Life Sciences 239 (2019) 116946

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

# LncRNA-MALAT1 promotes tumorogenesis of infantile hemangioma by competitively binding miR-424 to stimulate MEKK3/NF-κB pathway

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ARTICLE INFO

*Keywords:*
MALAT1
miR-424
MEKK3
Infantile hemangioma
IKK/NF-κB pathway

ABSTRACT

*Aims:* Infantile hemangioma (IH) is the most common vascular neoplasm in infant and young children. Long non-coding RNAs (lncRNAs) are known to be associated with IH. This study aims to investigate the role and underlying mechanism of lncRNA-MALAT1 in IH.

*Main methods:* qRT-PCR was used to quantify the expressions of MALAT1, miR-424, and MEKK3 in IH tissues. The cell proliferation, apoptosis, migration, invasion, and tube formation ability were assessed by MTT assay, colony formation assay, flow cytometric analysis, transwell assay and tube formation assay, respectively. The interaction among MALAT1, miR-424 and MEKK3 was evaluated by luciferase reporter assay. Immunohistochemistry (IHC) and Western blotting were utilized to evaluate the expression levels of MEKK3, Ki-67 and NF-κB pathway-related proteins both *in vitro* and *in vivo*.

*Key findings:* In IH tissues, MALAT1 and MEKK3 were overexpressed while miR-424 was down-regulated. Silencing MALAT1 or overexpression of miR-424 significantly inhibited the IH cell proliferation, migration and tube formation, but promoted the cell apoptosis. Knockdown of MALAT1 suppressed the expression of MEKK3 and inactivated the IKK/NF-κB pathway by sponging miR-424. Overexpression of MEKK3 in HemEcs reversed the impact of knockdown of MALAT1 and overexpression of miR-424 on the cell proliferation, apoptosis, migration, invasion and tube formation rate. The tumor xenografts experiments demonstrated that silencing MALAT1 significantly inhibited the tumor growth *in vivo* and Ki-67 in the tumor tissues was also significantly suppressed.

*Significance:* MALAT1 promoted the IH progression through inhibiting miR-424 to activate MEKK3-mediated IKK/NF-κB pathway, suggesting that MALAT1, miR-424 and MEKK3 could be used as potential targets to improve IH treatment efficiency.

## 1. Introduction

Infantile hemangioma (IH) is one of the most common vascular tumors for infant and children, which causes harm for 3%–10% newborn children [1]. Due to rapid growth and invasion, IH can damage normal tissue and organ severely, leading to life-threatening disease [2]. There are two different phases of IH, including proliferating and involuting phases. Proliferating IH was characterized by the unnormal proliferation of the immature endothelial cells. In involuting IH, fewer and larger capillary-like vessels surrounded by connective tissue were presented [3]. Even a lot of genes have been proved to be associated with the IH development, the pathogenesis and the mechanism of IH are still unclear [4–6]. It was reported that clonal expansion of endothelial cells and neovascularization played crucial roles in hemangiomagenesis [7]. The endothelial cells expressed CD133 and CD34 were discovered in the early proliferating IH [8]. However, the important roles of endothelial cells in the development of IH need further investigations for discovering new therapeutic strategies to improve the health of children.

Mitogen-activated protein kinase kinase kinase (MAP3K) is a family of serine/threonine protein kinase, which has been expressed in various mammalian tissues [9]. MAP3K involves in different endothelial

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https://doi.org/10.1016/j.lfs.2019.116946
Received 25 July 2019; Received in revised form 27 September 2019; Accepted 7 October 2019
Available online 11 October 2019
0024-3205/ 

cellular functions, such as cell proliferation and angiogenesis [10]. MEKKs are recognized as the family members of the serine/threonine protein kinase family, working as upstream regulators of MAP3Ks. The expression of MEKK1-3 was found to activate the expression of IκB kinase (IKK), which degraded IκB and activated NF-κB. Also, MEKK3 was important for the development of cardiovascular system. The deletion of MEKK3 in mice resulted in the embryonic death because of the failed angiogenesis, endothelial cell apoptosis and cardiac defects [11]. In several types of cancer, MEKK3 plays important role in the cancer cell proliferation, apoptosis, and metastasis by activating the IKK/NF-κB pathways [9,12,13]. For example, Liao et al. recently found that MEKK3 was aberrantly expressed in ovarian cancers. Silencing MEKK3 in ovarian cancer significantly improved the therapeutic efficiency [14]. Interestingly, Su et al. found that even MEKK3 was essential for endothelium function but was not necessary for tumor growth and angiogenesis [15]. However, the function and mechanism of the MEKK3 in infantile hemangioma is not discovered yet.

Long noncoding RNAs (lncRNAs) are a family of RNAs (longer than 200 nt) without ability to code proteins but possess important regulation functions in several biological processes. In the last decade, numerous lncRNAs have been discovered and involved in a number of biological processes [16]. For example, the cell proliferation, apoptosis, differentiation and initiation of cancer are related with the aberrant expression of lncRNAs [17,18]. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) has varies functions in different types of cancer and is a highly conserved lncRNA [19,20]. Feng et al. recently reported that MALAT1 promoted the lung cancer proliferation and drug resistance by regulating miR-200a as sponge [21]. In breast cancer, Wu et al. found that MALAT1 was significantly enhanced and they found that silencing MALAT1 in breast cancer cells significantly reduced the expression of vascular endothelial growth factor, suggesting that MALAT1 promoted angiogenesis in breast cancer [22]. In IH, MALAT1 was significantly up-regulated and knockout of MALAT1 lead to the apoptosis of endothelial cells and cellular cycle retardation at S stage [5]. These results demonstrated that MALAT1 might be important in the development of IH, which has not been uncovered yet.

MicroRNAs (miRNAs) are another group of non-coding RNAs, which is about 22 nucleotides in length. Through binding with the 3′ untranslated region (3′UTR), miRNA acts as a gene regulator at the post-transcriptional level. miR-424 has been recognized as a tumor suppressive gene in various types of cancers, such as cervical carcinoma [23], breast cancer [24], squamous cell carcinoma [25], ovarian cancer, [26] and hepatocellular carcinoma [27]. Moreover, miR-424 was down-regulated in infantile skin hemangioma [4]. Overexpression of miR-424 in HemECs cells significantly inhibited the proliferation and migration, as well as the tube formation [4]. Additionally, we found that the binding sequence of miR-424 in MALAT1 and 3′-UTR of MEKK3 was UGCUGCU through DIANA (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted) and Starbase (http://starbase.sysu.edu.cn/index.php) databases, respectively. Therefore, our hypothesis is that MALAT1 might bind with miR-424 to activate the MEKK3-induced IKK/NF-κB pathway, leading to the proliferation and migration of endothelial cells, and eventually the formation of IH.

In the present study, we aimed to investigate the role and functional mechanism of MALAT1 in IH. Our results indicated that MALAT1 and MEKK3 were overexpressed and miR-424 was down-regulated in IH. The cell proliferation, migration, invasion, and tube formation in IH were suppressed by silencing MALAT1 or overexpression of miR-424, which also promoted the cell apoptosis rate. However, these impacts could be reversed by overexpressing MEKK3 in HemECs. Through the mechanism investigation, we demonstrated that MALAT1 promoted IH development through regulating miR-424 to affect the MEKK3 and following IKK/NF-κB pathway, suggesting their critical roles in the development of IH. These results indicated that MALAT1 and miR-424 could be potential targets for IH treatment.

## 2. Materials and methods

### 2.1. Tissue specimens and cell culture

Three groups of the tissue specimens, including the infantile skin hemangioma in the proliferative stage and involuting stage, and normal subcutaneous tissues were obtained from patients (each group has 16 specimens) in the Henan Provincial People's Hospital. The specimens were immediately frozen in −80 °C or liquid nitrogen for storage and further analysis. Each patient agreed with the informed consent for the collection of samples. The study was approved by the ethics committee of the Henan Provincial People's Hospital.

The human hemangioma endothelial cells (HemECs) was obtained from the Biosci Biotechnology (Hubei, China), which were cultured in the petric dishes in OPTI-MEM medium with 10% fetal bovine serum (FBS) in a humidified chamber with 5% $CO_2$ at 37 °C.

