboBio Co., Ltd.) and either 50 nM miR-3613-3p mimic or miR-NC using Lipofectamine® 3000 at room temperature for 24 h. Firefly and *Renilla* luciferase

activities were quantified using the Dual-Luciferase reporter system (Promega Corporation) according to the manufacturer's protocol. The firefly luciferase activity was normalized to *Renilla* luciferase activity. All experiments were performed in triplicate; $n=3-6$ for each experiment.

Statistical analysis. Quantitative variables are presented as the mean \pm standard deviation. Student's t-test was used to calculate statistical significance between two groups. One-way ANOVA was used for multiple group comparisons followed by Tukey post hoc test. Statistical values were calculated using SPSS version 20.0 (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GO term analysis of miR-3613-3p. GO analysis results were presented in Fig. 1, and Tables I (data repository) and II. According to GO analysis of the 865 genes potentially regulated by miR-3613-3p, the top biological processes associated with the genes regulated by miR-3613-3p were associated with transcription. A number of the genes were also involved in cell proliferation and apoptosis.

KEGG pathway analysis of miR-3613-3p. Pathway analysis by KEGG revealed the signaling pathways affected by miR-3613-3p in heat-treated cells. The most significantly enriched pathways regulated by miR-3613-3p analyzed by KEGG analysis were presented in Fig. 2 and Table III. Enriched KEGG pathway analyses revealed that the top signaling pathways regulated by miR-3613-3p were centralized in cancer-associated terms, which participated in the regulation of apoptosis, proliferation and cell cycle. Additional identified signaling pathways were also associated with transcriptional misregulation in cancer and adherens junctions.

Table II. Top 20 significant GO biological processes of microRNA-3613-3p target genes.

GO ID	GO term	Enrichment	P-value	FDR
GO:0006351	Transcription, DNA-dependent	4.429852641	3.20039×10^{-56}	8.225×10^{-53}
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	5.752041467	1.42286×10^{-35}	1.82837×10^{-32}
GO:0006355	Regulation of transcription, DNA-dependent	4.150877171	1.52829×10^{-34}	1.30924×10^{-31}
GO:0045893	Positive regulation of transcription, DNA-dependent	6.299381822	3.20544×10^{-29}	2.0595×10^{-26}
GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	6.076613899	2.3074×10^{-28}	1.186×10^{-25}
GO:0045892	Negative regulation of transcription, DNA-dependent	6.125889879	2.8019×10^{-25}	1.20015×10^{-22}
GO:0010467	Gene expression	4.938916527	4.02629×10^{-25}	1.47822×10^{-22}
GO:0006366	Transcription from RNA polymerase II promoter	5.640723054	3.17439×10^{-16}	1.01977×10^{-13}
GO:0007165	Signal transduction	3.253151632	5.30103×10^{-16}	1.51374×10^{-13}
GO:0008380	RNA splicing	6.443742656	3.21943×10^{-15}	8.27393×10^{-13}
GO:0008284	Positive regulation of cell proliferation	4.766174616	1.15335×10^{-14}	2.69465×10^{-12}
GO:0043066	Negative regulation of apoptotic process	4.366833866	1.86122×10^{-14}	3.98612×10^{-12}
GO:0007173	Epidermal growth factor receptor signaling pathway	6.966208276	1.33741×10^{-13}	2.64395×10^{-11}
GO:0048011	Neurotrophin TRK receptor signaling pathway	5.55742861	4.69776×10^{-13}	8.62374×10^{-11}
GO:0006397	mRNA processing	6.873325499	5.87456×10^{-13}	1.00651×10^{-10}
GO:0006417	Regulation of translation	12.12939794	8.03991×10^{-13}	1.29141×10^{-10}
GO:0006468	Protein phosphorylation	4.727953191	5.1723×10^{-12}	7.8193×10^{-10}
GO:0007411	Axon guidance	4.715925224	2.91722×10^{-11}	4.16514×10^{-9}
GO:0006367	Transcription initiation from RNA polymerase II promoter	6.163579932	5.51489×10^{-11}	7.45962×10^{-9}
GO:0007399	Nervous system development	4.918192274	5.84827×10^{-11}	7.51503×10^{-9}

FDR, false discovery rate; GO, gene ontology; TRK, tropomyosin-related kinase.

