LncRNA Meg3 promotes oxygen and glucose deprivation injury by decreasing angiogenesis in hBMECs by targeting the miR-122-5p/NDRG3 axis

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Abstract. Oxygen-glucose deprivation (OGD) is widely used as an *in vitro* model for stroke. The present study aimed to explore the mechanisms of action of long non-coding RNA (lncRNA) maternally expressed gene 3 (Meg3) in angiogenesis following OGD. The human brain microvascular endothelial cell line, hCMEC/D3, was used to establish the OGD model. lncRNA Meg3 was highly expressed in hCMEC/D3 cells subjected to OGD. Furthermore, it was found that the overexpression of lncRNA Meg3 decreased the proliferation, migration and angiogenesis of hCMEC/D3 cells subjected to OGD, and increased cell apoptosis. Meg3 silencing exerted the opposite effects. Subsequently, lncRNA Meg3 increased the expression of NDRG family member 3 (NDRG3) by directly binding to miR-122-5p. The overexpression of miR-122-5p and the knockdown of NDRG3 reversed the inhibitory effects of Meg3 overexpression on the proliferation, migration and angiogenesis of hCMEC/D3 cells subjected to OGD, as well as the promoting effects of Meg3 overexpression on cell apoptosis. The present study demonstrated that lncRNA Meg3 functions as a competing endogenous RNA by targeting the miR-122-5p/NDRG3 axis in regulating OGD injury.

Introduction

Ischemic stroke (IS) is caused by the occlusion or stenosis of cerebral arteries, which in turn leads to the insufficient blood supply to the brain, resulting in the necrosis of brain tissue (1). At present, the clinical treatment of IS focuses on the reperfusion therapy of ischemic tissue. Recombinant tissue plasminogen activator (r-tPA) is the only thrombolytic drug approved by the US FDA for ultra-early cerebral infarction. However, due to the narrow treatment time window (<6 h), <10% of patients benefit from r-tPA treatment (2). It has been shown that the mortality and prognosis of patients with IS are closely related to the formation of new blood vessels in the ischemic area following IS (3). The effective promotion of angiogenesis following IS can directly restore the oxygen and nutrient supply to damaged brain tissue (4), ultimately leading to a reduced infarct volume, and promoting nerve function reconstruction (5). Thus, regulating angiogenesis in the ischemic area is one of the most promising strategies for the treatment of IS.

Long non-coding RNAS (lncRNAs) are a type of non-coding RNA that are commonly transcribed in the genome and are >200 nucleotides in length. A previous study showed that lncRNAs are critical regulators of various biological processes via several mechanisms, including chromatin looping regulation, epigenetic modification, RNA splicing and the modification of protein complexes (6). Notably, one major biological function of lncRNAs appears to be the sponging of microRNAs (miRNAs/miRs) (7). A number of studies have reported that the angiogenesis process is regulated by a bulk of aberrantly expressed lncRNAs in patients with IS or ischemia-insulted animals, such as HIF1A-AS2 (8), snhg1 (9) and XIST (10). Maternally expressed gene 3 (Meg3) is an imprinted gene on the DLK-Meg3 locus. Its post-transcription product is lncRNA Meg3, which has been reported to be highly expressed in the normal human brain and pituitary (11). As an important tumor suppressor, lncRNA Meg3 is absent in a number of tumors and cancer cells (12,13). Emerging evidence indicates that the down-regulation of Meg3 activates angiogenesis following ischemic brain injury by promoting the Notch signaling pathway (14). However, little is known about the functional role of Meg3 in the angiogenesis of human brain microvascular endothelial cell (hBMECs) injured by oxygen and glucose deprivation (OGD).

The present study investigated the biological functions and potential mechanisms of action of lncRNA Meg3 in hCMEC/D3

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cells subjected to OGD, including the effects of Meg3 on the proliferation, migration, apoptosis and angiogenesis of cells injured by OGD.

Materials and methods

Cell transfection and establishment of OGD model. The immortalized hCMEC/D3 was purchased from the American Type Culture Collection (ATCC). The cells were cultured (37˚C; 5% CO_2) in high-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone; Cytiva) with 10% fetal bovine serum (FBS; HyClone; Cytiva). The cells in the logarithmic growth stage were selected for use in the experiments. The cells subjected to OGD were slightly washed with sugar-free Earle's balanced salt solution (EBSS; MilliporeSigma) medium three times. The cells were then cultured with a sugar-free EBSS and under the conditions of oxygen deprivation (93% N_2, 5% CO_2) for 6, 12, 24 and 48 h, respectively. Normal control cells were cultured in a conventional CO_2 incubator. The transient transfection of pcDNA 3.1 vectors [pcDNA-empty vector (NC)/pcDNA-Meg3; final concentration, 100 nM], miRNA mimics (miR-122-5p mimic/NC mimic; final concentration, 50 nM), or short interfering (si)RNAs [Meg3-siRNA/NDRG family member 3 (NDRG3) NDRG3-siRNA/NC-siRNA; final concentration, 50 nM] was performed using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.) when the cell-density reached 70%. Cell transfection was performed for a duration of 48 h at 37˚C. The cells were examined 48 h post-transfection. miR-122-5p mimic (cat. no. miR10000421-1-5) and NC mimic (cat. no. miR1N0000001-1-5) were purchased from Guangzhou RiboBio Co., Ltd. pcDNA-NC (NR_002766.2), pcDNA-Meg3 (NR_002766.2), Meg3-siRNA, NDRG3-siR NA and NC-siRNA were purchased from Shanghai GenePharma Co., Ltd. The sequence of miR-122-5p mimics was 5'-UGGAGU GUGACAAUGGUGUUUG-3'. The sequence of NC mimics was 5'-UACUGAGAGACAUAAGUUGGUC-3'.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). The cell density was $0.2x10^6/cm^2$ for RNA extractions. TRIzol® (Thermo Fisher Scientific, Inc.) was used to extract total RNA from the hCMEC/D3 cells. Extracted RNA was reverse transcribed into cDNA for miRNA and mRNA detection using the SuperScrip™ IV First-Strand Synthesis system (Invitrogen; Thermo Fisher Scientific, Inc.) and Mir-X miRNA First-Strand Synthesis kit (Takara Bio, Inc.) according to the manufacturer's protocols, respectively. The expression of miR-122-5p, Meg3 and NDRG3 was determined by RT-qPCR using a Mir-X miRNA qRT-PCR TB Green kit or TB Green Fast qPCR mix (Takara Bio, Inc.) according to the manufacturer's protocols, respectively. The sequences of primers were as follows: Meg3 forward, 5'-TCATCCGTCCAC CTCCTTGTCTTC-3' and reverse, 5'-GTCCTCTTCATC CTTTGCCATCCTG-3', miR-122-5p forward, 5'-TGGAGT GTGACAATGGTGT-3' and universal antisense from Mir-X miRNA qRT-PCR TB Green kit (no. 638314; Clontech; Takara Bio, Inc.), NDRG3 forward, 5'-CTGGTGGAAGGTCTTGT-3' and reverse, 5'-GCGCCCATTATAGGAACCCA-3', β-actin forward, 5'-CTCCATCGTCCACCGCAAATGCTTCT-3' and reverse, 5'-GCTCCAACCGACTGCTGTCACCTTC-3', U6, Mir-X miRNA from qRT-PCR TB Green kit (cat. no. 638314; Takara Bio, Inc.). RT-qPCR was performed with a program of 5 min at 95˚C and then 45 cycles at 95˚C (30 sec), 95˚C (5 sec), 55˚C (30 sec), and 72˚C (30 sec). The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression (15). RT-qPCR reactions were performed in triplicates.