### 2.2. Cell transfection

To transfect HemECs cells, the shMALAT1, miR-424 mimic and miR-424 inhibitor were synthesized by GenePharma (Shanghai, China). The sequences of MEKK3 was coloned into the plasmid pcDNA3.1 with BamHI and EcoRI restriction enzymes (BersinBio, Guangzhou, China) to form pcDNA3.1-MEKK3. The empty pcDNA3.1 was used as a negative control. The HemECs cells were cultured in 6-well plates at 37 °C, 5% $CO_2$ incubator. When the cell density reached 60% confluency the next day, transfection was performed using shMALAT1, miR-424 mimic, miR-424 inhibitor, scramble controls, pcDNA3.1, or pcDNA3.1-MEKK3, with Lipofectamine 2000 (Invitrogen, USA) according to the manufactural protocol.

### 2.3. MTT assay

The cell viability of HemECs was evaluated by MTT assay. Briefly, the transfected HemECs were placed in 96-well plates in triplicate and cultured for 24, 48, 72, and 96 h. Then, the MTT reagent was incubated with the cells for another 4 h. 200 μL DMSO was added into each well after rinsing with the fresh medium. The absorbance at 490 nm was measured for each sample, resulting the growth curves based on the OD values.

### 2.4. Colony formation assay

The treated HemECs cells were seeded in a 6-well plate for 15 days. Then, the cells were fixed by methanol, followed by staining using 0.1% crystal violet. The visible colonies were imaged and counted to evaluate the colony formation rate for different treatments.

### 2.5. Apoptosis assay by flow cytometry

The HemECs were digested, centrifuged, and dispersed in a PBS buffer to form single-cell suspension. Then, Annexin V-FITC (BD Pharmingen, San Diego, CA) and PI (BD Pharmingen) were used to stain the cells for 30 min according to the manufacturers' instruction. The cells were analyzed by a FACScan flow cytometer (BD Bioscience, USA) with excitation laser of 488 nm. Triplicate for each sample were performed.

### 2.6. Transwell assay

The migration and invasion ability of HemECs with different treatments were evaluated by transwell assay. Typically, a two-chamber with a pore size of 8 μm membrane (BD Biosciences, USA) was used for migration investigation. 1000 cells was cultured in the top chamber in medium without serum. The bottom chamber was filled with 600 μL complete medium. After the incubation at 37 °C for 48 h,

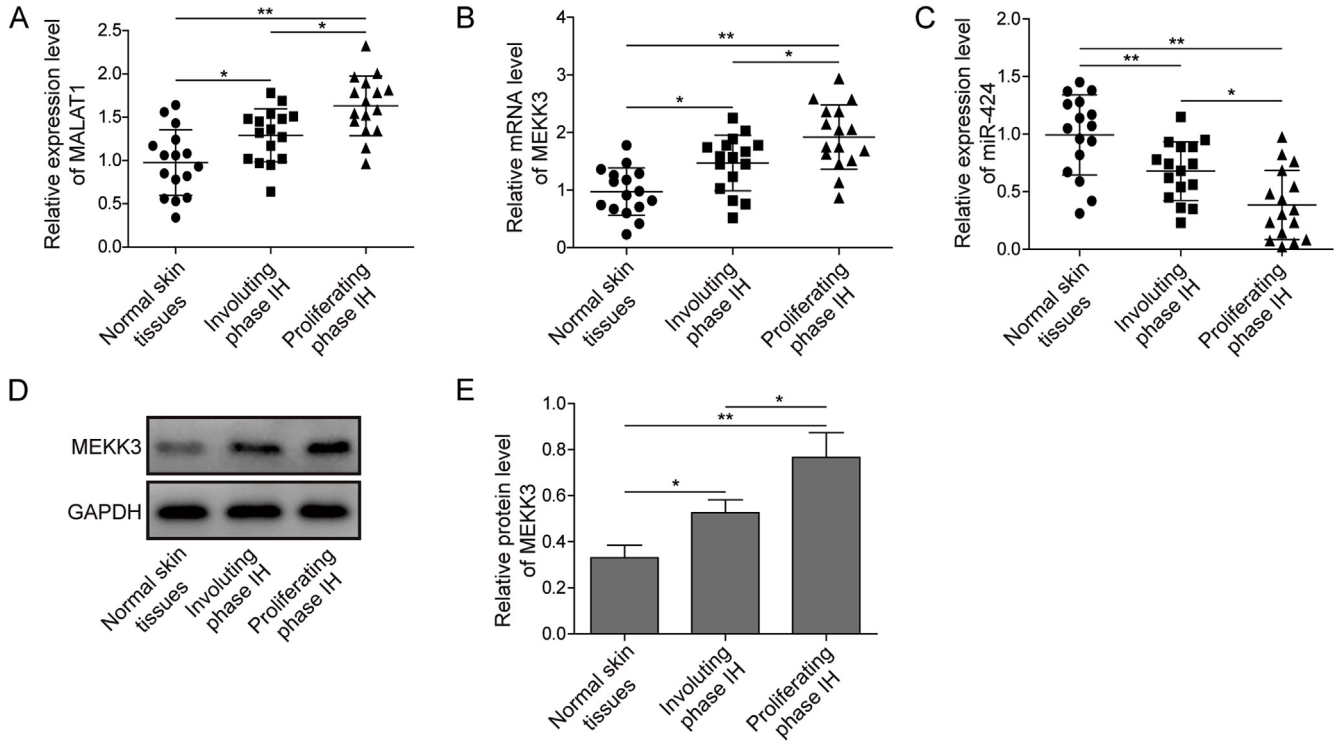


**Fig. 1. The expression level of MALAT1, miR-424 and MEKK3 in IH tissues.** The expression level of (A) MALAT1, (B) MEKK3 and (C) miR-424 in normal skin tissues, involuting phase IH, and proliferating phase IH by RT-PCR. (D) The expression level of MEKK3 in normal skin tissues, involuting phase IH, and proliferating phase IH by Western blotting. (E) The relative protein level compared with the GAPDH (internal control). The results were presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

the cells in the top chamber was removed. Methanol was used to fix the cells in the lower chamber. Then, the cells were stained by the 1% crystal violet before counting manually. The invasion assay was conducted similarly with the protocol of the migration assay, except that the chamber membrane was further coated with Matrigel (BD Bioscience, USA).

### 2.7. Tube formation assay

Matrigel melted overnight in a 4°C-ice bath was filled into 96-well plate on ice, which was incubated for 30–60 min at 37 °C. Then, the 96-well plates covered with Matrigel were seeded with the transfected HemECs in medium (2% FBS) with a density of $2.5 \times 10^4$ cells/well. After cultured for 48 h, the tubules were observed with a microscope and were counted in five individual fields.

### 2.8. Luciferase reporter assay

The interaction between MALAT1, miR-424, and MEKK3 was investigated by luciferase report assay. The wide-type and mutant of MALAT1 (MUT-MALAT1) and MEKK3 (MUT-MEKK3) were first inserted into the pmirGLO luciferase vector (Promega, Madison, WI). Lipofectamine 3000 (Invitrogen) was used to transfect the 293T cells with miR-424 mimics, mimics NC, miR-424 inhibitor, inhibitor NC, and the constructed pmirGLO luciferase vectors. With a dual-Glo Luciferase Assay System (Promega, USA), the luciferase activities were recorded after 48 h incubation.

### 2.9. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total RNA was first extracted from the tissues and cells, followed by the reverse transcription to form cDNA with a reverse transcription kit (Invitrogen) according to the instruction. qRT-PCR analysis was carried out by GoTaq@qPCR Master Mix (Promega) coupled with SYBR Green (Invitrogen). The StepOne Realtime PCR System (Applied Biosystems, Foster City, CA) was utilized to perform the qRT-PCR. The primers sequences were: miR-424: 5′-CGGCAGCAGCAATTCATGT-3' (sense) and 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGA TACGACTTCAAA-3' (antisense); MEKK3: 5′-TTGAAGGCTTACGGTGC TCT-3' (sense) and 5′-CAAAGTCCCCCAGCTTTACA-3' (antisense); MALAT1: 5′-AAAGCAAGGTCTCCCCACAAG-3' (sense) and 5′-GGTCTG TGCTAGATCAAAAGGCA-3' (antisense). U6: 5′-CTCGCTTCGGCAGC ACA-3' (sense) and 5′-AACGCTTCACGAATTTGCGT-3' (antisense); GAPDH, 5′-CCAGGTGGTCTCCTCTGA-3' (sense) and 5′-GCTGTAGCCA AATCGTTGT-3' (antisense). The relative expression of targets was calculated by $2^{-\Delta\Delta Ct}$ method.

