SCIENTIFIC ARTICLE

Role of Junction-Mediating and Regulatory Protein in the Pathogenesis of Glucocorticoid-Induced Endothelial Cell Lesions

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Objective: Nontraumatic osteonecrosis of the femoral head (ONFH) is one of the most common diseases in orthopaedics. The damage to vascular endothelial (VE) cells caused by glucocorticoids (GC) has been reported as a possible mechanism of pathogenesis for ONFH. Junction-mediating and regulatory protein (JMY), originally identified as a p53 coactivator, plays prominent roles in the DNA damage response and in cell motility. This study aimed to discover the role of JMY in the pathogenesis of GC-induced endothelial cell lesions.

Methods: High-throughput RNA sequencing was performed to identify the differentially expressed genes between GC-treated human umbilical vein endothelial cells (HUVEC) and control cells. JMY knockdown and overexpressing HUVEC lines were treated with GC. Cell proliferation was examined with a survival cell count assay (Cell Counting Kit-8, CCK-8); cell apoptosis was measured by flow cytometry; a scarification assay was used to detect the capability of cell migration; a Transwell chamber assay was done to detect the cell motility . Differential expression of cell protein was detected by western blot.

Results: A total of 1561 differential genes were obtained through transcription sequencing, of which 789 mRNA were upregulated and 772 mRNA were downregulated in the GC-treated HUVEC compared with the control cells. CCK-8 assay results showed that: without GC treatment, overexpression or knockdown of JMY did not affect the proliferation activity of HUVEC. In the presence of GC treatment, the proliferation activity of HUVEC in the JMY knockdown group was significantly higher than that in the control group ($P < 0.01$). The proliferation activity of HUVEC in the overexpression JMY group was significantly lower than that in the control group ($P < 0.01$). The results of flow cytometry showed that without GC treatment, overexpression or knockdown of JMY did not affect the apoptosis proportion of HUVEC. With GC treatment, the apoptosis proportion of HUVEC in the JMY knockdown group was significantly lower than that in the control group ($P < 0.01$), and the apoptosis proportion of HUVEC in the overexpression JMY group was significantly higher than that in the control group ($P < 0.01$). Western blot results showed that with GC treatment, the JMY expression level of HUVEC increased with the reaction time. Moreover, the distribution of JMY was mainly concentrated in the nucleus. The expression level of Bax also increased with the reaction time. With GC treatment, overexpression of JMY could significantly increase the expression of Bax in HUVEC. JMY knockdown could reduce the expression of Bax in HUVEC. In the absence of GC treatment, HUVEC overexpression or knockdown of JMY did not affect the expression of Bax. The results of scarification and Transwell chamber assays showed that: without GC treatment, JMY knockdown could significantly decrease the cell motility and increase the expression level of VE-cadherin in HUVEC; with GC treatment, JMY knockdown in HUVEC had lower cell motility compared with the control group ($P < 0.01$).

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Conclusion: Glucocorticoids can induce the HUVEC apoptosis, and reduce its proliferation, cell motility. Our results mainly confirmed the role of JMY in the pathogenesis of GC-inducing lesions in HUVEC. GC act on HUVEC, inducing cell damage. Following the event of cell damage, JMY levels upregulate in the nucleus to induce transcription of Bax, triggering apoptosis. JMY can also regulate HUVEC motility *via* its regulation of VE-cadherin levels.

Key words: Endothelial cell; Glucocorticoid; Junction-mediating and regulatory protein (JMY); Osteonecrosis of the femoral head; Pathogenesis

Introduction

Osteonecrosis of the femoral head (ONFH) is a common refractory disease in orthopaedics, which is often caused by the use of high-dose glucocorticoid (GC). To date, the detailed mechanism of ONFH, including apoptosis of osteocytes and osteoblasts, increased intraosseous pressure in bone marrow adipocytes, intraosseous microvascular thrombosis, and injury of endothelial cells, has not been clarified. The hypothesis of blood circulation assumes that coagulation, fibrinolysis, and endothelial cell dysfunction are the key factors that promote microcirculation dysfunction in bone, which is one of the initial steps for the occurrence and development of ONFH[1–3].

As early as 1934, Phemister *et al.* proposed that thrombosis and embolism caused by abnormal microvascular function are important reasons for the occurrence and development of ONFH[4]. Chandler *et al.* (2001) first compared the ONFH with myocardial infarction, and proposed that the femoral head and the heart have similar blood supply structure, with tortuous nutrient vessels in various regions and limited anastomotic branches. The degree of degeneration and the effectiveness of repair depend on the degree of microcirculatory vascular injury and the establishment of sufficient collateral circulation[5]. The blood supply of the femoral head is affected by many endogenous and exogenous factors. It is generally believed that all these factors lead to focal thrombosis and ONFH through various channels and mechanisms[6]. The histological study of Hirano *et al.* showed that in the early stage of necrosis of the femoral head, there was severe lumen stenosis in the return vein lumen of the femoral head and part of the femur[7].

Research shows that the endothelial cell is not only a simple semi-permeable membrane barrier between blood and vascular smooth muscle but also a highly active endocrine organ tissue, which regulates the local physiological function of blood vessels by releasing vasoconstrictor and vasodilator substances[8]. Endothelial cells are also targets of local regulatory factors, especially the interaction with steroid hormones that are directly exposed to blood circulation. Endothelial cells and their secreted products play a regulatory role in the bone microenvironment and metabolism. This is reflected in neovascularization being a prerequisite for osteogenesis and the close interaction between the vascular system and osteoblasts and osteoclasts during embryonic development and growth[9]. Several kinds of cells, such as endothelial cells, osteoblasts, and osteoclasts, exist in an intraosseous microenvironment composed of a complex regulatory network, in which vascular endothelial (VE) cells play a substantial role[8,9]. Clinical studies have shown that in the absence of thrombosis, ONFH is associated with endothelial cell dysfunction[10]. Therefore, damage to VE cells may be the initial step and the key factor in ONFH and may also be involved in the whole process of ONFH. Studies have indicated that numerous diseases related to the imbalance of osteogenesis and osteoclasts may be connected with metabolic disorders of the intraosseous microenvironment as a result of damaged VE cells[8,10].