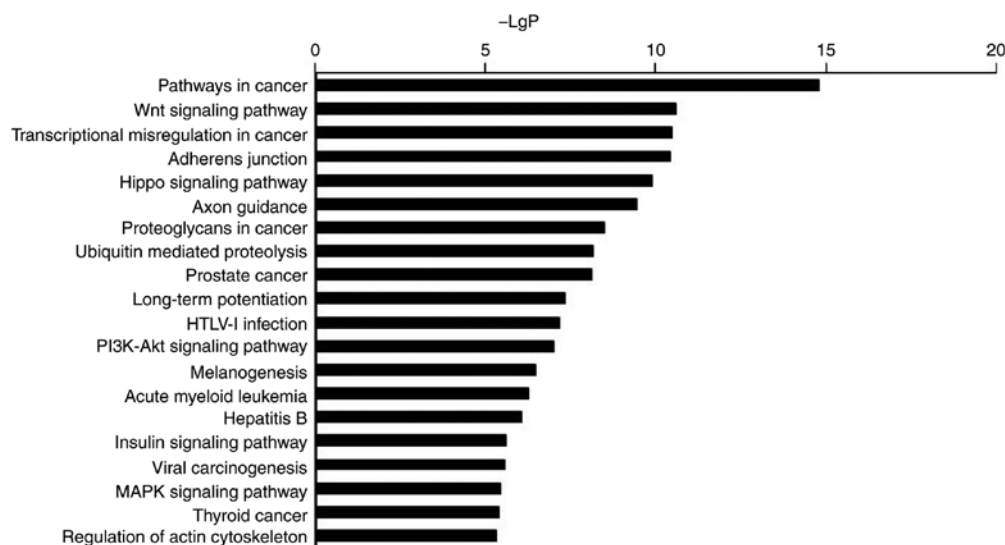


Figure 2. Kyoto Encyclopedia of Genes and Genomes pathway analyses of miR-3613-3p. Top 20 significantly enriched pathways are presented in a descending order. The vertical axis denotes the pathway category, and the horizontal axis denotes the degree of enrichment of the pathway. HTLV, human T-cell leukemia virus; LgP, negative logarithm of P-value; MAPK, mitogen-activated protein kinase.

Table III. Top 20 significant KEGG pathways of microRNA-3613-3p target genes.

KEGG ID	Pathway name	Enrichment	P-value	FDR
05200	Pathways in cancer	5.517577809	1.69483×10^{-15}	3.25407×10^{-13}
04310	Wnt signaling pathway	7.209781992	2.54375×10^{-11}	1.8035×10^{-9}
05202	Transcriptional misregulation in cancer	6.300548374	3.54805×10^{-11}	1.8035×10^{-9}
04520	Adherens junction	10.59245368	3.75729×10^{-11}	1.8035×10^{-9}
04390	Hippo signaling pathway	6.608966826	1.29264×10^{-10}	4.96376×10^{-9}
04360	Axon guidance	7.083198034	3.87582×10^{-10}	1.24026×10^{-8}
05205	Proteoglycans in cancer	4.996029548	3.30873×10^{-9}	9.07539×10^{-8}
04120	Ubiquitin mediated proteolysis	6.350355081	7.1811×10^{-9}	1.63216×10^{-7}
05215	Prostate cancer	8.108979522	7.65076×10^{-9}	1.63216×10^{-7}
04720	Long-term potentiation	8.712666126	4.7221×10^{-8}	9.06642×10^{-7}
05166	HTLV-I infection	4.231711595	7.15157×10^{-8}	1.24827×10^{-6}
04151	PI3K-Akt signaling pathway	3.713972712	1.06017×10^{-7}	1.69628×10^{-6}
04916	Melanogenesis	6.635140952	3.41775×10^{-7}	5.04776×10^{-6}
05221	Acute myeloid leukemia	9.043849341	5.42797×10^{-7}	7.44407×10^{-6}
05161	Hepatitis B	5.224656207	8.84732×10^{-7}	1.13246×10^{-5}
04910	Insulin signaling pathway	5.154994125	2.62577×10^{-6}	3.15092×10^{-5}
05203	Viral carcinogenesis	4.233570054	2.90592×10^{-6}	3.28198×10^{-5}
04010	MAPK signaling pathway	3.767111091	3.92363×10^{-6}	4.1852×10^{-5}
05216	Thyroid cancer	12.44308927	4.33983×10^{-6}	4.38551×10^{-5}
04810	Regulation of actin cytoskeleton	4.076041866	4.92093×10^{-6}	4.7241×10^{-5}