### 2.10. Western blotting

The cellular proteins in the clinical samples and HemECs were collected using RIPA lysis buffer (Solarbio Science & Technology, Beijing, China) and the concentrations of protein were measured by a Bradford Protein Assay kit (Beyotime, China) according to the manufacturers' protocols. 10% SDS-PAGE was used to separate the proteins and then the proteins were transferred to PVDF membranes (Millipore Corporation, USA). The primary antibodies (all purchased from Abcam, USA), including antibodies for MEKK3, p-p65, p-IKKα/β, p-IκBα, IκBα, c-Myc, CyclinD1, Ki-67, Bax, Bcl-2 and GAPDH were incubated at 4 °C overnight with the membrane blocked by 5% nonfat milk. After rinse with buffer, the secondary antibodies labeled by peroxidase (goat anti-rabbit, ZB-2301, 1:5000; goat anti-mouse, ZDR5307; ZSGB-Bio, Beijing, China) were incubated with the membrane for another 2 h. An ECL detecting system (Thermo Scientific, USA) was utilized to quantify the expression levels of proteins. GAPDH was used as loading control.

### 2.11. Tumor xenografts experiments in vivo

BALB/c nude mice (six-week old) were obtained from SJA

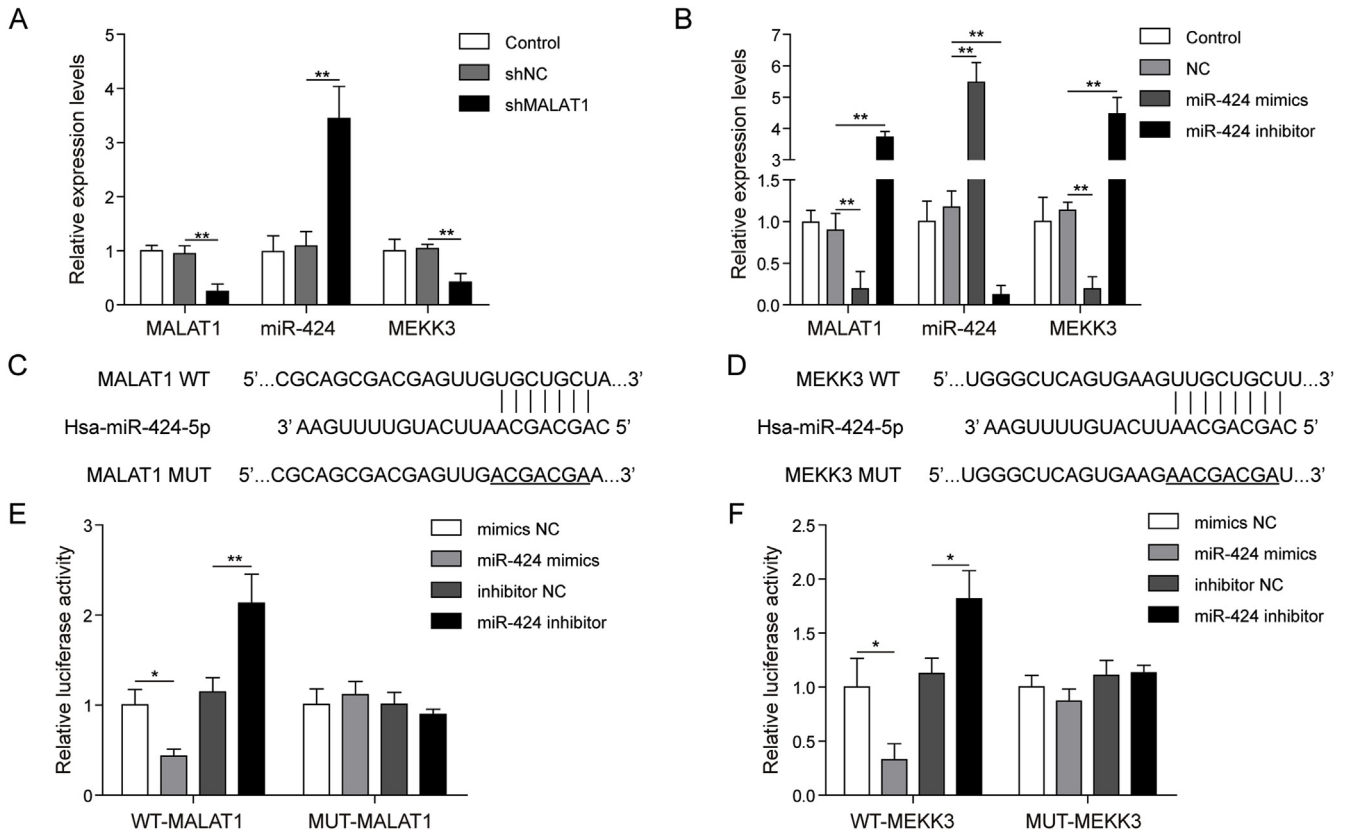


**Fig. 2. MALAT1 promoted MEKK3 expression via sponging miR-424.** (A) MALAT1, miR-424 and MEKK3 was quantified by qRT-PCR in HemECs transfected with shMALAT1 or shNC. (B) miR-424 and MEKK3 was quantified by qRT-PCR in HemECs transfected with miR-424 mimics or miR-424 inhibitor. (C) The binding sites were analyzed between two types of MALAT1 (wide type and mutant type) and miR-424. (D) The sites for binding were predicted between MEKK3 (wide type and mutant type) and miR-424. (E) Dual-luciferase assay was performed to study the interaction between MALAT1 and miR-424. (F) Dual-luciferase assay was conducted to confirm the interaction between MEKK3 and miR-424. The results were presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

Laboratory Animal Co., Ltd (Hunan, China). The HemECs transfected with shNC or shMALAT1 were subcutaneously injected into BALB/c nude mice. Once the xenografts were generated, the tumor volumes were monitored every five days until the day 30. Every five days, the tumor length (L) and width (W) were measured. The tumor volume (V) was calculated according to the equation of $V = 0.5 \times L \times W^2$. After 30 days, the tumor samples were collected with scarification of mice.

### 2.12. Immunohistochemistry (IHC) assay

The tumor tissues from the xenografted mice were prepared as 3-μm slices. Then, the sections were deparaffinized by xylene and hydrated in gradient alcohol. Sodium citrate buffer (pH 6.0) was used to retrieve the antigen. The endogenous peroxidase activity was quenched by adding 3% $H_2O_2$ at room temperature for 10 min, followed by treatment with 5% BSA for 1 h to minimize the nonspecific binding. Then, the sections were treated by an anti-Ki-67 antibody (Proteintech, 1:800) at 4 °C overnight. The secondary antibody working solution (Dako, GK500705) was used to incubated with the sections for 1 h at 37 °C. Finally, a DAB Substrate Kit (Invitrogen, USA) was utilized to stain the specimens for 5 min to observe the expression of Ki-67.

### 2.13. Statistical analysis

The data were shown as the Means ± SD and analyzed by SPSS 13.0 software. Student's t-test or one-way ANOVA was performed to evaluate the statistically significant differences. (*p < 0.05 and **p < 0.01).

## 3. Results

### 3.1. MALAT1 and MEKK3 were highly expressed and miR-424 was down-regulated in IH tissues

To uncover the roles of MALAT1, MEKK3 and miR-424 and their relationships in IH development, the expressions of these three elements in normal skin tissues, involuting phase IH, and proliferating phase IH were analyzed by qRT-PCR and Western blotting. As shown in Fig. 1A and B, MALAT1 and MEKK3 were greatly up-regulated in the IH tissues than that in the normal skin. The expressions of MALAT1 and MEKK3 were even higher in the proliferating phase IH than that in the involuting phase IH (Fig. 1A and B). In contrast, miR-424 was significantly down-regulated in the IH tissues than that in the normal skin tissues (Fig. 1C). Furthermore, Western blotting was used to examine the protein expression of MEKK3 in these tissues. The results showed that the expression of MEKK3 was significantly enhanced in the proliferating phase IH than that in the normal skin tissues and involuting phase IH (Fig. 1D and E). All these results demonstrated that in IH tissues, the expression of MALAT1 had a reverse correlation with the expression of miR-424, but a positive correlation with the expression of MEKK3.