Glucocorticoids are endogenous stress hormones that have been therapeutically exploited for more than six decades. GC are often used at dosages that exceed the physiological levels[11]. Studies have shown that GC and their metabolites inhibit capillary growth and regeneration, and promote capillary degeneration. Tetrahydrocortisone, a metabolite of cortisone, is the most representative steroid vascular growth inhibitor in nature. The mechanism of action of these steroids is based on their ability to specifically change the basement membrane conversion in capillary growth[12]. A growing amount of evidence indicates that GC regulate multiple aspects of endothelial physiology[13]. In the past decade, scientists have focused on the relationship between GC-induced ONFH and endothelial cell dysfunction because it has been demonstrated that the use of high-dose GC can induce endothelial cell apoptosis and decrease cell motility[14–16]. It has been reported that the dysfunction of endothelial cells caused by high-dose GC is related to oxidative stress response; oxidative stress can damage VE cells of arterioles, and excessive oxidative stress may lead to ischemic osteonecrosis[17,18]. Kuribayashi *et al.* point out that vitamin E and other antioxidants can reduce the damage from GC to VE cells by reducing the oxidative stress response, and can reduce the occurrence of osteonecrosis in animal models[19]. Vogt *et al.* injected Wistar rats with dexamethasone at a concentration of 0.5 mg/kg for 5 days. The number of necrotic endothelial cells in mesenteric arterioles and venules in the GC-treatment group was significantly higher than that in the control group. Confocal analysis showed that the nuclear fragmentation and nuclear volume of dead endothelial cells were consistent with the process of apoptosis[12]. However, the mechanism of endothelial cell dysfunction caused by high-dose GC is still unclear.

Junction-mediating and regulatory protein (JMY) was originally identified as a CBP/p300 co-factor that regulates

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p53 activity. When DNA damage occurs, JMY interacts with p300 and STRAP to form a complex and recruits PRMT5 into the coactivator complex, triggering the p53 response[20–23]. JMY also prominently influences cell motility by affecting actin nucleation and cell adhesion[24,25]. To our knowledge, the role of JMY in the pathogenesis of GC-induced endothelial cell lesions has not been reported. Therefore, the aim of the present study was to explore: (i) the effects of GC on the phenotype and the function of HUVEC; (ii) the differentially expressed genes of HUVEC under the treatment of GC; and (iii) the role of JMY in GC-induced dysfunction of HUVEC.

Materials and Methods

Cell Culture and Treatment

Human umbilical vein endothelial cells were purchased from OBiO Technology. (Shanghai, China). The cell lines were cultured in DMEM supplemented with 10% FBS, 100 units/mL of penicillin, and 100 μg/mL streptomycin (Thermo, USA) at 37 °C in a humidified atmosphere of 5% CO_2. To assess the effects of GC, HUVEC were incubated in the presence or absence of 1 mg/mL hydrocortisone.

mRNA Library Construction and Sequencing

Total RNA from control cells and GC-treated (hydrocortisone, 1 mg/mL) HUVEC was isolated using TRIzol Reagent and treated with DNase I (37°C, 1 h, followed by heat inactivation). The concentration and purity of the total RNA were analyzed using a Bioanalyzer 2100 and the RNA 6000 Nano LabChip Kit (Agilent Technologies, USA). Next, 10 μg of total RNA was mixed with poly-T oligo-attached magnetic beads (Invitrogen, USA) to extract poly(A) mRNA. Following mRNA capture, the mRNA was fragmented into small pieces with fragmentation buffer. The cleaved RNA fragments were reverse-transcribed (with random hexamers primer) to construct a cDNA library using the mRNA-Seq Sample Prep Kit (Illumina, USA). Then, the cDNA library was generated by paired-end sequencing on the Illumina HiSeq 4000 sequence platform (LC Sciences, USA) following the manufacturer's instructions.

Construction of Lentivirus Overexpressing Junction-Mediating and Regulatory Protein

The gene sequence of JMY was chosen according to the NCBI (Accession No. NM_152405). The lentiviral vector pLenti-CMV-MCS-3FLAG-PGK-Puro and the EcoR I/BamHI restriction endonuclease were purchased from OBiO Technology. The human JMY gene was cloned from the human genome with the following primers: JMY forward primer, 5′-CGCAAATGGGCGGTAGGCGTG-3′ and JMY reverse primer, 5′-CAGCGGGGCTGCTAAAGCGCATGC-3′. The lentivirus vector plasmid pLenti-CMV-MCS-3FLAG-PGK-EGFP (OBiO Technology, China) and the polymerase chain reaction (PCR) product of the JMY gene were digested by EcoRI and BamHI restriction enzymes (Thermo, USA) and

then ligated by T4 DNA ligase (Thermo, USA). The resulting DNA product was transformed into the E. coli Stbl3 strain (New England Biolabs, USA). A recombinant lentiviral vector was detected by colony PCR and confirmed by Sanger sequencing. ThepLenti-CMV-JMY-3FLAG-PGK-Puro plasmid was cotransfected with two packaging plasmids (pSPAX2 and pMD2.GpHelp 1.0 and pHelp 2.0) into HEK 293 cells to produce the recombinant lentivirus expressing JMY. Negative control containing empty vector was used as a lentivirus control group.

Human umbilical vein endothelial cells were infected with a multiplicity of infection (MOI) of 40 at 80% confluence. Subsequently, the expression of enhanced green fluorescent protein (EGFP) was observed using a fluorescence microscope at magnification of 200× (IX71; Olympus, Tokyo, Japan).

Construction of A Lentiviral Vector Expressing Junction-Mediating and Regulatory Protein-Specific shRNA

Three different JMY-specific target sequences were chosen based on the siRNA tools provided by Invitrogen (http://www.invitrogen.com/rnai). Double-stranded DNA containing the interference sequences was synthesized and then inserted into a linearized pLKD-CMV-Puro-U6-shRNA viral vector (OBiO Technology, Shanghai, China). All three constructs were confirmed by Sanger sequencing and then noted as Y8976, Y8977, and Y8978. The three lentiviral vectors were separately transfected into HEK 293 cells to evaluate the knockdown effects. The effects of Y8978 were found to be the most significant. Thus, the Y8978 vector was transfected into HEK 293 cells with psPAX2 and pMD2.G at a ratio of 4:3:1 to produce the lentivirus. The lentivirus (LV) produced with Y8978 was denoted as Ri-JMY, and the empty vector was also packaged and used as a negative control (noted as NC). Viral supernatant was harvested 48 h after transfection, filtered through a 0.45-μm polyvinylidene fluoride (PVDF) filter (Cat# SLHV033RB, Millipore, USA), and frozen at −80°C. Viral concentrations were determined by serial dilution of the concentrated vector stocks in HEK 293 cells in 96-well plates.