FDR, false discovery rate; HTLV, human T-cell leukemia virus; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK, mitogen-activated protein kinase.

miRNA expression levels are dysregulated in HUVECs during heat stress. Based on previous miRNA microarray analysis, miR-3613-3p expression levels were decreased in HUVECs under heat stress (19). To confirm the downregulation of miR-3613-3p in heat-treated cells, miR-3613-3p expression was analyzed by RT-qPCR, and the results verified that miR-3613-3p expression was reduced in HS-treated HUVECs compared with control cells ($P < 0.05$; Fig. 3).

miR-3613-3p affects apoptosis in HUVECs during heat stress. Previous bioinformatics analysis demonstrated that miR-3613-3p negatively regulates apoptosis (19). To confirm this, miR-3613-3p mimic was transfected into HUVECs, which increased the expression levels of microRNAs-3613-3p (Fig. 4A). Annexin V-FITC/PI flow cytometry was then used to evaluate the effects of different expression levels of miR-3613-3p on the apoptotic rates of HUVECs. The results revealed that miR-3613-3p expression decreased in HUVECs following heat stress and that the miR-3613-3p mimic increased the level of miR-3613-3p in heat-treated and untreated cells ($P < 0.05$; Fig. 4A). In addition, heat stress induced apoptosis in HUVECs, whereas the miR-3613-3p mimic transfection significantly decreased the apoptotic rate under heat stress ($P < 0.05$; Fig. 4B). These results suggested that the level of miR-3613-3p may affect HUVEC apoptosis during heat stress.

miR-3613-3p affects the expression of MAP3K2 in HUVECs during heat stress. Previous bioinformatics analysis indicated

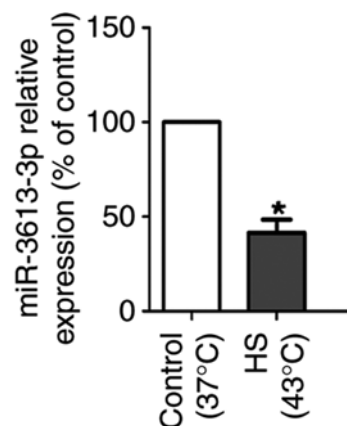


Figure 3. Expression of miR-3613-3p is dysregulated in HUVECs during heat stress. Reverse transcription-quantitative PCR analysis was performed to analyze miRNA expression levels. miR-3613-3p was significantly down-regulated following heat shock compared with the control group. * $P < 0.05$ vs. control (37°C). HS, heat shock; HUVECs, human umbilical vein endothelial cells; miR, microRNA; miRNA, microRNA.

that MAP3K2 may be a target gene of miR-3613-3p (19). To investigate the association between miR-3613-3p and MAP3K2, HUVECs were transfected with miR-3613-3p mimic for 24 h, and western blotting was performed. As presented in Fig. 5, the protein level of MAP3K2 was significantly reduced in the miR-3613-3p mimic-transfected cells under heat treatment, compared with cells only treated