### 3.2. MALAT1 promoted the expression of MEKK3 by sponging miR-424 in HemECs

The reverse correlation between MALAT1 and miR-424 in IH tissues proposed that MALAT1 might act as a sponge for miR-424. In order to test this hypothesis, we firstly investigated the impact of silencing MALAT1 on HemECs. As demonstrated in Fig. 2A, the expression of

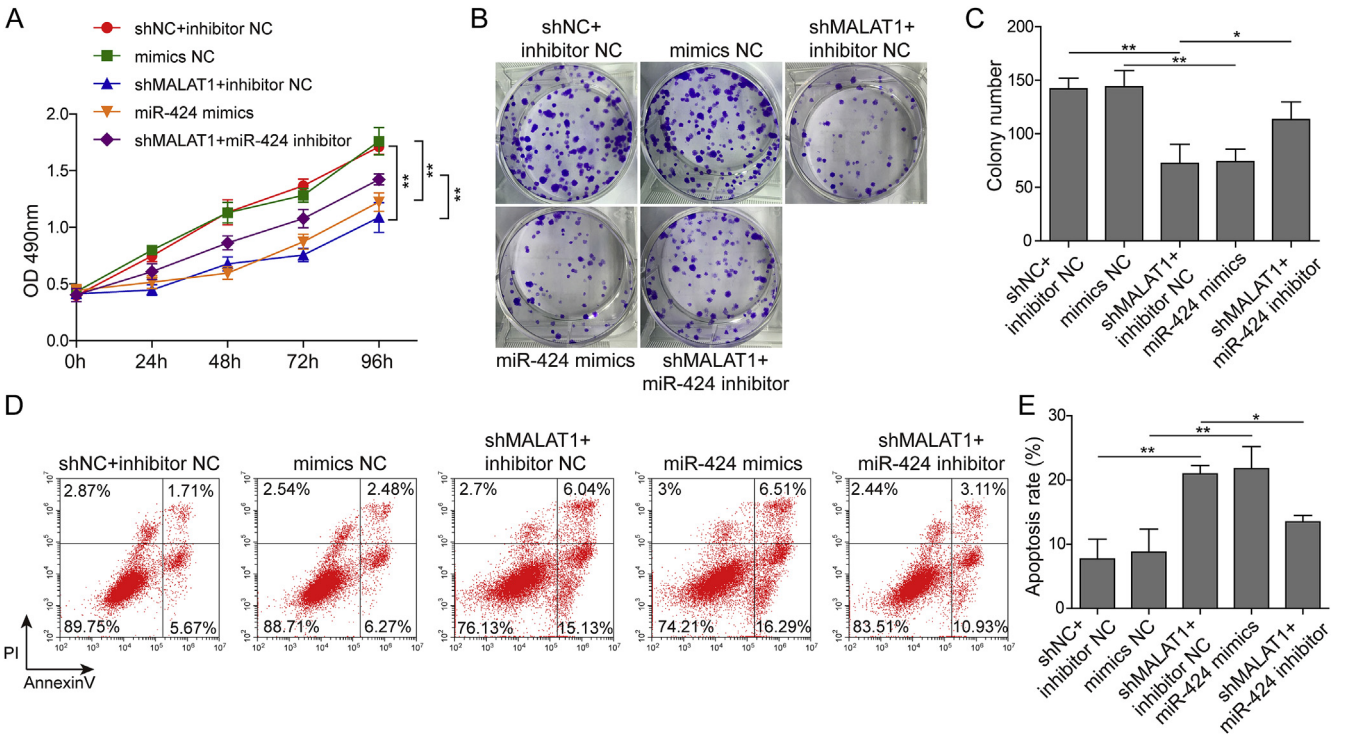


**Fig. 3. MALAT1 regulated the HemECs proliferation and apoptosis through miR-424.** (A) Cell viability of HemECs was examined by MTT assay after transfected with shMALAT1, miR-424 mimics or miR-424 inhibitor. (B–C) Colony formation assay was utilized to examine the cell proliferation ability of HemECs transfected with shMALAT1, miR-424 mimics or miR-424 inhibitor. (D–E) The HemECs cells tranfected with shMALAT1, miR-424 mimics or miR-424 inhibitor were stained by Annexin V and propidium iodide (PI), followed by the analysis of apoptosis using flow cytometry. The results were presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

MALAT1 and MEKK3 were clearly inhibited by silencing MALAT1 in HemECs, but the expression of miR-424 was significantly enhanced by silencing MALAT1. Through transfecting with miR-424 mimics in HemECs, the expression of miR-424 was increased but the expression of MALAT1 and MEKK3 were decreased (Fig. 2B). In contrast, the transfection of miR-424 inhibitor in HemECs blocked the expression of miR-424 but increased the expression of MALAT1 and MEKK3 (Fig. 2B). Furthermore, a dual luciferase assay was performed to investigate the relationship between MALAT1, miR-424, and MEKK3. The bioinformatic analysis indicated that both MALAT1 and MEKK3 could bind with miR-424 (Fig. 2C and D). The results from the luciferase activity assay showed that the wide-type MALAT1 and wide-type MEKK3 could be cut down by miR-424 mimics (Fig. 2E and F). In contrast, in the mutated MALAT1 and MEKK3 groups, the luciferase activities with all the treatments showed the similar levels compared with the mimics NC group. Taken together, the results demonstrated that MALAT1 could directly sponge miR-424 to counteract its suppression on MEKK3, serving as a positive regulator of MEKK3 in IH.

### *3.3. Silencing MALAT1 suppressed the proliferation but promoted apoptosis of HemECs by up-regulating miR-424*

In order to discover the impacts of silenced MALAT1 on the proliferation of HemECs cells, MTT assay and colony formation assay were conducted in HemECs cells. The MTT results demonstrated that the cell viability of HemECs cells was greatly inhibited by shMALAT1 or miR-424 mimics (Fig. 3A). The miR-424 inhibitor recovered the impact of shMALAT1 in the cell viability (Fig. 3A). Similarly, after the HemECs cells were treated with shMALAT1 or miR-424 mimics, the clone counts were significantly decreased (Fig. 3B and C). Furthermore, the cell apoptosis of HemECs cells after different treatment were analyzed by flow cytometry. The results indicated that silencing MALAT1 or transfection of miR-424 mimics promoted the cell apoptosis (Fig. 3D and E). In the clone counts assay and apoptosis assay, the impact of shMALAT1 was partially reversed by introducing miR-424 inhibitor (Fig. 3B–E). All these results indicated that silence of MALAT1 in IH significantly suppressed the cell proliferation but promoted the cell apoptosis through over-expressing miR-424 in IH.

### *3.4. Knockdown of MALAT1 inhibited cell migration, invasion and tube formation of HemECs*

The function of MALAT1 in HemECs cells was further analyzed by transwell assay and tube formation assay. As shown in Fig. 4A–C, the number of migrated and invaded cells was significantly suppressed by the treatments of shMALAT1 and miR-424 mimics in HemECs compared with the control group treated with shNC with inhibitor NC, or mimics NC. However, the introduction of miR-424 inhibitor partially reversed the impact of shMALAT1 on the cell migration and invasion ability (Fig. 4A–C). Furthermore, the impact of MALAT1 and miR-424 on the tube formation ability of HemECs was analyzed. The results showed that silencing MALAT1 or transfecting miR-424 mimics inhibited the formation of tubule branches in HemECs cells (Fig. 4D and E). In contrast, the treatment of miR-424 inhibitor significantly attenuated the effect of silencing MALAT1 in HemECs. Therefore, these results demonstrated that the cell migration and invasion ability, as well as the tube formation ability of HemECs could be significantly suppressed by silencing MALAT1.