Human umbilical vein endothelial cells were transduced with shRNA-expressing lentivirus at a MOI of 40 particles/cell in serum-free growth medium and selected with 2 μg/mL puromycin.

Quantitative Reverse Transcription Polymerase Chain Reaction

Four differentially expressed mRNA, including JMY, P53, Mdm2, and P300, were selected to assess the accuracy of quantitative reverse transcription PCR (RT-qPCR) using SYBR Green fluorescence. The RT-qPCR was repeated three times for each sample. The relative abundance of each gene was calculated with the comparative C_T ($\Delta\Delta C_T$) method.

Western Blotting

Samples were collected and then lysed by using RIPA buffer (Beyotime, China). Denatured protein samples (20 μg per lane) were separated by using a 10% SDS-PAGE gel and then transferred to PVDF membranes (EMD Millipore, USA). The following primary antibodies were used: JMY (1:500, ab190212; Abcam, USA), P53 (1:1000, ab32389; Abcam, USA), Bax (1:500, ab53154; Abcam, USA), VE-cadherin (VE-cad) (1:5000, ab33168; Abcam, USA), and actin (1:3000, ab8226; Abcam, USA). Horseradish peroxidase-linked secondary antibodies were used and blots were visualized with the Clarity Western ECL Substrate Kit (Bio-Rad, USA).

Survival Cell Count Assay (Cell Counting Kit-8)

Proliferation activity was measured using the Cell Counting Kit-8 (CCK-8) (Cat#ab228554, Abcam, USA). HUVEC were plated in 96-well plates (3×10^3 cells/well) and treated with GC (1 mg/mL) for 48 h. Next, 100-μL culture medium with 10 μL WST-8 was added and incubated in a 37 °C, 5% CO_2 incubator. The absorbance results were then measured at 460 nm using a multifunctional microplate reader (ELX800, BioTeK, USA). The absorbance of the blank wells with only growth medium was subtracted from the values for the wells with cells.

Flow Cytometry Analysis

After GC treatment, the cells were washed with phosphate-buffered saline (PBS) solution and then resuspended in FACS buffer. The stained cells were analyzed using a flow cytometry staining buffer (BD Biosciences, USA). These assays were performed with an apoptosis kit (eBioscience, USA) according to the manufacturer's instructions.

Transwell Chamber Assay

To determine the effects of JMY and GC on the motility of endothelial cells, a migration chamber of 24 Transwells (Cat#3422, Costar, USA) with an 8 μm pore was used. HUVEC were pretreated with FBS-free medium in the presence or absence of GC for 24 h. Then, 5×10^4 cells were resuspended with 200-μL FBS-free medium and plated on the top chamber, and 500 μL medium containing FBS was added into the bottom chamber to allow migration. After incubation at 37 °C, 5% CO_2 for 24 h, cells on the top surface of the membrane were scraped with a cotton swab, while cells on the bottom surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet dye (Cat# ab143095, Abcam, USA) for 30 min. Next, 33% ethylic acid was applied to extract the violet staining and we measured its absorbance at 570 nm.

Scarification Assay

Human umbilical vein endothelial cells were cultured in 12-well plates until confluency in the appropriate medium and then washed twice with PBS. After wounding the cells, 3 mL of medium with or without GC was added. After 24 h, the distances that the cells migrated were measured and expressed as migratory velocity (μm/h) using ImageJ software. At least three independent experiments were performed.

Cell Grouping Method

Definitions: Con, blank control group; NC, empty vector control group; JMY, JMY overexpression group; Con + GC, GC treatment control group; NC + GC, empty vector with GC treatment group; JMY + GC, JMY overexpression with GC treatment group; Ri-JMY, JMY knockdown group; Ri-JMY + GC, JMY knockdown with GC treatment group.

Statistical Analysis

All *in vitro* experiments were repeated at least three times. The final data are reported as means ± standard deviation and were analyzed using SPSS 19.0 statistical software (SPSS, USA). Statistical analyses were performed by one-way analysis of variance (ANOVA) or Student *t*-test. A *P*-value of <0.05 was considered statistically significant.

Results

Glucocorticoid Treatment Caused Transcriptome Changes in Human Umbilical Vein Endothelial Cells

The mRNA expression profiles of HUVEC in the control group and GC-treated group were determined by Illumina HiSeq 4000. A fold-change of >2 and a *P*-value of <0.05 were used as the threshold for identifying the significant differentially expressed mRNA. From the sequencing and analysis results, we found that compared to the control group, 789 genes were upregulated and 772 genes were downregulated in the GC-treated group (Fig. 1A). We also performed Gene Ontology (GO) terms analysis of RNA-Seq data to distinguish the classifications of the changed genes, and the results indicated that genes related to apoptotic processes were markedly enriched (Fig. 1B). In addition to GO term analysis, we still performed GO functional analysis (Fig. 1C), which showed that membrane proteins and binding proteins were distinctly affected.

To confirm the sequencing results, four genes (JMY, P53, Mdm2, and P300) that may be associated with the GC treatment were chosen for validation by RT-qPCR. The quantitative results confirmed that the altered expression of these four mRNA observed with RT-qPCR was consistent with that observed with RNA-Seq (Fig. 1D). Both JMY and P53 were upregulated, and Mdm2 was downregulated. In addition, the expression level of P300 showed no significant change, which was consistent with the sequencing results.