Western blot analysis. Total proteins were collected from hCMEC/D3 cell lysates using RIPA buffer (Cell Signaling Technology, Inc.). The protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology). Total protein (30 μg/sample) was separated via 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk powder overnight at 4˚C. The corresponding protein antibodies used were as follows: NDRG3 (cat. no. ab131266, 1:1,000), Bax (cat. no. ab32503, 1:1,000), Bcl-2 (cat. no. ab32124, 1:1,000), VEGFA (cat. no. ab46154, 1:1,000), VEGF receptor 2 (VEGFR2, cat. no. ab134191, 1:1,000) and β-actin (cat. no. ab8227, 1:2,000) (all from Abcam). The incubation conditions for the primary antibodies were 37˚C for 1 h. Subsequently, they were incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (cat. no. 1706515, Bio-Rad Laboratories, 1:2,000) for 30 min at 37˚C. The bands were visualized and quantitated using the ECL system (Thermo Fisher Scientific, Inc.). β-actin is used as an internal control. For densitometry analysis of Western blot data ImageJ 1.48 software (National Institutes of Health) was used.

Luciferase reporter assay. StarBase (https://starbase.sysu.edu.cn/starbase2/index.php) was applied to predict the binding sites between the Meg3 3'-untranslated region (3'-UTR) and miR-122-5p. TargetScan (https://www.targetscan.org/vert_71/) was applied to identify the potential target genes of miR-122-5p. The analyzed data were downloaded from StarBase and TargetScan. A fragment of Meg3 or the NDRG3 3'-UTR, containing the predicted miR-122-5p binding site, was generated by PCR using specific primers. The resulting PCR amplicons were cloned into the pmiR-RB-REPORT TM luciferase reporter plasmid (Shanghai GeneChem Co., Ltd.). 293T cells were purchased from Procell Life Science & Technology Co., Ltd. (cat. no. CL-005). The 293T cells were seeded in 96-well plates at a density of 50-70% cells/well, and transfected with the Meg3-wt, Meg3-mut, NDRG3 3'UTR-wt/mut, together with miR-122-5p/NC mimic using the Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific, Inc.). At 24 h following transfection, the luciferase activities were recorded with a plate-style luminometer using the Promega Dual-Luciferase system (Promega Corporation), with *Renilla* luciferase as normalization.

Cell Counting Kit-8 (CCK-8) assay The viability of the hCMEC/D3 cells was confirmed using a CCK-8 assay. The cultures were supplemented with CCK-8 solution (Dojindo Laboratories, Inc.) and incubated for 1 h at 37˚C. Cell proliferation was measured at a wavelength of 450 nm using a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Inc.).

Tube formation assay. A total of 150 μl dissolved Matrigel matrix adhesive was added to each pre-cooled 24-well plate and

placed at 37˚C for 30 min and allowed to solidify. hCMEC/D3 cells ($4x10^5$) were added to each well and cultured for 12 h in an incubator at 37˚C. Lumen formation was observed under a Leica DMI6000B light microscope (Leica Microsystems, Inc.), and five fields were randomly selected for each group to obtain images.

Wound healing assay. The migration of hCMEC/D3 cells was assessed using a wound healing assay. The cells were seeded into a 6-well plate with serum-free medium. Once the cells reached 90% confluency, a scratch was made across the surface of the well using a sterile 200 μl micropipette tip. The cells were cultured at 37˚C for 24 h, and the distance between the two edges was observed.

Flow cytometric assay. The apoptosis of the hCMEC/D3 cells was analyzed using Annexin V, according to the manufacturer's protocol. Briefly, the cells were washed with PBS (Invitrogen; Thermo Fisher Scientific, Inc.) and the cell concentration was adjusted to $1.0x10^6$ cells/ml. The cells were subsequently suspended in 150 μl buffering solution. Subsequently, the cells were stained with 10 μg/ml Annexin V-FITC (Roche Diagnostics GmbH) and 5 μl PI (Roche Diagnostics GmbH) at 4˚C for 20 min in the dark. Apoptotic cells were then analyzed using a BD FACSCelesta flow cytometer with FACSDiva software v8.0.1.1 (Becton-Dickinson and Company). Apoptotic rate (%)=percentage of early + percentage of late apoptotic cells.