### *3.5. Knockdown of MALAT1 suppressed the IKK/NF-κB pathway by targeting miR-424*

The mechanism of the influence of MALAT1 on IH tumorogenesis was further investigated by Western blotting in investigating the protein levels of IKK/NF-κB pathway. After the knockout of the MALAT1 or transfection of miR-424 mimics, the expressions of MEKK3, p-p65, p-

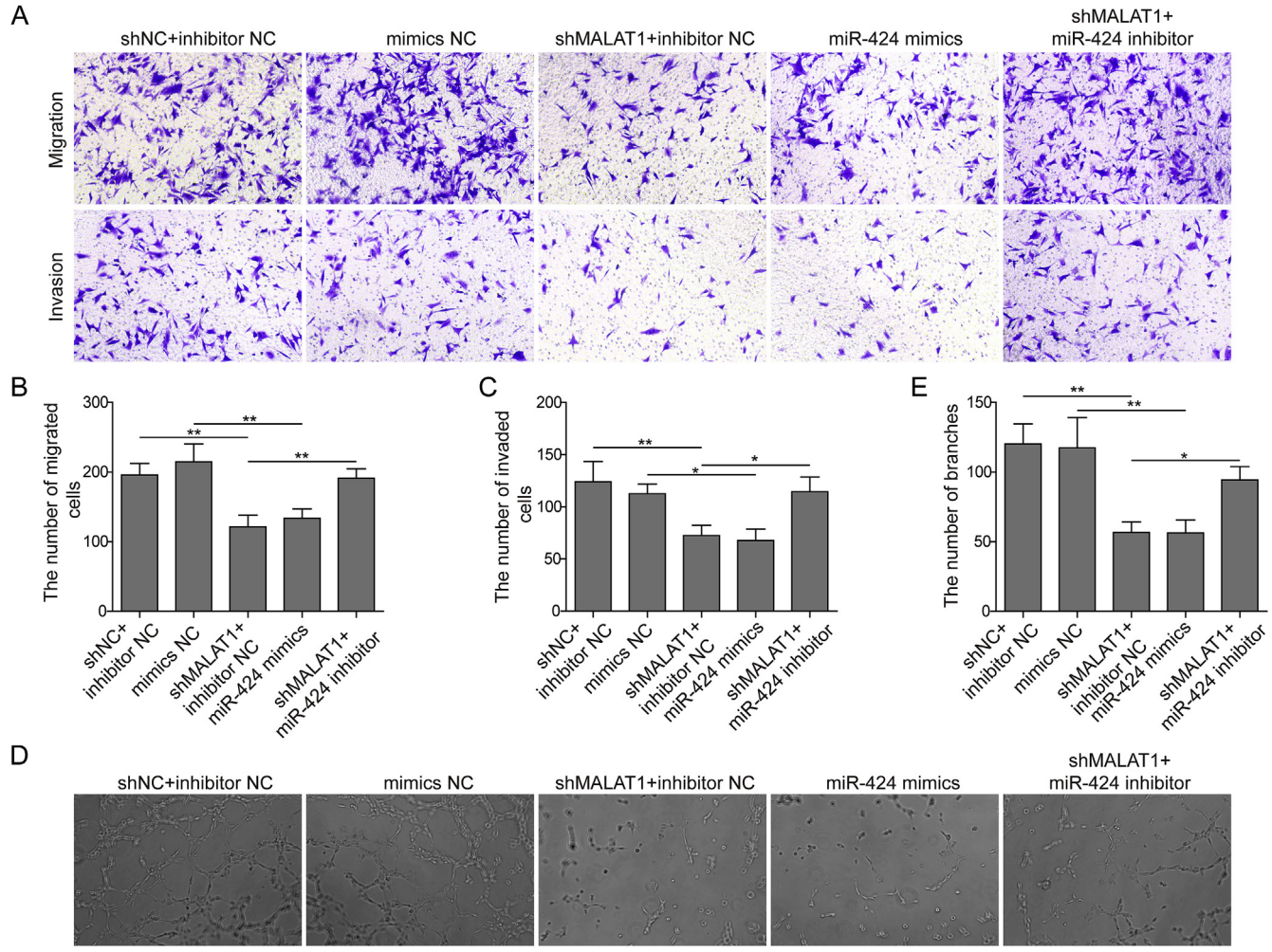


**Fig. 4. MALAT1 regulated migration, invasion and vasoformation of HemECs through miR-424.** (A–C) Transwell assay was performed to investigate the migration and invasion in HemECs cells treated by shMALAT1, miR-424 mimics or miR-424 inhibitor, respectively. Panel A shows the representative images of migrated and invaded cells. Panel B represents the quantification of cell migration. Panel C demonstrates the quantification of cell invasion. (D–E) Tube formation assay was performed to investigate the tube formation ability in HemECs transfected with shMALAT1, miR-424 mimics or miR-424 inhibitor. Panel D shows the images of the tube formation and panel E shows the number of branches. The results were presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

IKKα/β, p-IκBα were significantly inhibited but the expression of IκBα was enhanced (Fig. 5A and B), indicating that knockout of MALAT1 or overexpression of miR-424 could repress IKK/NF-κB signaling pathway. The treatment of miR-424 inhibitor reversed the impact of shMALAT1 on the expressions of these proteins (Fig. 5A and B). Furthermore, the proliferation and apoptosis related proteins, including c-Myc, CyclinD1, Ki-67, Bcl2, and Bax were also analyzed after different treatments (Fig. 5C and D). The results showed that silencing MALAT1 or overexpression miR-424 suppressed the expression of c-Myc, CyclinD1, Ki-67, and Bcl2, but promoted the expression of Bax (Fig. 5C and D). Interestingly, the co-treatment of shMALAT1 with miR-424 inhibitor reversed the effect of shMALAT1 (Fig. 5C and D). All these results indicated that MALAT1 could regulate the IKK/NF-κB pathway and control IH tumorogenesis through regulating miR-424.

### *3.6. Overexpression of MEKK3 reversed the effects of knockdown of MALAT1 and overexpression of miR-424 on HemECs function*

Next, we explored the role of MEKK3 in IH tumorogenesis. Using the qRT-PCR and Western blotting assay, we determined that the overexpression of MEKK3 in HemECs was successful by transfecting with pcDNA3.1-MEKK3 (Fig. 6A and B). We then investigated the impact of MEKK3 on the cell proliferation and apoptosis rate of HemECs by colony formation assay and apoptosis assay. As shown in Fig. 6C–F, knockdown of MALAT1 or overexpression of miR-424 demonstrated lower colony formation rate and higher apoptosis rate. However, the overexpression of MEKK3 in these cells significantly increased the colony formation rate and decreased the apoptosis rate. Moreover, we found out that the cell migration rate and cell invasion rate were relatively low when the MALAT1 was knockdown or miR-424 was overexpressed in HemECs cells (Fig. 7A–C). In contrast, the overexpression of MEKK3 in these cells significantly reversed the impact of the knockdown of MALAT1 or overexpression of miR-424, causing the significantly increase cell migration and invasion rate (Fig. 7A–C). We also investigated the tube formation of the HemECs after the overexpression of MEKK3. The results in Fig. 7D and E showed that the decreased tube formation rated caused by knockdown of MALAT1 or overexpression of miR-424 was significantly recovered by overexpression of MEKK3. Taking together, overexpression of MEKK3 could reverse the impact of knockdown of MALAT1 or overexpression of miR-424 in HemECs on cell proliferation, apoptosis, migration, invasion and tube formation rate.