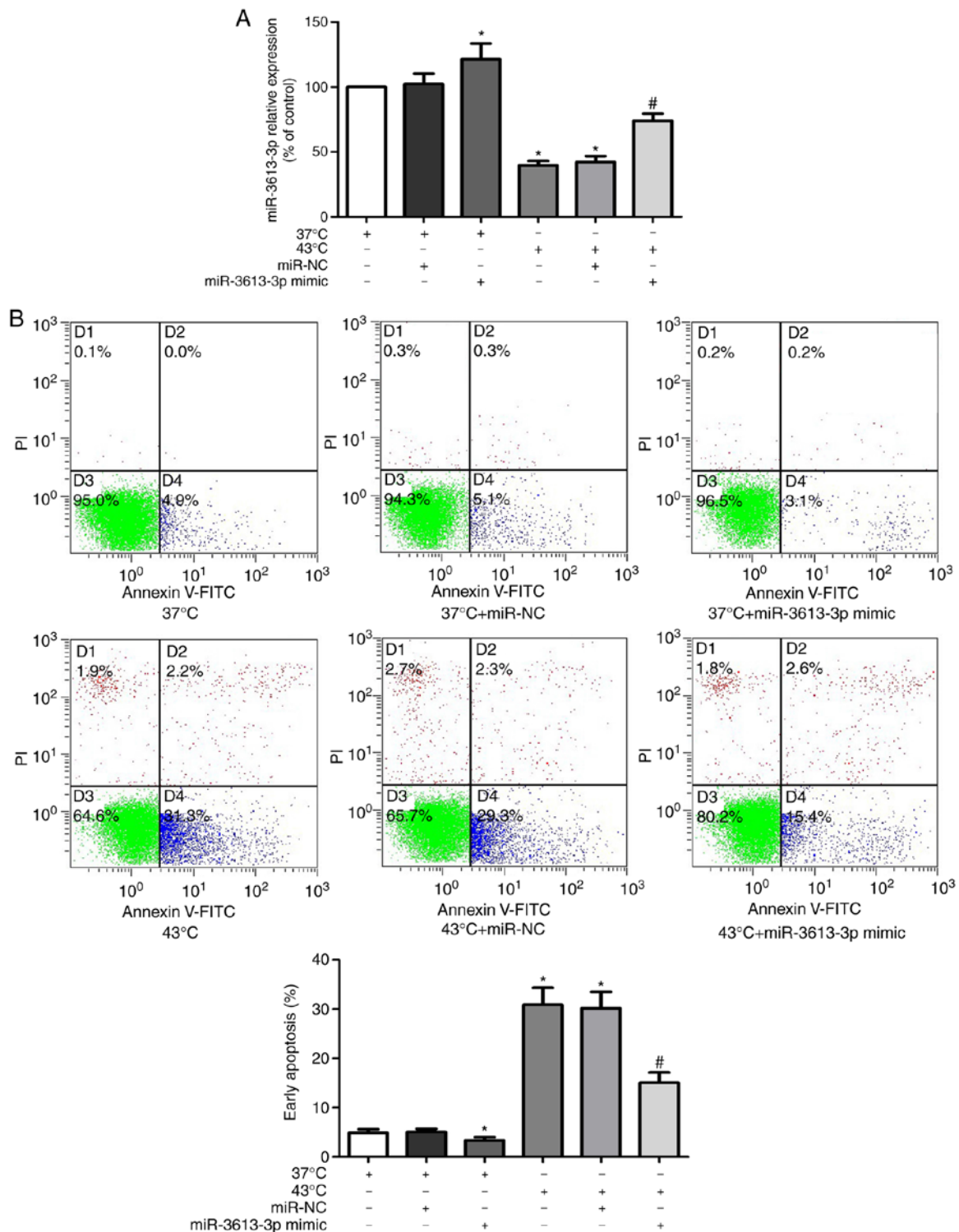


Figure 4. miR-3613-3p affects apoptosis in HUVECs during heat stress. (A and B) HUVECs were transfected with a miR-3613-3p mimic or miR-NC. (A) Expression of miR-3613-3p was decreased in HUVECs under heat stress, and the miR-3613-3p mimic partly reversed the heat stress-induced decline in miR-3613-3p. (B) Annexin V-FITC/PI flow cytometry was used to detect the effect of miR-3613-3p on the apoptotic rate of HUVECs. Heat stress induced apoptosis in HUVECs, and the miR-3613-3p mimic partially reversed the effects of heat stress. * $P < 0.05$ vs. 37°C; # $P < 0.05$ vs. 43°C. HUVECs, human umbilical vein endothelial cells; miR, microRNA; NC, negative control; PI, propidium iodide.