Statistical analysis. The data are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS software (version 19.0; IBM Corp.). One-way analysis of variance with Tukey's post hoc test and the unpaired Student's t-test were used for comparisons between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

lncRNA Meg3 regulates OGD injury in hCMEC/D3 cells subjected to OGD. First, the present study investigated the expression of lncRNA Meg3 in the OGD model using hCMEC/D3 cells. It was found that the expression of lncRNA Meg3 was increased in a time-dependent manner in response to hypoxia and OGD when compared with normal conditions (Fig. 1A). To verify the transfection efficiency, the expression of Meg3 in Meg3 siRNA (si-Meg3) or pcDNA-Meg3-transfected-hCMEC/D3 cells was detected. lncRNA Meg3 expression was successfully silenced or upregulated in OGD-exposed hCMEC/D3 cells, respectively (Fig. 1B). In addition, Meg3 siRNA decreased Meg3 expression, while pcDNA-Meg3 transfection increased Meg3 expression in OGD-exposed hCMEC/D3 cells (Fig. 1C). Moreover, the data demonstrated that hCMEC/D3 cell proliferation, migration and angiogenesis were markedly enhanced in the si-Meg3 + OGD-treated group compared with the group exposed to OGD only (Fig. 1D-H). Meg3 silencing inhibited the apoptosis of OGD-exposed hCMEC/D3 cells (Fig. 1I and J). By contrast, pcDNA-Meg3 transfection exerted the opposite effects (Fig. 1D-J). In addition, western blot analysis revealed an increase in Bax, and a decrease in Bcl-2, VEGFA and VEGFR2 expression in the OGD-exposed hCMEC/D3 cells compared with the control cells (Fig. 1K-L). However, these expression levels were significantly reversed after Meg3 silencing (Fig. 1K-L). Thus, the aforementioned data indicated that Meg3 knockdown promoted hCMEC/D3 cell proliferation, migration and angiogenesis, as well as inhibiting cell apoptosis in response to OGD-induced injury.

miR-122-5p overexpression reverses the deteriorating effects of lncRNA Meg3 overexpression on OGD injury. Subsequently, the present study aimed to identify the possible target miRNAs of Meg3. It was found that miR-122-5p expression was decreased in a time-dependent manner in the OGD model of hCMEC/D3 cells (Fig. 2A). Therefore, it was hypothesized that lncRNA Meg3 could interact with miR-122-5p. A Meg3-wt luciferase reporter vector and a Meg3-mut luciferase reporter vector with mutations on a predicted miR-122-5p binding site in Meg3 were constructed (Fig. 2B). A luciferase reporter assay was then conducted. miR-122-5p mimic markedly decreased the luciferase activity of 293T cells transfected with Meg3-wt; however, miR-122-5p mimic did not alter the luciferase activity of 293T cells transfected with Meg3-mut compared with the NC mimic group (Fig. 2C). Moreover, in miR-122-5p mimic-transfected hCMEC/D3 cells, the RNA level of Meg3 was significantly decreased (Fig. 2D). Furthermore, as shown in Fig. 2E, Meg3 knockdown promoted the miR-122-5p level in OGD-exposed hCMEC/D3 cells, and the overexpression of Meg3 exerted the opposite effect.

To further explore whether miR-122-5p mediates the regulatory effects of Meg3 on OGD-induced cell damage, the hCMEC/D3 cells were co-transfected with miR-122-5p mimic/anti-miR-122-5p and pcDNA-Meg3 (Meg3) prior to OGD. It was found that miR-122-5p overexpression restored hCMEC/D3 cell proliferation, migration and angiogenesis inhibited by pcDNA-Meg3, while miR-122-5p knockdown exerted the opposite effects (Fig. 3A-D). In addition, compared with the control group, the OGD-exposed hCMEC/D3 cells exhibited a marked induction in cell apoptosis (Fig. 3F-G). However, this induction in apoptosis was abolished after miR-122-5p silencing (Fig. 3F-G). Western blot analysis of the Bax, Bcl-2, VEGFA and VEGFR2 expression levels revealed consistent changes with the expression levels in hCMEC/D3 cells following the OGD challenge and transfection with pcDNA-Meg3 + miR-122-5p mimic or pcDNA-Meg3 + anti- miR-122-5p (Fig. 3H and I)

lncRNA Meg3 promotes NDRG3 expression by interacting with miR-122-5p. Subsequently, online database searches were performed to predict the downstream target genes of miR-122-5p. Notably, NDRG3 was found to be a potential target gene of miR-122-5p (Fig. 4A). To investigate whether miR-122-5p directly targets the 3'UTR of NDRG3, luciferase reporter assays were performed. The data indicated that miR-122-5p mimic markedly reduced the luciferase activity of 293T cells transfected with NDRG3 3'UTR-wt and miR-122-5p mimic (Fig. 4B). Moreover, miR-122-5p mimic markedly suppressed NDRG3 expression (Fig. 4C and D). Furthermore, Meg3 knockdown inhibited NDRG3 expression in OGD-exposed hCMEC/D3 cells, and Meg3 overexpression exerted the opposite effects (Fig. 4E and F). Moreover, the