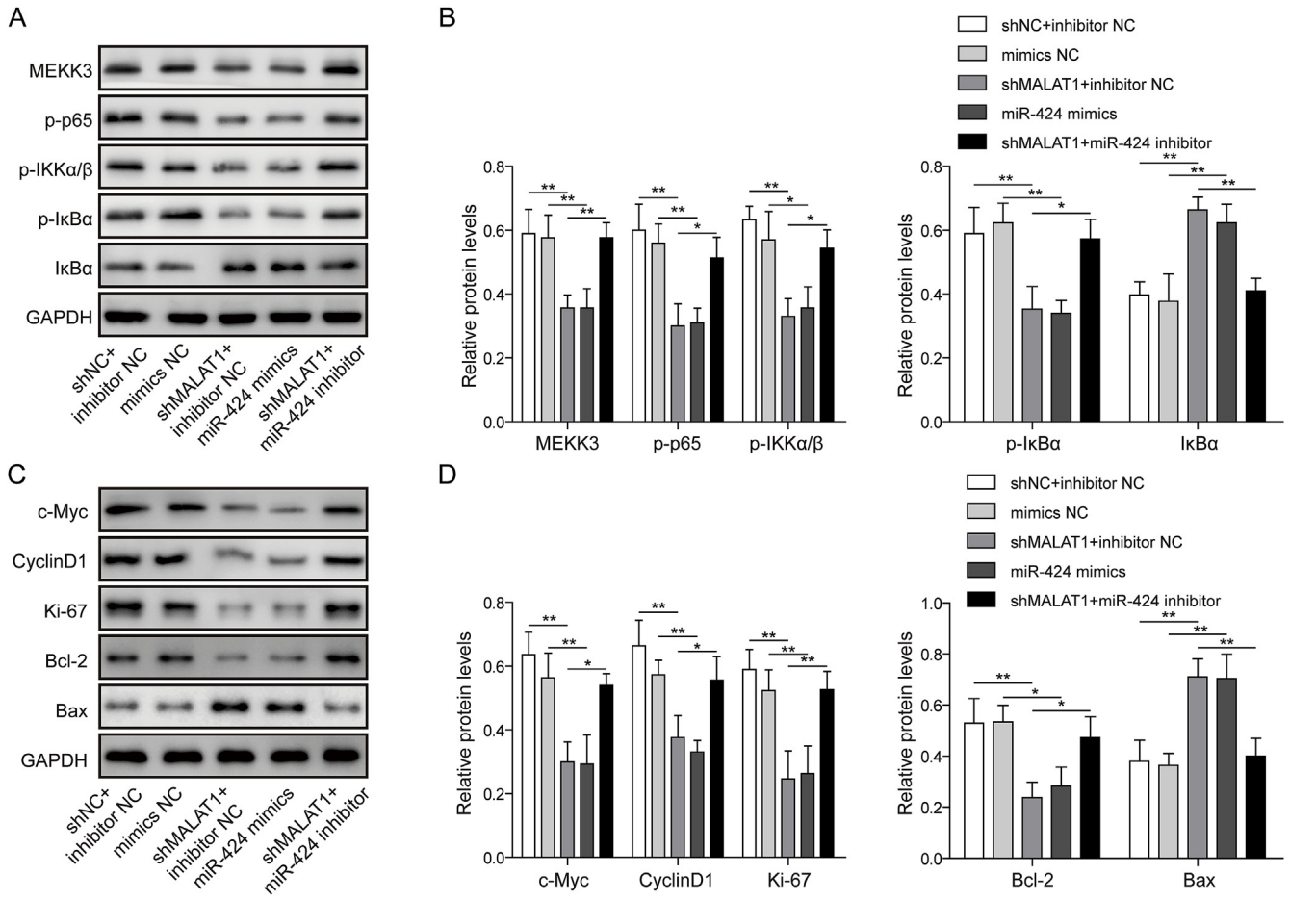


**Fig. 5. MALAT1 regulated the IKK/NF-κB pathway by influencing miR-424.** (A) The protein expression levels of MEKK3, p-p65, p-IKKα/β, p-IκBα and IκBα in HemECs after transfecting shMALAT1, miR-424 mimics or miR-424 inhibitor were quantified by Western blotting. (B) Using GAPDH as an internal control, the relative proteins level was quantified. (C) The expression levels of c-Myc, CyclinD1, Ki-67, Bcl-2, and Bax of HemECs transfected with shMALAT1, miR-424 mimics or miR-424 inhibitor were investigated by Western blotting. (D) Using GAPDH as an internal control, the relative proteins level was quantified. The results were presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

### *3.7. Knockdown of MALAT1 inhibited xenograft growth via regulating miR-424/MEKK3 axis in vivo*

In order to confirm the function of MALAT1 in IH development *in vivo*, HemECs cells transfected with shNC or shMALAT1 were injected subcutaneously into nude mice for examining the development of the tumor. The results showed the growth of the IH tumor after silencing MALAT1 was significantly inhibited (Fig. 8A–C), indicated by the decreased tumor growth rate and lower tumor weight at day 30 in the group treated with shMALAT1. Furthermore, through the qRT-PCR analysis, we found that MALAT1 and MEKK3 were inhibited by silencing MALAT1, but the expression of miR-424 was up-regulated (Fig. 8D). Western blotting analysis demonstrated that the IKK/NF-κB pathway was significantly inactivated by silencing MALAT1, indicating by the down-regulation of MEKK3, p-p65, p-IKKα/β, p-IκBα, Ki-67 and PCNA, but enhanced the expression of IκBα (Fig. 8E and F). Furthermore, the expression of Ki-67 was significantly down-regulated when the tumor xenograft was treated by shMALAT1 by the immunohistochemistry analysis (Fig. 8G). Overall, these results indicated that MALAT1 could promote IH tumor growth by inhibiting miR-424.

## 4. Discussion

In infants and young children, IH is the most frequent benign vascular lesion, in which the endothelial cells grow abnormally [28]. IH mainly occurs in females, preterm children, and children with placental abnormalities during pregnancy [29]. In the IH development, lncRNAs were reported to be associated with cell proliferation, apoptosis and metastasis. For example, Ou et al. found that the tumorigenesis of IH was inhibited by MEG3 through regulating miR-494 and further controlling PTEN/PI3K/AKT pathway [30]. However, the role of other lncRNAs was seldomly known in the development of IH. In the present study, we demonstrated that MALAT1 was elevated in IH tissues, especially in proliferating phase IH. Knockdown of MALAT1 not only suppressed the proliferation, but also inhibited migration, invasion and tube formation ability. In contrast, the apoptosis of HemECs was promoted. The mechanism investigation demonstrated that MALAT1 sponged on miR-424 to regulate MEKK3, resulting in the activation of IKK/NF-κB pathway.

Previous reports indicated that MALAT1 played vital roles in various types of cancer. MALAT1 impacts many biological processes, including cellular proliferation, migration, and apoptosis [19,20]. For example, Tong et al. found that MALAT1 worked as a ceRNA to regulate the vimentin expression in hepatocellular carcinoma by targeting miR-30–5p. MALAT1 was significantly upregulated in HCC cancer tissues and HCC cells, inducing enhanced proliferation, migration and invasion of the HCC cell [20]. MALAT1 involved in the angiogenesis and regulation of endothelial cells. For instance, Lu et al. found that MALAT1 promoted the angiogenesis in brain microvascular endothelial cells by targeting miR-145 [31]. Moreover, MALAT1 prevented the apoptosis of vascular endothelial cells, which was induced by oxygen-glucose deficiency, promoting the tumor development [32]. In colorectal cancer