Effects of Glucocorticoids on Human Umbilical Vein Endothelial Cell Function

The CCK-8 assay results showed that GC treatment had a time-dependent growth inhibition effect in HUVEC. The absorbance values of GC-treated and blank control groups were 0.236 ± 0.004 *vs* 0.358 ± 0.006 and 0.38 ± 0.008 *vs* 0.686 ± 0.005 (*P* < 0.01) at 24 h and 48 h, respectively

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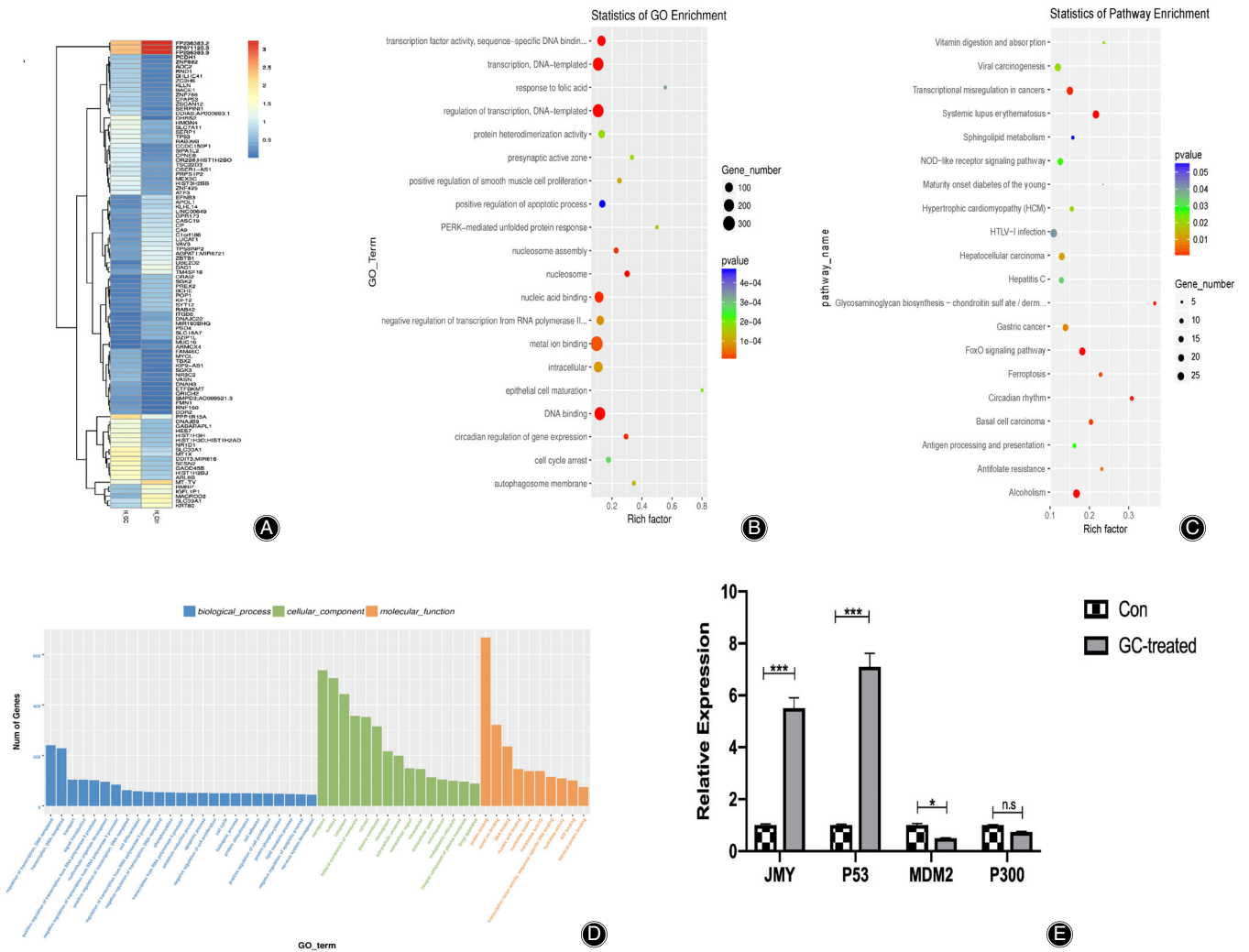
Fig. 1 The differentially expressed genes between the control and glucocorticoid (GC)-treated human umbilical vein endothelial cell (HUVEC) groups. (A) Clustering analysis of differentially expressed genes by RNA-Seq. A dendrogram of correlation between genes is shown to the left of the heat map. Each column represents an experimental sample, and each row represents a gene. Expression differences are shown in different colors. Red indicates high expression and blue indicates low expression. (B) Statistics of Gene Ontology (GO) enrichment analysis. The colorful circle indicates the gene number. The color indicates the P-value. (C) Statistics of Kyoto Encyclopedia of Genes and Genomes (KEEG) pathway enrichment analysis. The colorful circle indicates the gene number. The color indicates the P-value. (D) Function classifications of Gene Ontology (GO) terms of all Muscovy duck transcripts. The results are summarized in three main categories: biological process, cellular component, and molecular function. The X-axis indicates the subcategories, and the Y-axis indicates the number of genes in the same category. (F) Real-time polymerase chain reaction was performed to analyze the mRNA levels of JMY, P53, MDM2, and P300 in the control and GC-treated group. Data are presented as $2^{(-\Delta\Delta CT)}$.

(Fig. 2A). The results of flow cytometry showed that GC could induce HUVEC apoptosis, and the proportions of apoptotic cells in GC-treated and blank control groups were 31.33% ± 5.17% *vs* 8.91% ± 2% (P < 0.01) (Fig. 2B). Meanwhile, GC treatment significantly reduced cell motility. Scarification assay results show that the healing rates of scratches in GC-treated and blank control groups were 18.29% ± 1.32% *vs* 38.22% ± 0.57% (P < 0.01) (Fig. 2C). The results of the Transwell chamber assay showed that the number of

metastatic cells in GC-treated and blank control groups were 46.67 ± 3.21 and 125.67 ± 8.33 (P < 0.01) (Fig. 2D).