with heat stress ($P < 0.05$). These results indicated that miR-3613-3p overexpression may downregulate MAP3K2 expression.

MAP3K2 is a direct target of miR-3613-3p in HUVECs. To further demonstrate whether MAP3K2 was a direct target

of miR-3613-3p, MAP3K2 wild-type and mutant 3'UTR were cloned into a luciferase reporter vector and transfected into HUVECs (Fig. 6A). The effects of miR-3613-3p were determined using the luciferase reporter assay. The results demonstrated that upregulation of miR-3613-3p significantly reduced the luciferase activity of pGL3-MAP3K2 3'UTR WT

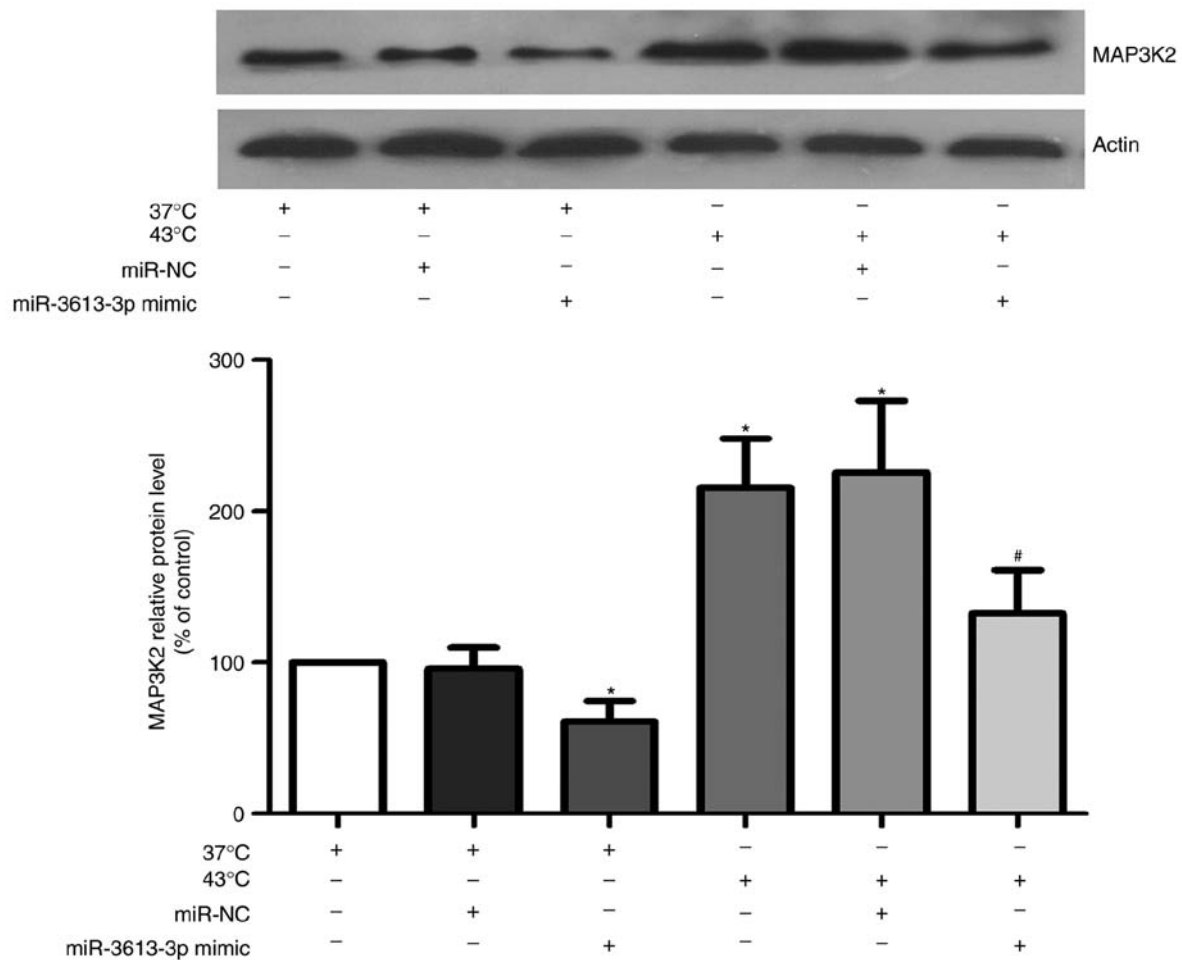


Figure 5. miR-3613-3p affects the expression of MAP3K2 in HUVECs during heat stress. HUVECs were transfected with a miR-3613-3p mimic or miR-NC, and expression of MAP3K2 was analyzed by western blotting using actin as an internal control. MAP3K2 protein expression was increased in heat-treated HUVECs, whereas upregulation of miR-3613-3p