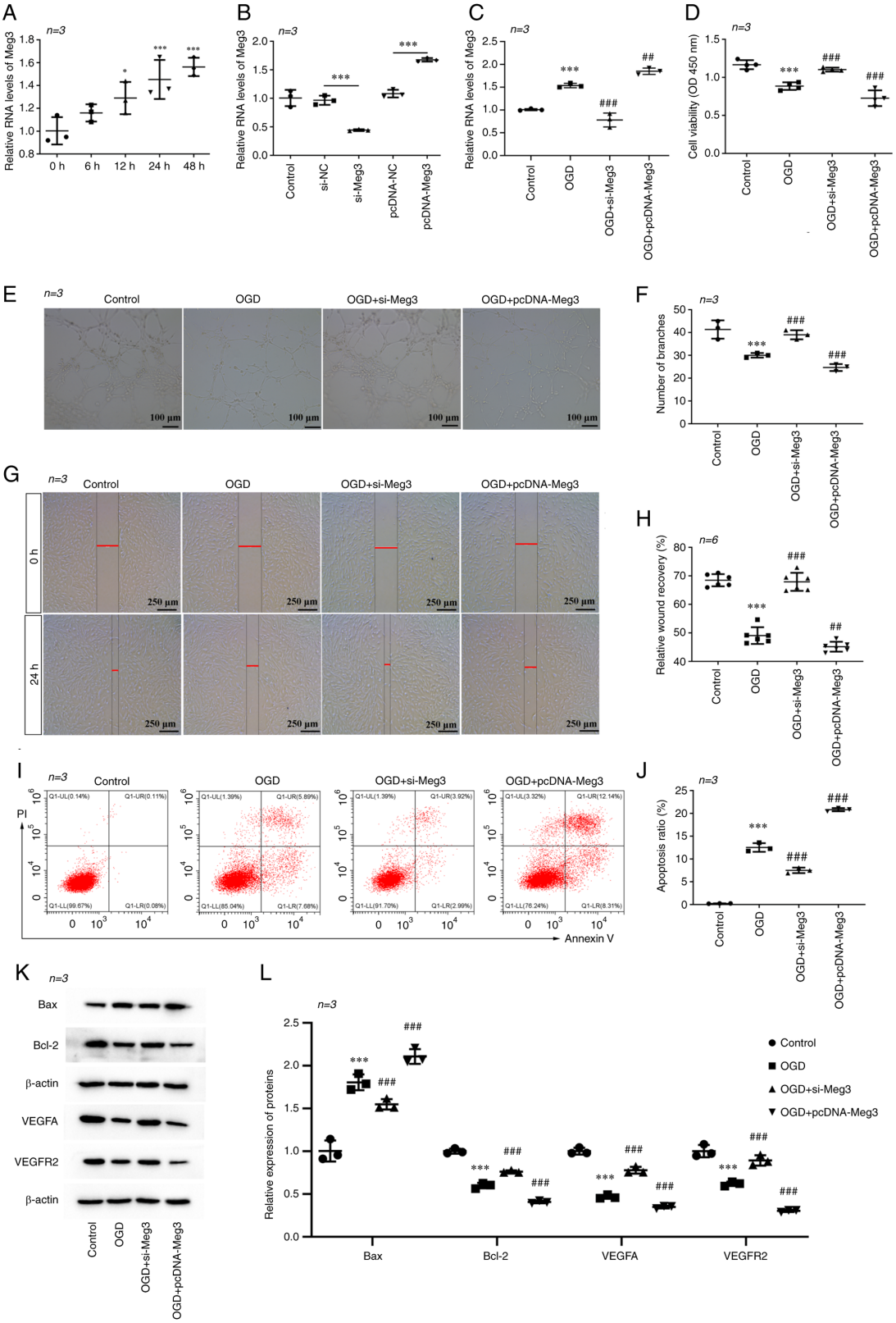

Figure 1. lncRNA Meg3 regulates OGD-induced injury in hCMEC/D3 cells. (A) hCMEC/D3 cells were exposed to OGD for 6, 12, 24 and 48 h. RT-qPCR analysis was performed to test lncRNA Meg3 expression. n=3. *P<0.05 vs. 0 h in one-way ANOVA, ***P<0.001 vs. 0 h in one-way ANOVA. (B) hCMEC/D3 cells were examined using RT-qPCR for Meg3 mRNA; n=3. (C) The expression of Meg3 in hCMEC/D3 cells under normal and OGD conditions for 24 h was measured using RT-qPCR; n=3. (D) The proliferation of hCMEC/D3 cells was evaluated using a CCK-8 assay. hCMEC/D3 cells underwent OGD for 24 h. n=4. (E and F) Tube formation assay of cells transfected with si-Meg3 or pcDNA-Meg3 when cultured in normal and OGD conditions for 24 h (magnification, x100); n=3. (G and H) Representative images of cell migration following transfection with si-Meg3 or pcDNA-Meg3 when cultured in normal and OGD conditions at 0 and 24 h (magnification, x50); n=6. (I and J) Apoptotic rates of hCMEC/D3 cells following the OGD challenge for 24 h and transfection with pcDNA-Meg3 or si-Meg3 estimated using flow cytometry; n=3. (K and L) The relative protein expression levels of Bax, Bcl-2, VEGFA, and VEGFR2 were measured using western blot analysis in hCMEC/D3 cells exposed to OGD for 24 h; n=3. ***P<0.001 vs. control in t-test; $^{\#\#}$P<0.01 vs. OGD in one-way ANOVA; $^{\#\#\#}$P<0.001 vs. OGD in one-way ANOVA. lncRNA, long non-coding RNA; OGD, oxygen-glucose deprivation; RT-qPCR, reverse transcription-quantitative PCR; si, short interfering.