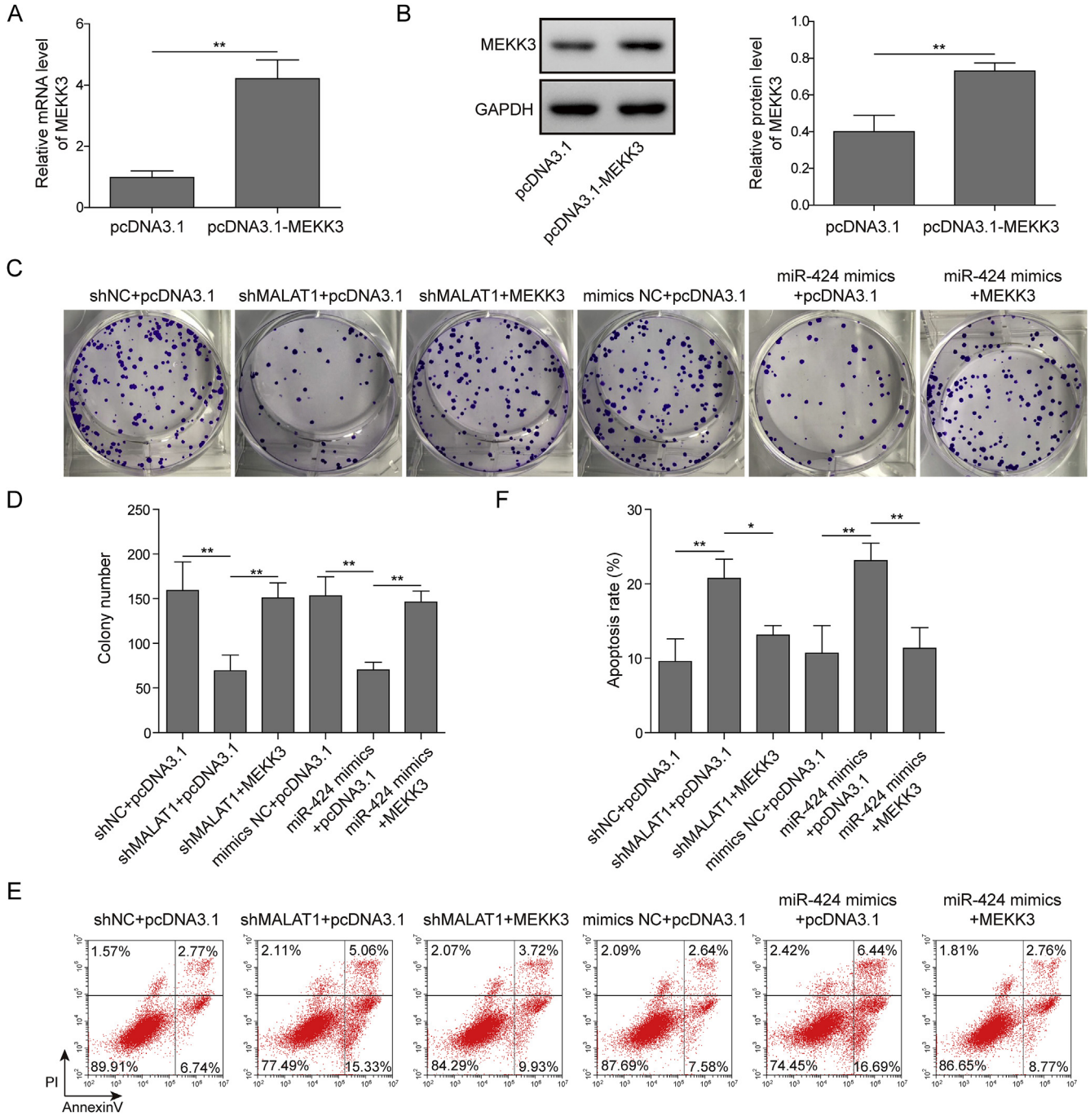


**Fig. 6. Overexpression of MEKK3 reversed the impact of knockdown of MALAT1 or overexpression of miR-424 on cell proliferation and apoptosis in HemECs.** (A) MEKK3 was quantified by qRT-PCR in HemECs transfected with pcDNA3.1 or pcDNA3.1-MEKK3. (B) MEKK3 was quantified by Western blot in HemECs transfected with pcDNA3.1 or pcDNA3.1-MEKK3. (C–D) Colony formation assay was utilized to investigate the cell proliferation ability of HemECs transfected with shMALAT1, miR-424 mimics or pcDNA3.1-MEKK3. (E–F) The HemECs cells tranfected with shMALAT1, miR-424 mimics or pcDNA3.1-MEKK3 were stained by Annexin V and propidium iodide (PI), followed by the analysis of apoptosis using flow cytometry. The results were presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

and breast cancer, MALAT1 was also reported to promote the angiogenesis [22,33]. However, the role and mechanism of MALAT1 in IH remains unknown. In our study, we found that MALAT1 was significantly up-regulated in IH tissues and cells than that in the normal skin. Furthermore, it was reported that MALAT1 could play important role in the development of IH, causing the abnormal proliferation of vascular endothelial cells [34]. Our results demonstrated that after silencing MALAT1, the proliferation, migration, invasion and tube formation ability was significantly suppressed, but the cell apoptosis of HemECs was promoted.

The molecular mechanism of MALAT1 in HemECs was further investigated. miRNAs, especially the miR-15 superfamily, were reported to regulate angiogenesis [35]. miR-424, as a member of miR-15 superfamily member, which locates on human chromosome Xq26.3, is acting as tumor suppressor gene [27]. Nakashima et al. and Ruan et al. both investigated the role of miR-424 in the development of hemangioma [4,36]. The former found that miR-424 acted as a potential inhibitory miRNA in senile hemangioma. Meanwhile, the later found

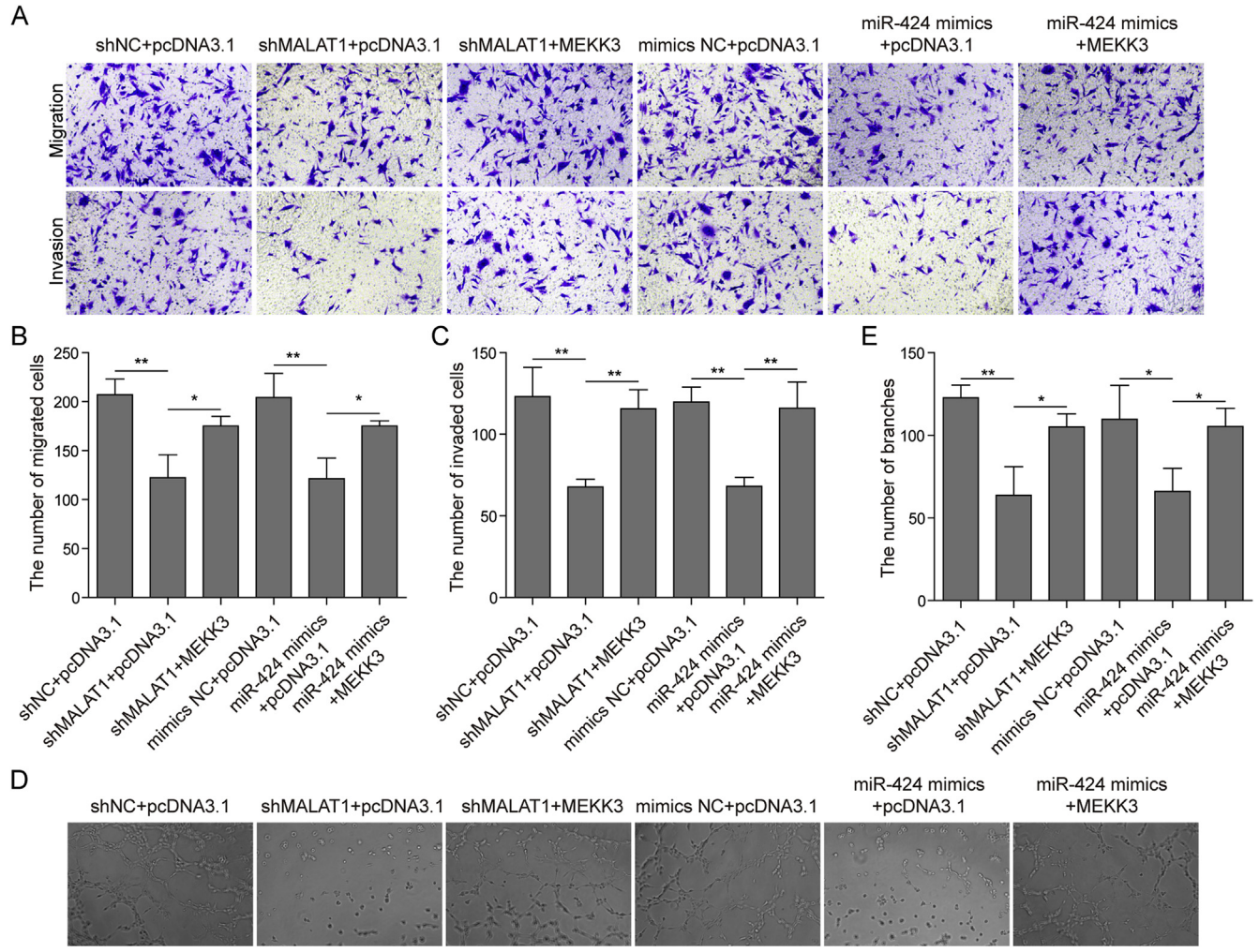


**Fig. 7. Overexpression of MEKK3 reversed the impact of knockdown of MALAT1 or overexpression of miR-424 on cell migration, invasion and tube formation of HemECs.** (A–C) Transwell assay was performed to investigate the migration and invasion in HemECs cells treated byshMALAT1, miR-424 mimics or pcDNA3.1-MEKK3. Panel A shows the representative images of migrated and invaded cells. Panel B represents the quantification of cell migration. Panel C demonstrates the quantification of cell invasion. (D–E) Tube formation assay was performed to investigate the tube formation ability in HemECs transfected with shMALAT1, miR-424 mimics or pcDNA3.1-MEKK3. Panel D shows the images of the tube formation and panel E shows the number of branches. The results were presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