partly reversed the increase in MAP3K2 induced by heat stress. * $P < 0.05$ vs. 37°C; # $P < 0.05$ vs. 43°C. HUVECs, human umbilical vein endothelial cells; MAP3K2, mitogen-activated protein kinase kinase kinase 2; miR, microRNA; NC, negative control.

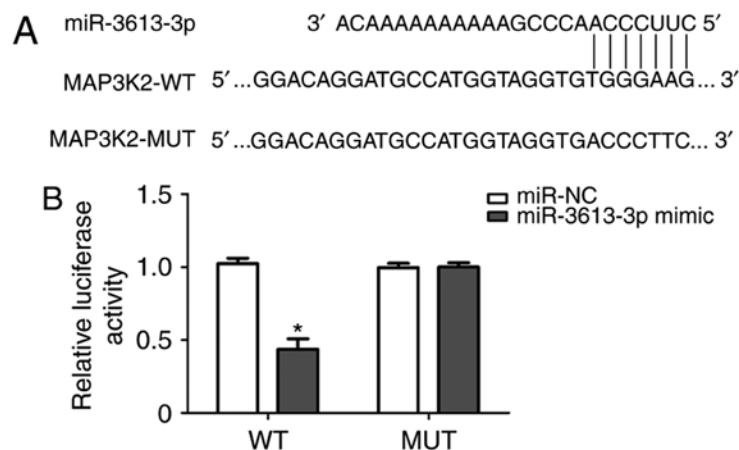


Figure 6. MAP3K2 is a target gene for miR-3613-3p. (A) Schematic representation of the putative miR-3613-3p target site in the MAP3K2 3'untranslated region. (B) Analysis of relative luciferase activity of MAP3K2-WT, MAP3K2-MUT in HUVECs. * $P < 0.05$ vs. miR-NC + WT. HUVECs, human umbilical vein endothelial cells; MAP3K2, mitogen-activated protein kinase kinase kinase 2; miR, microRNA; MUT, mutant; WT, wild type.

($P < 0.05$), but the effects of miR-3613-3p was not observed with the mutation of the miR-3613-3p target site in the MAP3K2 3'UTR (Fig. 6B). These results suggested that MAP3K2 may be directly and negatively regulated by miR-3613-3p.