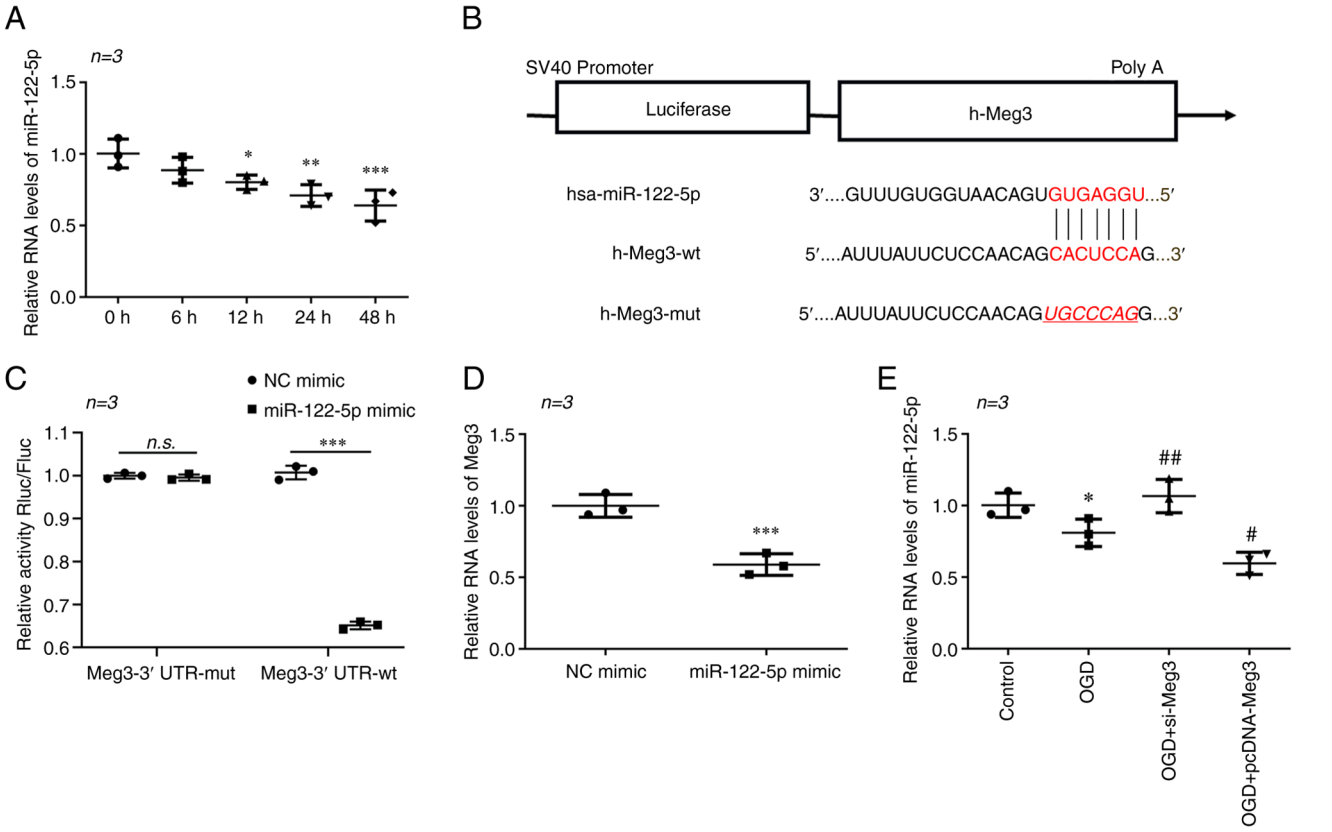

Figure 2. miR-122-5p is a potential target of Meg3. (A) The expression of miR-122-5p was measured using RT-qPCR. *P<0.05 vs. 0 h in one-way ANOVA; **P<0.01 vs. 0 h in one-way ANOVA; ***P<0.001 vs. 0 h in one-way ANOVA. n=3. (B) Online database was used to demonstrate the binding sites of miR-122-5p and Meg3. (C) Luciferase reporter assay was used to assay the interaction of miR-122-5p and Meg3 sequences. n.s., not significant, ***P<0.001 vs. NC mimic in t-test; n=3. (D) Meg3 level was tested by RT-qPCR. ***P<0.01 vs. NC mimic in t-test; n=3. (E) miR-122-5p expression was detected using RT-qPCR in hCMEC/D3 cells exposed to OGD for 24 h; n=3. *P<0.05 vs. control in t-test; #P<0.05 vs. OGD in one-way ANOVA; ##P<0.01 vs. OGD in one-way ANOVA. NC, normal control; OGD, oxygen-glucose deprivation; RT-qPCR, reverse transcription-quantitative PCR.

increase in NDRG3 expression at both the mRNA and protein level induced by Meg3 overexpression was significantly attenuated by miR-122-5p mimic and enhanced by a miR-122-5p inhibitor (Fig. 4G and H). Overall, these results suggested that NDRG3 was a target gene of miR-122-5p, and that Meg3 promoted NDRG3 expression by sponging miR-122-5p.

Knockdown of NDRG3 expression reverses the deteriorating effects of lncRNA Meg3 overexpression on OGD-induced injury. To confirm that NDRG3 is a functional target gene of lncRNA Meg3, efficient siRNA was designed to knock down NDRG3 expression at both the RNA and protein level (Fig. 5A and B). Subsequently, knockdown experiments were performed by silencing NDRG3 expression in hCMEC/D3 cells transfected with pcDNA-Meg3 (Meg3). The exacerbating effect of Meg3 overexpression on OGD-induced injury was abolished by NDRG3 knockdown, including an increase in the proliferation, migration and angiogenesis, as well as a decrease in the apoptosis of hCMEC/D3 cells subjected to OGD (Fig. 5C-K).

Discussion

Emerging evidence suggests that a number of lncRNAs, such as MALAT1 (16), GAS5 (17) and H19 (18) are abnormally expressed in cerebral ischemic animals and/or cells subjected to OGD. In the present study, increased expression of lncRNA Meg3 was observed following OGD. It has been demonstrated that Meg3 plays a key role in embryonic development, and the deletion of the Meg3 gene in mice has been shown to lead to skeletal muscle defects and perinatal mortality (19). lncRNA Meg3 is widely involved in tumor progression, including the suppression of cancer cell proliferation, migration and angiogenesis (20-22). Additionally, the inactivation of Meg3 results in an enhanced expression of vascular endothelial growth factor pathway genes and microvessel formation in the brain (23). In rat models of middle cerebral artery occlusion (MCAO), Meg3 knockdown has been shown to ameliorate microvessel density and neurological impairment by activating the Wnt/β-catenin signaling pathway (24). Furthermore, Meg3 serves a crucial role in endothelial cell proliferation and angiogenesis by negatively regulating miR-9 as a miRNA sponge (25). These results also suggested that the silencing of Meg3 protected hCMEC/D3 cells from OGD-induced cell injury, including promoting proliferation, migration and angiogenesis, as well as inhibiting cell apoptosis, as opposed to Meg3 overexpression.

Studies have revealed that Meg3 serves as a competing endogenous RNA (ceRNA), reducing the regulatory effects of miRNAs on target genes. Meg3 binds to miRNA-17 (26) or miRNA-125a-5p (27), ultimately affecting the Treg/Th17 balance in the pathogenesis of asthma. Moreover, Meg3 can sponge miR-499-5p (28), miR-147 (29), or miR-494 (30)