Glucocorticoid Treatment Regulates Junction-Mediating and Regulatory Protein and Related Proteins

We also observed a concomitant increase in JMY at the protein level when HUVEC were treated with GC (Fig. 3A). In consideration of the distribution of JMY, we also checked the protein level of JMY in its main subcellular locations

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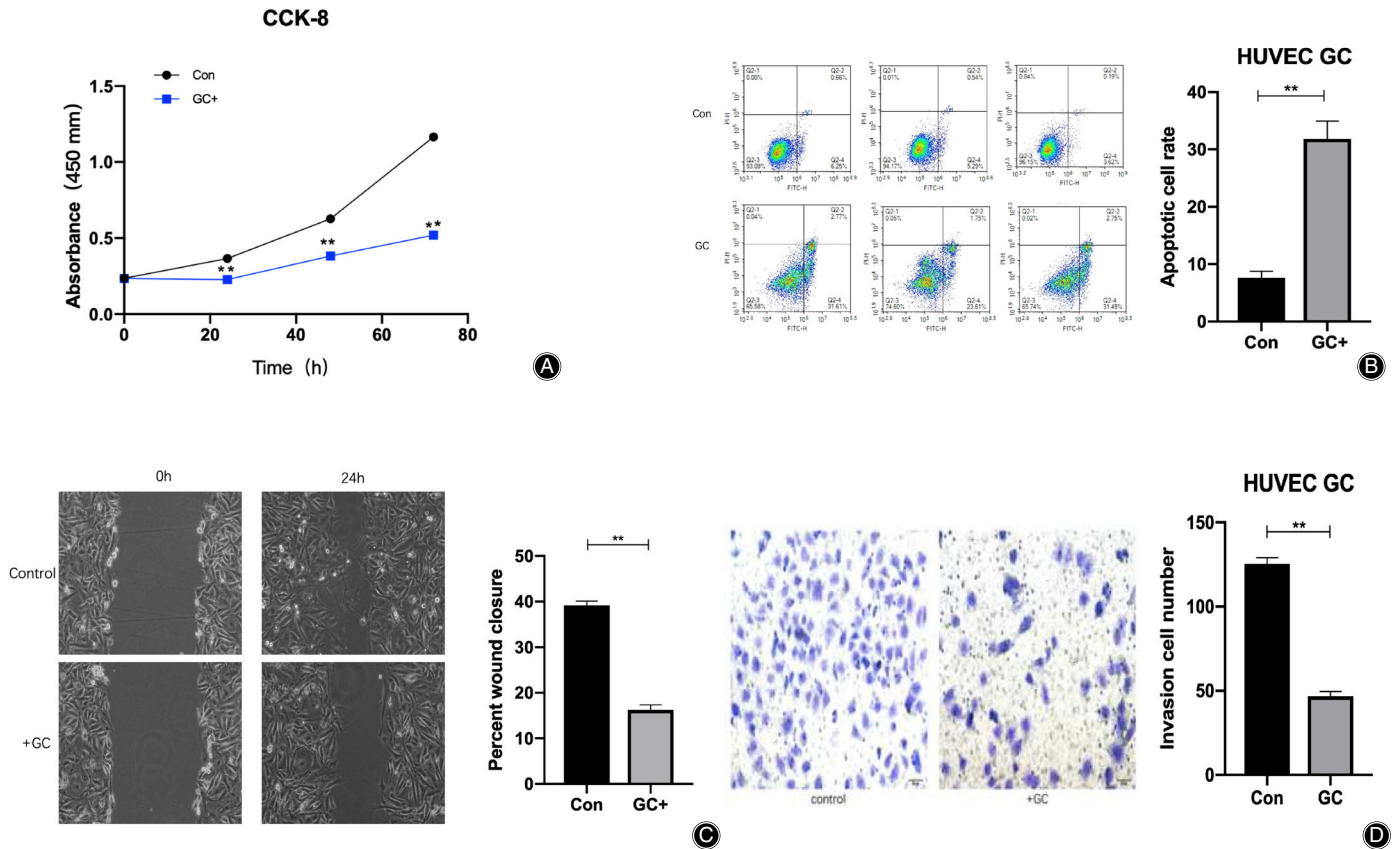
Fig. 2 Effects of glucocorticoids (GC) on human umbilical vein endothelial cell (HUVEC) phenotype function. * indicates $P < 0.05$, ** indicates $P < 0.01$, and n.s indicates no significance. (A) Detection of cell proliferative activity using the Cell Counting Kit-8 in the control and GC-treated group. The proliferation activity of HUVEC in the GC-treated group decreased significantly. (B) Detection of apoptosis by flow cytometry analysis in the control and GC-treated groups. The apoptotic cell proportion of HUVEC in the GC-treated group increased significantly. (C) Detection of cell migration by scarification assay in the control and GC-treated groups. The migration ability of HUVEC in the GC-treated group decreased significantly. (D) Detection of cell motility by Transwell chamber assay in the control and GC-treated groups. The cell motility of HUVEC decreased significantly in the GC-treated group.

(cytoplasm and nucleus). The results of western blotting detection demonstrated that JMY was mainly distributed in the cytoplasm without GC treatment and concentrated in the nucleus with GC treatment (Fig. 3B). In addition to changes in JMY, GC treatment also caused a concomitant increase in Bax at the protein level (Fig. 3C,D). GC treatment also increased the protein level of VE-cad, which suggests that GC treatment may regulate the cell–cell adhesions and motility of HUVEC (Fig. 3E).

Role of Junction-Mediating and Regulatory Protein in Glucocorticoid-Induced Human Umbilical Vein Endothelial Cell Apoptosis

There was no significant difference between blank control and empty vector control groups in HUVEC proliferation activity and apoptotic cell proportion ($P > 0.05$) (Fig. 4A–D). The results showed that lentivirus vectors did not affect the proliferation and apoptosis of HUVEC. Without GC treatment, overexpression or knockdown of JMY will neither affect

the cell proliferative activity nor the apoptosis. Without GC treatment, the absorbance values of the JMY overexpression and blank control groups were 0.356 ± 0.003 vs 0.361 ± 0.004 and 0.551 ± 0.006 vs 0.552 ± 0.007 ($P > 0.05$) at 24 and 48 h, respectively (Fig. 4B). Without GC treatment, the absorbance values of the JMY knockdown and blank control groups were 0.536 ± 0.005 vs 0.544 ± 0.004 and 1.163 ± 0.047 vs 1.184 ± 0.043 ($P > 0.05$) at 24 and 48 h, respectively (Fig. 4A). Without GC treatment, the proportion of apoptotic cells in the JMY overexpression and blank groups were $6.7\% \pm 0.55\%$ vs $7.08\% \pm 1.85\%$ ($P > 0.05$) (Fig. 4D).