Discussion

Among the miRNAs identified and target genes predicted in our previous study, miR-3613-3p was identified as an miRNA

downregulated in heat-treated cells compared with non-treated control (19); it was also demonstrated to potentially regulate 865 genes. Similar results were observed in a previous bioinformatics prediction, which suggested that miRNAs regulate >30% of protein-coding genes and that one miRNA may have hundreds or even thousands of potential gene targets (15). Indeed, the wide target range of miRNAs appears to be an important biological mechanism that enables them to mediate different cellular responses to environmental changes and is likely to significantly advance our knowledge of miRNA biological functions. For example, several studies have demonstrated that miR-3613-3p is upregulated in lung adenocarcinoma (28) and in gastric (29), colon (30), prostate (31) and thyroid papillary (32) cancers. In addition, the high level of miR-3613-3p expression in gastric cancer cells was reduced by cinobufagin (33), which is used clinically to treat patients with solid malignant tumors. Thus, miR-3613-3p might serve an important role in cancer by acting as an oncogene. In addition, by targeting pain-associated genes, including γ -aminobutyric acid receptor type A subunit $\beta 3$, N-methyl-D-aspartate 3A, transient receptor potential vanilloid-1, neuropeptide Y receptor Y1, and sodium channel protein type 9 subunit α , miR-3613-3p may be involved in severe axial pain after motor vehicle collision in African Americans (34). Upregulation of miR-3613-3p was observed in the left atrial appendage in rheumatic mitral valve disease with atrial fibrillation (35). Another report indicated that decreased expression of miR-3613-3p may regulate B cell activation through the Wnt pathway and participate in the pathogenesis of immunoglobulin A nephropathy (36). Therefore, it was speculated that miR-3613-3p may have different functions and regulate different target genes in various tissues under different conditions. This may also explain the large number of target genes of miR-3613-3p. Overall, the effects of miR-3613-3p in ECs under heat stress need to be clarified.

According to GO analysis, there was potential significant enrichment of miR-3613-3p in transcription-related functions (8 of 10 terms, 80%), and RNA polymerase II may be a key regulatory point. This finding was expected as the transcriptional status of cells changes markedly in response to heat stress. Transcriptional activity is commonly considered to decrease globally with transcriptional activation of certain genes that are necessary for heat stress-related cellular responses (37). However, there is no clear understanding of the exact mechanism of transcriptional repression. RNA polymerase II activity reduction is mediated by Alu RNAs, which are non-coding RNAs representing transcripts of short interspersed elements (SINE) in humans (38). Under heat stress, Alu RNAs are rapidly upregulated (39,40) and bound directly to RNA polymerase II to form kinetically stable complexes, thereby interfering with the binding of RNA polymerase to DNA and subsequent formation of the closed complex (41). To mediate repression, small repressing RNAs bind to RNA polymerase prior to formation of the closed complex (42), which prevents the mechanism from interfering with the transcription of heat shock protein (HSP) genes that are associated with RNA polymerase II molecules under non-heat shock conditions (43,44); this results in HS-induced increase in HSP expression despite a global decline in cell transcription. However, it is unclear whether SINE-dependent transcriptional

repression is universal in cells under heat stress. The data from the present study suggested that miR-3613-3p may perform a complex regulatory function in transcription during heat stress. miR-3613-3p may be involved in regulating a variety of transcription-related processes and appears to simultaneously regulate opposing functions. For example, miR-3613-3p was found to be involved not only in 'positive regulation of transcription' but also in 'negative regulation of transcription'. It is not clear how miR-3613-3p participates in the regulation of transcription and whether it depresses transcription via SINE-dependent mechanisms. Thus, miR-3613-3p may serve a complex role in the regulation of transcription through unknown and complicated mechanisms.

KEGG pathway analysis provided a more detailed view of the integrated relationship between the regulation of miR-3613-3p and target genes. The pathways identified by KEGG analysis serve crucial roles in regulating diverse processes of ECs, including proliferation, apoptosis, survival, migration and polarity, tight junction integrity and monocyte-EC adhesion enhancement, mostly through a series of downstream transcription factors (45-53). These data suggested that miR-3613-3p may facilitate individual biological reactions not by targeting an isolated pathway, but rather by targeting a complete network.