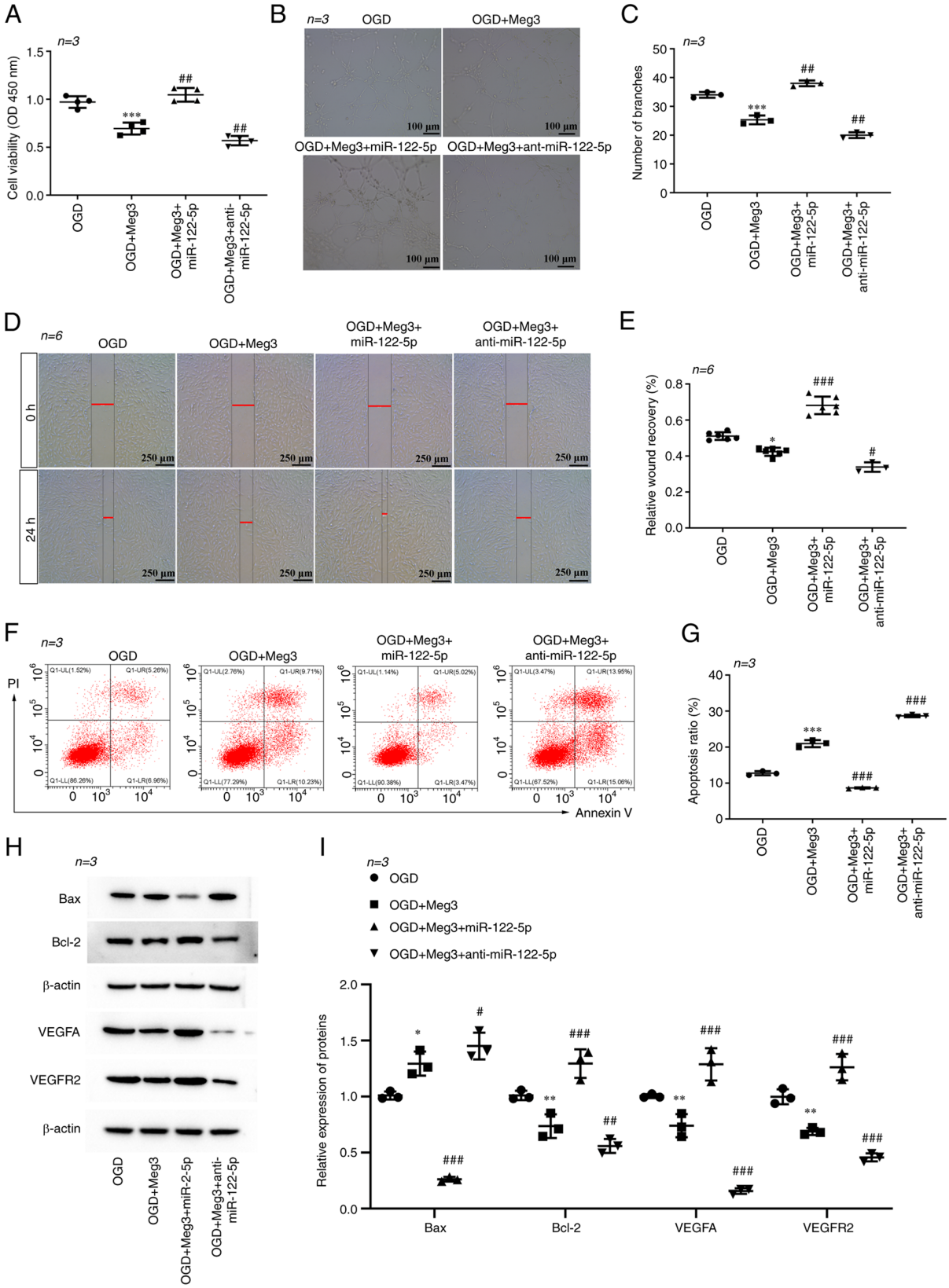

Figure 3. miR-122-5p overexpression reverses the deteriorating effects of lncRNA Meg3 overexpression on OGD-induced injury. (A) The proliferation of hCMEC/D3 cells was evaluated using CCK-8 assay. hCMEC/D3 cells were subjected to OGD for 24 h; n=4. (B and C) Tube formation of cells co-transfected with pcDNA-Meg3 (Meg3) and miR-122-5p mimic (miR-122-5p)/anti-miR-122-5p cultured under OGD conditions for 24 h (magnification, x100); n=3. (D and E) Representative images of cell migration following co-transfection with (Meg3) and miR-122-5p mimic (miR-122-5p)/anti-miR-122-5p and culture under OGD conditions for 0 and 24 h (magnification, x50); n=6. (F and G) The apoptotic rates of hCMEC/D3 cells exposed to OGD for 24 h and transfected with pcDNA-Meg3 (Meg3)/miR-122-5p mimic (miR-122-5p)/anti-miR-122-5p were estimated using flow cytometry; n=3. (H and I) The relative protein expression levels of Bax, Bcl-2, VEGFA, and VEGFR2 were measured using western blot analysis in hCMEC/D3 cells exposed to OGD for 24 h; n=3. *P<0.05 vs. OGD in one-way ANOVA; **P<0.01 vs. OGD in one-way ANOVA; ***P<0.001 vs. OGD in one-way ANOVA; $^{\#}$P<0.05 vs. OGD + Meg3 in one-way ANOVA; $^{\#\#}$P<0.01 vs. OGD + Meg3 in one-way ANOVA; $^{\#\#\#}$P<0.001 vs. OGD + Meg3 in one-way ANOVA. miR, microRNA; OGD, oxygen-glucose deprivation; lncRNA, long non-coding RNA.