The proportions of apoptotic cells in the JMY knockdown group and the blank control group were: $7.79\% \pm 1.16\%$ vs $6.56\% \pm 1.31\%$ ($P > 0.05$) (Fig. 4C). With GC treatment, overexpression of JMY can significantly inhibit the proliferation of HUVEC and increase the proportion of apoptotic cells. Knockdown of JMY can alleviate the proliferation inhibition of HUVEC and reduce the proportion of apoptotic cells. With GC treatment, the absorbance values of the JMY
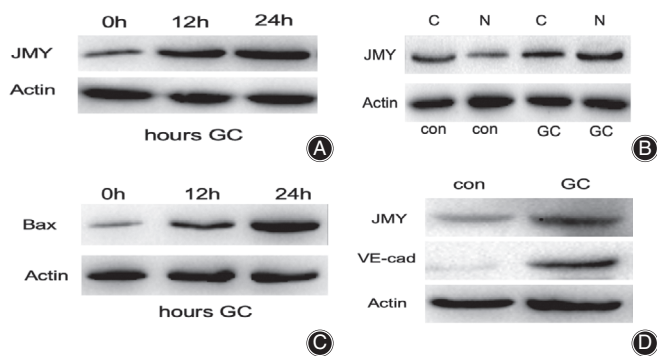
Fig. 3 Glucocorticoids (GC) regulate the protein levels of junction-mediating and regulatory protein (JMY), Bax, and vascular endothelial (VE)-cadherin in human umbilical vein endothelial cells (HUVEC). (A) Western blot analysis of JMY in GC-treated HUVEC. The expression of JMY in GC-treated HUVEC increased significantly. (B) HUVEC were subjected to fractionation to obtain nuclear and cytoplasmic extracts with or without GC treatment. C, cytoplasmic; N, nuclear. High expression of JMY was concentrated in the nucleus. (C) Western blot analysis of Bax in GC-treated HUVEC. Cell extracts were collected at the indicated time points. The expression of Bax in GC-treated HUVEC increased significantly. (D) Western blot analysis of JMY and VE-cadherin levels in control and GC-treated HUVEC. The expression of JMY and VE-cadherin in GC-treated HUVEC increased significantly.

overexpression group and the GC treatment control group were 0.180 ± 0.004 vs 0.227 ± 0.004 and 0.192 ± 0.009 vs 0.378 ± 0.005 ($P < 0.01$) at 24 and 48 h, respectively (Fig. 4B); the absorbance values of the JMY knockdown group and the GC treatment control group were 0.367 ± 0.012 vs 0.238 ± 0.005 and 0.531 ± 0.004 vs 0.377 ± 0.005 ($P < 0.01$) at 24 and 48 h, respectively (Fig. 4A).

With GC treatment, the proportion of apoptotic cells in the overexpression group and GC treatment control group were: $49.2\% \pm 1.67\%$ vs $31.82\% \pm 3.12\%$ ($P < 0.01$) (Fig. 4D). The proportion of apoptotic cells in the JMY knockdown group and the GC treatment control group were $17.04\% \pm 1.78\%$ vs $30.88\% \pm 2.11\%$ ($P < 0.01$, with significant difference) (Fig. 4D). When Bax expression increases with the treatment of GC, knockdown of JMY can significantly reduce the Bax expression in HUVEC induced by GC, and the overexpression of JMY can increase the Bax expression in endothelial cells induced by GC (Fig. 4E,F).

Role of Junction-Mediating and Regulatory Protein in Glucocorticoid-Induced Human Umbilical Vein Endothelial Cell Motility Reduction

There was no significant difference in HUVEC motility between blank control and empty vector control groups ($P > 0.05$, no statistical difference) (Fig. 5A–D). The results showed that lentivirus vectors did not affect the cell motility of HUVEC. Without GC treatment, the percentage of scratch healing and the number of metastatic cells in the JMY

knockdown group and blank control group were, respectively: $21.21\% \pm 2.05\%$ vs $36.84\% \pm 0.44\%$ and 70.33 ± 1.53 vs 123.67 ± 5.51 ($P < 0.01$) (Fig. 5A,C). The results showed that without GC treatment, knockdown of JMY in HUVEC can significantly reduce the cell motility and increase the expression of VE-cad (Fig. 5E). With GC treatment, the healing ratio and the number of invasive cells of HUVEC in the JMY knockdown and GC treatment control groups were: $11.55\% \pm 1.48\%$ vs $17.92\% \pm 1.33\%$ and 31 ± 2.65 vs 51 ± 5 ($P < 0.01$). The results showed that with GC treatment, knockdown of JMY can further reduce the cell motility and the expression of VE-cad in HUVEC (Fig. 5E). Under the circumstances with GC treatment, the overexpression of JMY can neither reduce the expression of VE-cad in cells nor prevent the cell motility reduction induced by GC ($P > 0.05$) (Fig. 5F).

Discussion

To date, numerous studies have shown that the use of high-dose GC can significantly affect endothelial cells by inducing apoptosis and inhibiting cell motility[14–16]. Our experimental results proved this point as well. However, the specific mechanism of endothelial cell function induced by high-dose GC has not yet been clearly reported. In this study, we compared the differentially expressed genes between the GC-treatment group and the control group by using high-throughput RNA sequencing. Then the function annotation and enrichment analysis of the differential genes were carried out for each database. According to the existing reports and TCGA database analysis, we selected the JMY gene related to apoptosis and cell motility.