Through the bioinformatics analysis in the present study, 'negative regulation of apoptotic process' was identified as a potential function of miR-3613-3p. Apoptosis is implicated in the physiological and pathological processes of ECs under heat stress (54-56). *In vitro* experiments on the inhibitory effect of miR-3613-3p on apoptosis indicated that heat stress induced miR-3613-3p repression and promoted apoptosis. In addition, reintroduction of miR-3613-3p partly reversed the promoting effect of heat stress on apoptosis, which suggested that miR-3613-3p may be involved in regulating the apoptotic process in ECs during heat stress; these results may provide new insight into the treatment of HS.

Our previous miRNA-gene network analysis revealed a series of hub target genes, and MAP3K2 appeared in the central position of the network regulated by many miRNAs (19). MAP3K2 is a component of the mitogen-activated protein kinase (MAPK) signaling pathway, which preferentially regulates JNK and ERK5 pathways by phosphorylating and activating MAP2K5 and MAP2K7 (57,58). In addition, MAP3K2 participates in the regulation of apoptosis through a number of downstream targets (59,60). In the present study, bioinformatics analysis indicated that MAP3K2 was a target gene of miR-3613-3p. Therefore, miR-3613-3p may be involved in EC apoptosis by targeting the MAP3K2 gene during heat stress. In the current study, increased expression of MAP3K2 was observed in ECs under heat stress accompanied by reduced expression of miR-3613-3p compared with non-treated cells, whereas overexpression of miR-3613-3p partly reversed MAP3K2 augmentation, which suggested that MAP3K2 may be partially regulated by miR-3613-3p. Additionally, miR-3613-3p directly inhibited the expression of MAP3K2 by binding to its 3'UTR. It is noteworthy that MAP3K2 may induce distinct effects on apoptosis under varying external conditions (61-63), which may be due to distinct downstream molecules. The MAP2K5/ERK5 pathway is required for normal cardiovascular development and

vascular integrity, and it improves EC viability and reduces apoptosis (64,65); conversely, JNK MAPKs promote apoptosis in ECs under most conditions (66). MAK3P2 coordinately activates signaling through MEK5/ERK5 and MEK7/JNK protein kinases to respond to different stimuli. Therefore, the effects of MAP3K2 on EC apoptosis during heat stress may be induced by JNK MAPKs. The present study demonstrated that heat stress may decrease the activation of miR-3613-3p and promote the apoptotic effect on HUVECs by MAP3K2 through targeted binding to 3'-UTR, which leads to the inhibition of MAP3K2 expression.

There were certain limitations to the present study. First, HUVECs cannot completely reflect the biological characteristics of vascular ECs as venous endothelial function may differ substantially from arterial or capillary endothelial function. It remains to be determined whether similar regulatory mechanisms are involved *in vivo* and in other cellular systems. Second, the heat stress cell model used in the present study did not ideally simulate the clinical course of HS, and interactions between ECs and other cells were ignored. Third, some of the bioinformatics results may have been affected by technical deficiencies, such as technical artifacts introduced by the smaller scale of experimentally validated data and other associated biological data, deficiencies in effective integration of diverse datasets or the different computational models (67,68).

In conclusion, bioinformatics analysis revealed a range of target genes of miR-3613-3p, and a number of functions and pathways involved. Additionally, 'negative regulation of apoptotic process' was predicted to be a potential function of miR-3613-3; *in vitro* analysis revealed that miR-3613-3p may suppress apoptosis in ECs under heat stress, potentially by directly targeting MAP3K2. These results shed new light on the underlying mechanisms of heat stress in ECs, although further studies are needed to explore the roles of miR-3613-3p in the pathogenesis of heat stress in ECs.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Table I is published in the following repository: <https://figshare.com/s/81268025c7dac8b67bd2> (DOI:10.6084/m9.figshare.7435958).

Authors' contributions

QL and ZT conceived and designed all the experiments, performed data analysis for the study, and contributed towards

overall supervision of the project, and the drafting and editing the manuscript. JL was involved in the design of the study, performed experiments and data analysis, and was a major contributor to the writing of the manuscript. SL and GZ analyzed and interpreted the data regarding miR-3613-3p. XH performed reverse transcription-quantitative PCR for miR-3613-3p. All authors read and approved the final 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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