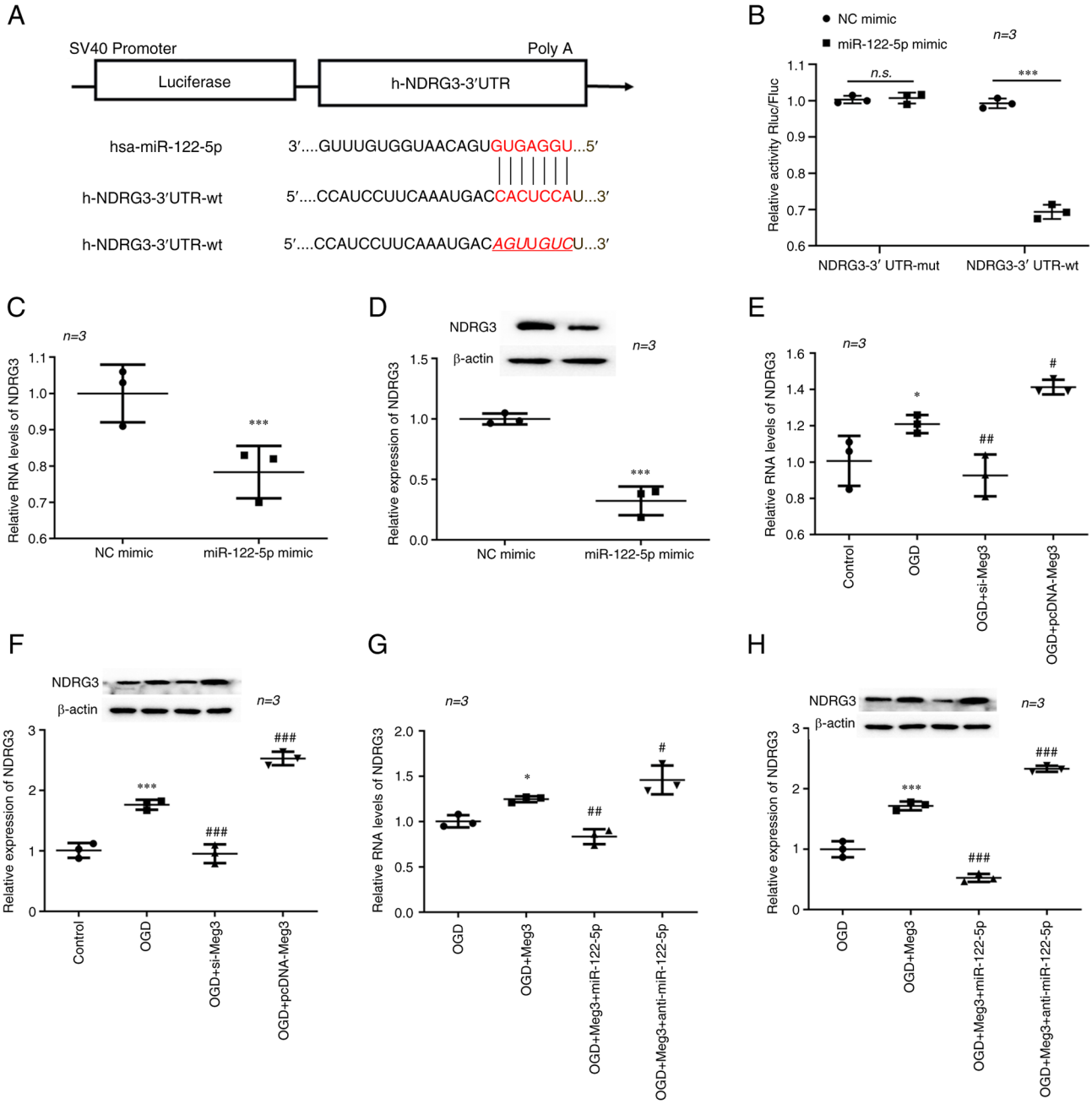

Figure 4. lncRNA Meg3 promotes NDRG3 expression by interacting with miR-122-5p. (A) Diagram of the predicted miR-122-5p binding site in the 3'UTR sequences of NDRG3. (B) Online database predicted binding sites between miR-122-5p and NDRG3 3'UTR sequences. n=3. (C) The RNA level of NDRG3 was examined using RT-qPCR in normal hCMEC/D3 cells; n=3. (D, F and H) The protein level of NDRG3 was examined using western blot analysis in hCMEC/D3 cells subjected to OGD for 24 h. β-actin was used as a loading control; n=3. (E and G) The mRNA levels of NDRG3 in hCMEC/D3 cells under normal and OGD conditions for 24 h was measured using RT-qPCR; n=3. (B-D) n.s., not significant, ***P<0.001 vs. NC mimic in t-test. (E and F) *P<0.05 vs. control in t-test, ***P<0.001 vs. control in t-test; $^{\#}$P<0.05 vs. OGD in one-way ANOVA; $^{\#\#}$P<0.01 vs. OGD in one-way ANOVA; $^{\#\#\#}$P<0.001 vs. OGD in one-way ANOVA. (G and H) *P<0.05 vs. OGD in one-way ANOVA; ***P<0.001 vs. OGD in one-way ANOVA; $^{\#}$P<0.05 vs. OGD + Meg3 in one-way ANOVA; $^{\#\#}$P<0.01 vs. OGD + Meg3 in one-way ANOVA, $^{\#\#\#}$P<0.001 vs. OGD + Meg3 in one-way ANOVA. lncRNA, long non-coding RNA; NDRG family member 3; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; OGD, oxygen-glucose deprivation; NC, normal control.

to suppress the proliferation and invasion of tumors. In the present study, luciferase reporter assay verified that Meg3 directly interacted with miR-122-5p to inhibit its expression in hCMEC/D3 cells. These findings illustrated a novel Meg3-mediated ceRNA network in the OGD model. miR-122-5p has been widely reported to participate in the occurrence and development of various types of cancer, such as ovarian cancer (31), gastric cancer (32) and glioma (33). In a previous study, RT-qPCR chips detected the abnormal expression of various miRNAs in peripheral blood cells from patients with IS. Among these, downregulated miR-122-5p was predicted to regulate the expression of various genes in the pathogenesis of IS (34). Furthermore, miR-122-5p overexpression has been proven to protect against neuronal cell death in IS by targeting the FOXO3 gene (35). In addition, increasing the expression of miR-122-5p in blood has been shown to improve the prognosis of rats subjected to MCAO by attenuating direct target genes (e.g., Vcam1, Nos2 and Pla2g2a)