Role of Junction-Mediating and Regulatory Protein in Glucocorticoid-Induced Human Umbilical Vein Endothelial Cell Apoptosis

Junction-mediating and regulatory protein was originally identified as a p53 coactivator, which localizes to the nucleus during DNA damage[20–22]. When HUVEC is treated with GC, JMY is upregulated at the protein level, and our study also showed that the high expression of JMY at this time is mainly related to the accumulation of JMY in the nucleus. In the nucleus, the majority of the JMY is sequestrated by the Cp300/strap/p53 complex[22–24,26,27]. Under the circumstances without GC treatment, overexpression or knockdown of JMY will neither affect the cell proliferative activity and the apoptosis nor the Bax expression in HUVEC. Similar to the results of Coutts et al., JMY is found to be a damage responsive protein[21]. However, under the circumstances with GC treatment, overexpression of JMY can increase the proportion of apoptotic cells, while knockdown of JMY can reduce the proportion of apoptotic cells in HUVEC induced by GC. When Bax expression increases with the treatment of GC, knockdown of JMY can significantly reduce the Bax expression in HUVEC induced by GC, and the overexpression of JMY can increase the Bax expression in HUVEC induced by GC. Combined with the results of previous experiments, it can be suggested that the increase of
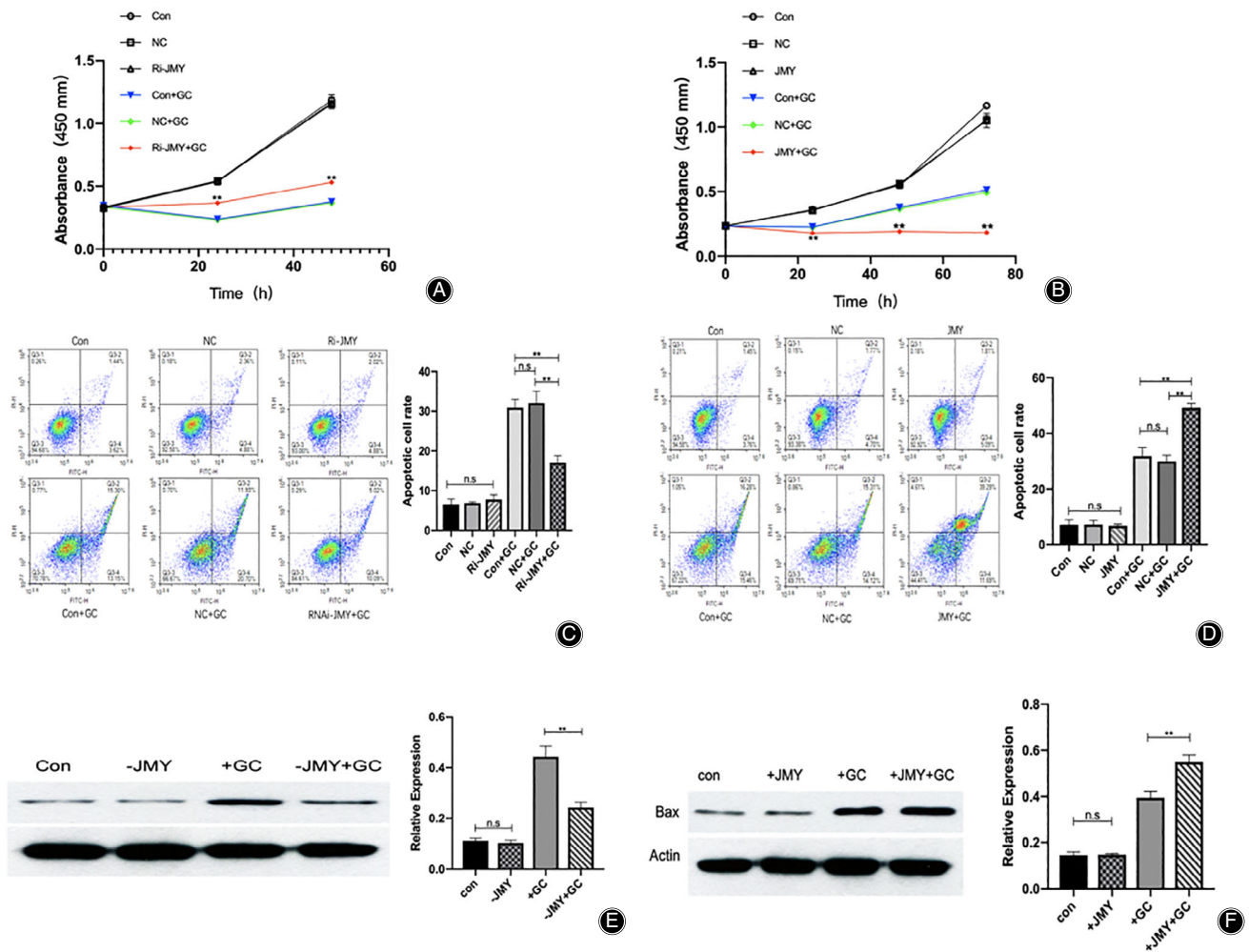
Fig. 4 The role of junction-mediating and regulatory protein (JMY) in glucocorticoids (GC)-induced human umbilical vein endothelial cell (HUVEC) apoptosis. * indicates $P < 0.05$, ** indicates $P < 0.01$, and n.s indicates no significance. (A and B) Analysis of cell proliferative activity in each group. With GC-treatment, the Ri-JMY + GC group had higher proliferation activity and JMY + GC group had lower proliferation activity. (C and D) Analysis of cell apoptosis by flow cytometry in each group. With GC-treatment, the Ri-JMY + GC group had a lower apoptosis ratio, and the JMY + GC group had a higher apoptosis ratio. (E and F) Western blot analysis of Bax in each group. With GC treatment, overexpression of JMY can increase the expression of Bax, and knockdown of JMY can reduce the expression of Bax.