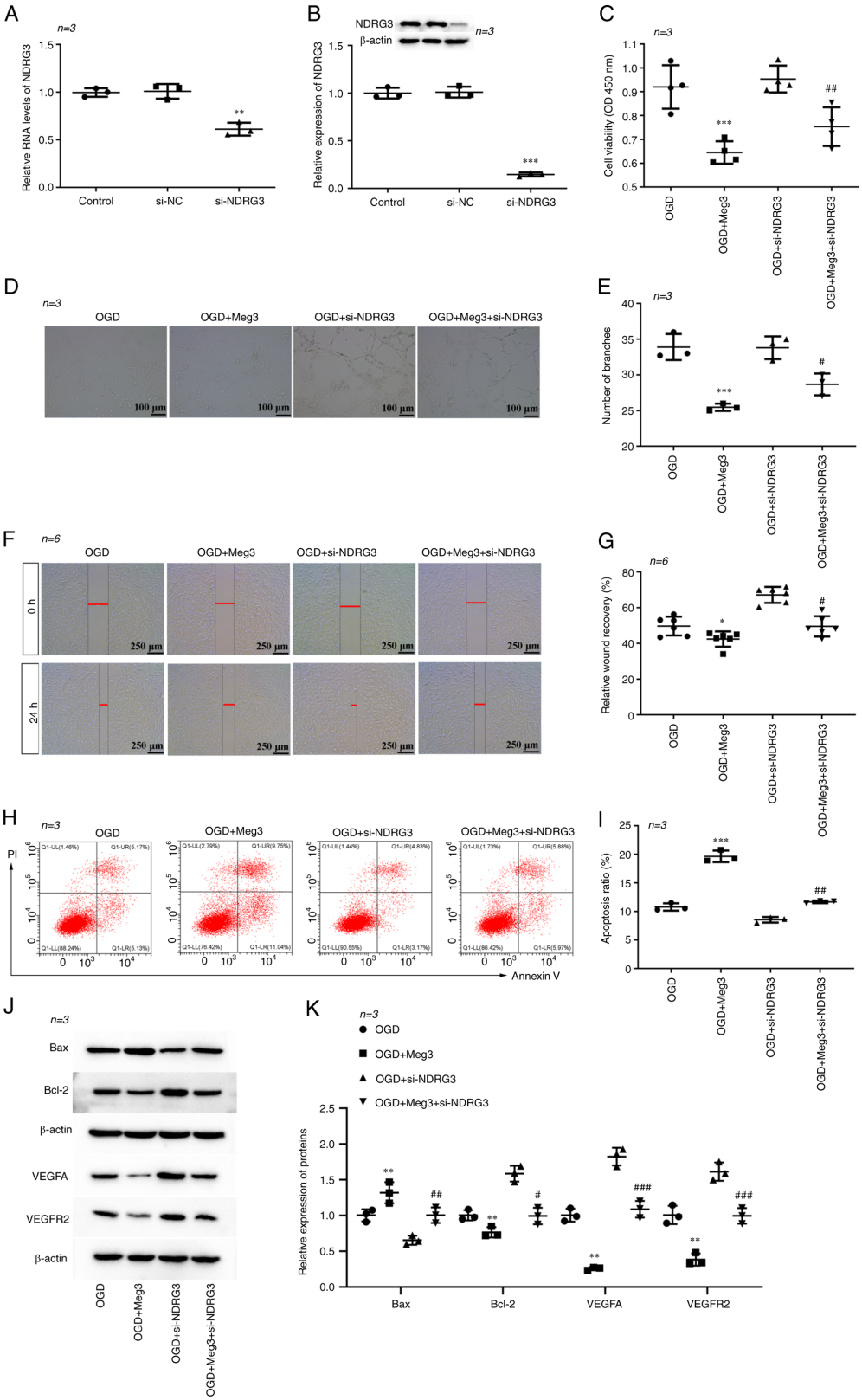

Figure 5. Knockdown of NDRG3 expression reverses the deteriorating effects of lncRNA Meg3 overexpression on OGD-induced injury. (A) The RNA level of NDRG3 was examined using RT-qPCR; n=3. (B) The expression of NDRG3 protein was determined using western blot analysis. β-actin was used as a loading control; n=3. (C) hCMEC/D3 cells were subjected to OGD for 24 h. The proliferation of hCMEC/D3 cells was evaluated using a CCK-8 assay; n=4. (D and E) Tube formation of cells transfected with pcDNA-Meg3 (Meg3) and/or si-NDRG3 when cultured under OGD conditions for 24 h (magnification, x100); n=3. (F and G) Representative images of cell migration following transfection with pcDNA-Meg3 (Meg3) and/or si-NDRG3 when cultured in OGD conditions at 0 and 24 h (magnification, x50); n=6. (H and I) The apoptotic rates of hCMEC/D3 cells subjected to OGD for 24 h and transfected with pcDNA-Meg3 (Meg3) and/or si-NDRG3 were estimated using flow cytometric analysis; n=3. (J and K) The relative protein expression levels of Bax, Bcl-2, VEGFA and VEGFR2 were measured using western blot analysis in hCMEC/D3 cells subjected to OGD for 24 h; n=3. *P<0.05 vs. OGD in one-way ANOVA; **P<0.01 vs. OGD in one-way ANOVA; ***P<0.001 vs. OGD in one-way ANOVA; $^{\#}$P<0.05 vs. OGD + Meg3 in one-way ANOVA; $^{\#\#}$P<0.01 vs. OGD + Meg3 in one-way ANOVA; $^{\#\#\#}$P<0.001 vs. OGD + Meg3 in one-way ANOVA. NDRG family member 3; lncRNA, long non-coding RNA; OGD, oxygen-glucose deprivation; RT-qPCR, reverse transcription-quantitative PCR; NC, normal control.

and indirect target genes (e.g., Alox5, Itga2b, Timp3, Il1b, Il2 and Mmp8) (36). Consistently, the present study demonstrated that miR-122-5p overexpression eliminated the inhibition of hCMEC/D3 cell proliferation, migration and angiogenesis induced by Meg3, as well as Meg3-induced cell apoptosis following OGD. Moreover, the findings of the present study demonstrated that miR-122-5p overexpression attenuated NDRG3 expression in OGD-exposed hCMEC/D3 cells. As an apoptosis and autophagy-related gene, the differential expression of NDRG3 is associated with traumatic brain injury, the hypoxic response of cerebral ischemia and nervous system cancers (37). Recently, as an oxygen-regulated pathway, let-7f/NDRG3 signaling was found to mediate neuron viability in rat cerebral ischemia (38). According to a previous study, NDRG3 protein expression and the downstream Raf-ERK axis were associated with angiogenesis and cell growth under hypoxic conditions (39). In the present study, it was observed that hCMEC/D3 cells with a decreased NDRG3 expression following transfection with si-NDRG3 reversed the suppression of cell proliferation, migration and angiogenesis, as well as the promotion of cell apoptosis induced by OGD and Meg3 overexpression.

In conclusion, the present study, to the to the best of the authors' knowledge, is the first to report a novel mechanism of lncRNA MEG3 as a ceRNA by targeting the miR-122-5p/NDRG3 signaling pathway to regulate the viability and angiogenesis of hCMEC/D3 cells subjected to OGD. These findings provide insight for future intervention studies on IS.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZL, TG and WT conceived and designed the study. ZL, TG and WL performed the experiments. ZL and TG analyzed the data. ZL and TG wrote the manuscript. ZL and TG confirm the authenticity of all the raw data. All authors have 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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