JMY during the cell damage response caused by GC can further induce the proapoptotic pathway by upregulating the apoptotic protein Bax.
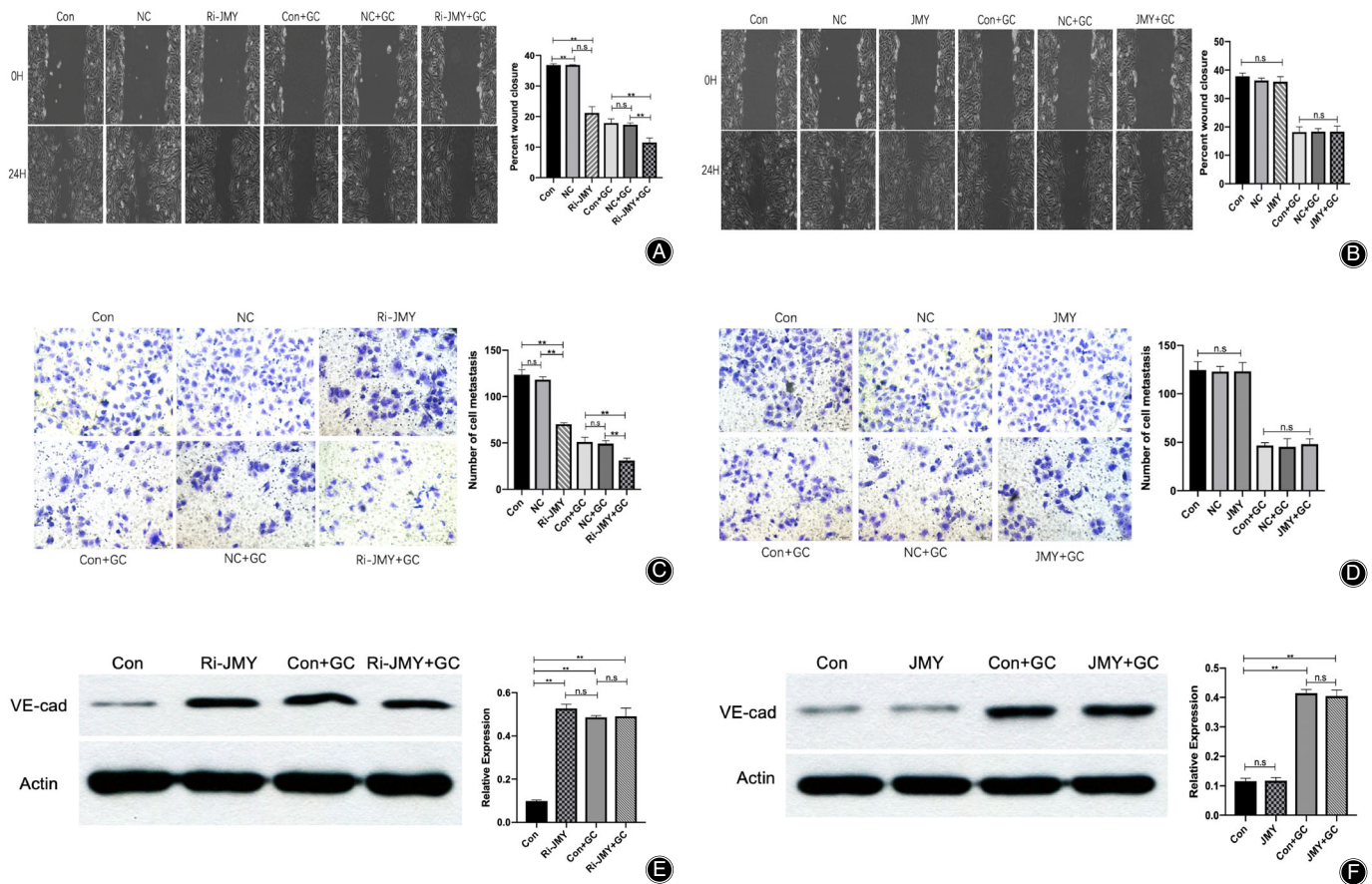
Role of Junction-Mediating and Regulatory Protein in the Regulation of Cell Motility of Human Umbilical Vein Endothelial Cells

Under the circumstances without GC treatment, knockdown of JMY in HUVEC separately can significantly reduce the cell motility and increase the expression of VE-cad. VE-cad is the main component of the endothelial cell adhesion junction and the most basic endothelial cell-specific cadherin involved in the formation and regulation of the endothelial cell junction. In addition to maintaining the integrity of endothelial cells and regulating the integrity of junction sites, VE-cad can regulate a variety of signal transduction processes to affect the behavior of endothelial cells and the expression of other junction components (such as proliferation, migration, invasion, and vascular lumen formation), participate in the regulation of vascular permeability, and participate in tumor growth and metastasis[28,29]. We reasoned that the upregulation of VE-cad might be involved in the ability of JMY to influence cell motility of HUVEC. However, when HUVEC is treated with GC, the expression of JMY increases, while the expression of VE-cad increases and cell motility decreases.

The research of Coutts *et al.* reveals a possible explanation for why JMY expression increases under the stimulus of stress reaction, and JMY plays its role through the transfer

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Fig. 5 The role of junction-mediating and regulatory protein (JMY) in glucocorticoid (GC)-induced human umbilical vein endothelial cell (HUVEC) motility. * indicates $P < 0.05$, ** indicates $P < 0.01$, and n.s indicates no significance. (A and B) Scarification assay in each group. With GC-treatment, the Ri-JMY + GC group had lower migration ability. Without GC-treatment, the Ri-JMY group had lower migration ability. (C and D) Transwell chamber assay in each group. With GC treatment, the Ri-JMY + GC group had lower cell motility . Without GC treatment, the Ri-JMY group had lower cell motility. (E and F) Western blot analysis of VE-cadherin in each group. Without GC treatment, the expression level of VE-cadherin in HUVEC can be significantly reduced by knocking out JMY.

from cytoplasm to nucleus, so that the JMY in cytoplasm decreases, leading to the increase of VE-cad expression and the decrease of cell motility[22]. Meanwhile, the research of Adighibe *et al.* suggests that the JMY content in cytoplasm is the major factor affecting the adhesion expression[30]. In the presence of GC treatment, knockdown of JMY can further reduce the expression of VE-cad in cells as well as the cell motility.

Conclusion
In conclusion, our results mainly confirmed the role of JMY in the pathogenesis of GC-inducing lesions in HUVEC. GC act on HUVEC, inducing cell damage. Following the event of cell damage, JMY levels upregulate in the nucleus where JMY, as a cofactor of P53, increases the ability of p53 to induce transcription of Bax triggering apoptosis. JMY can also regulate HUVEC motility *via* its regulation of VE-cad levels.

Limitations
According to the established experimental cell model, we carried out high-throughput transcriptome sequencing and selected one of many differentially expressed genes for related research. Therefore, we cannot fully and comprehensively present the complex mechanism of cellular pathophysiological changes. The selected research direction reveals part of the mechanism of GC-induced HUVEC dysfunction. The main work of this study is cell phenotype function, however the molecular mechanisms are not thoroughly explored. For this study, we preliminarily explored the role of JMY in GC-induced HUVEC dysfunction. However, a series of physiological and biochemical processes related to the expression of JMY and the expression of upstream and downstream proteins are still worthy of further exploration. In addition, the results of *in vitro* cell experiments need to be verified in animal experiments.

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