that miR-424 inhibited the cell proliferation, migration and tube formation capabilities in IH by regulating ERK1/2 phosphorylation. However, the impact of miR-424 on the angiogenesis and the function of endothelial cells in IH were rarely reported. Herein, we discovered that miR-424 was one of the targets of MALAT1. The effects of knockout of MALAT1 in HemECs on cell proliferation, migration, invasion and apoptosis were attenuated by the miR-424 inhibitor. miR-424 was working as a tumor suppressor gene in IH by regulating the angiogenesis process, which was agree with the previous reports.

Furthermore, MEKK3 is one of the important factors that regulates angioegenesis, which is the major reason of IH [37]. Deletion of MEKK3 causes angiogenic failure [38]. The bioinformatic analysis indicated that MALAT1 and MEKK3 shared the same response elements of miR-424, indicating that miR-424 might also bind with MEKK3. The analysis of the IH tissues and HemECs demonstrated that MEKK3 also had a significant overexpression compared with the normal tissues. Knockdown of MALAT1 or overexpression of miR-424 significantly decreased the expression of MEKK3. The dual-luciferase assay also confirmed that MEKK3 directly bonded to miR-424. Moreover, we found that the impact of the silence of MALAT1 and overexpression of miR-424 on HemECs cell proliferation, apoptosis, migration, invasion and tube formation rate was partially reversed by the overexpression of MEKK3. In many types of cancer, MEKK3 was found to be related with the increased basal IKK kinase and NF-κB activity [14]. MEKK3 was highly expressed in ovarian carcinoma, correlated with NF-κB activity, which was reported to protect cancer cells from apoptosis, causing drug and radiation resistance [39,40]. Our results also demonstrated that the knockdown of MALAT1 significantly down-regulated MEKK3, p-p65, p-IKKα/β, p-IκBα, Ki-67, PCNA, but enhanced the expression of IκBα, indicating the inactivation of IKK/NF-κB pathway through regulating MEKK3. The inactivation of IKK/NF-κB pathway induced the slower tumor growth for IH. However, we also found that when HemECs cells were treated with shMALAT1 and miR-424 inhibitor, the cell defects were only partially rescued, while the MEKK3 protein level restored to the control level. These results implied that the MALAT1/miR-424 might have other pathways to affect the IH development besides MEKK3. Xu et al. found that miR-424 overexpression inhibited the cervical cancer cell growth through regulating the expression of protein checkpoint kinase (Chk1) and phosphorylated Chk1 (p-Chk1) [41]. In senile hemangioma, Nakashima et al. demonstrated the miR-424 controlled cell proliferation, apoptosis and angiogenesis by regulating the expression of MEK1 and cyclin E1 [42]. Therefore, MEK1/cyclin E1 pathway might be another target that MALAT1/miR-424 could functionalized. Furthermore, miR-424 was found to work on BCR-ABL

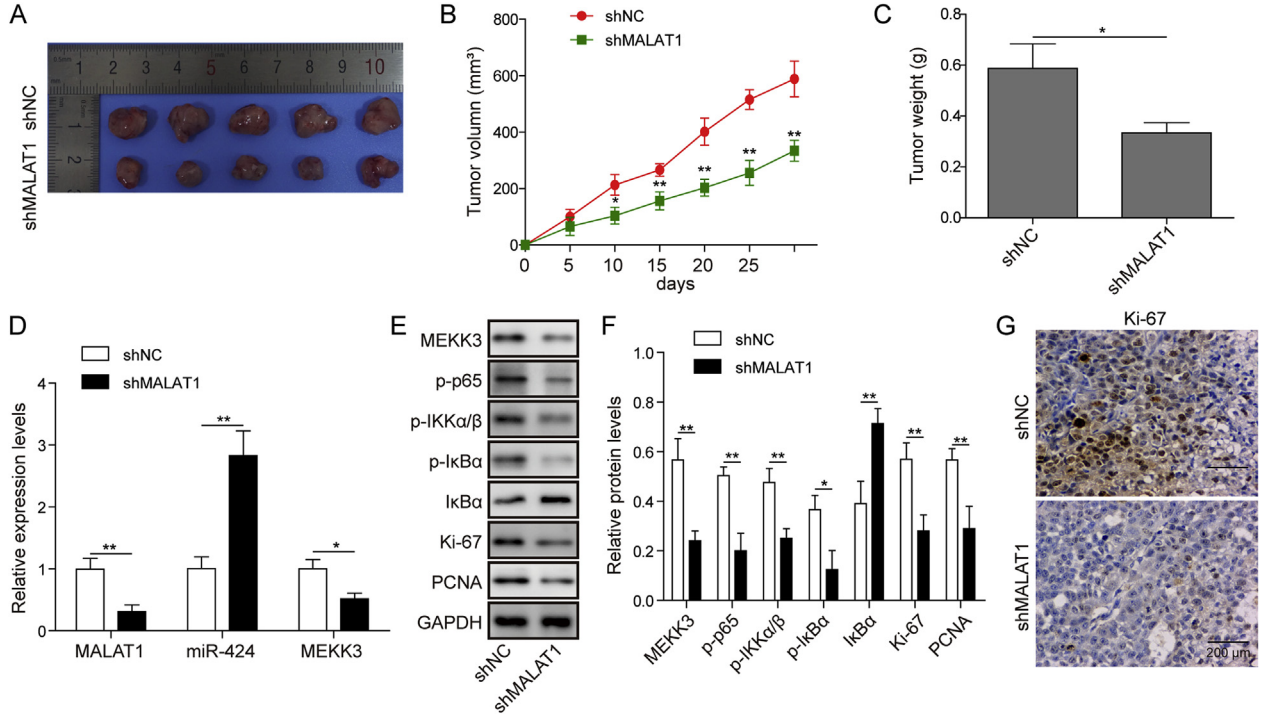


**Fig. 8. MALAT1 regulated tumor growth through miR-424-mediated MEKK3 signal.** (A) The xenograft tumor tissues were collected and presented. (B) Tumor growth was recorded by analyzing tumor volume for 30 days. (C) The weights of xenograft tumor tissues at day 30. (D) MALAT1, miR-424, and MEKK3 in the xenograft tumor tissues were analyzed by qRT-PCR. (E) The proteins expression levels of MEKK3, p-p65, p-IKKα/β, p-IκBα, IκBα, Ki-67, and PCNA were analyzed by Western blotting in the xenograft tumor tissues. (F) Using GAPDH as an internal control, the relative proteins level was quantified. (G) Immunohistochemistry analysis of the expression of Ki-67 in the xenograft tumor tissues. The results were presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01.

expression and activity in chronic myeloid leukemia (CML) cell lines, indicating another possible pathway of MALAT1/miR-424 axi [43]. Therefore, besides regulating MEKK3/NF-κB signaling pathway, MALAT1/miR-424 may also control other signaling pathways to regulate IH tumorigenesis, which deserves further study.

## 5. Conclusion

In summary, our work demonstrates that MALAT1 and MEKK3 are overexpressed in IH, which is negatively correlated with the expression of miR-424. MALAT1 promotes the cell proliferation, migration, invasion and tube formation through suppressing miR-424 to regulate the MEKK3 expression, which finally regulates the IKK/NF-κB pathway in IH. As an upstream regulator of IKK/NF-κB pathway, MALAT1, miR-424 and MEKK3 may be potential therapeutic targets for IH treatment.

## Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Declaration of competing interest

None declared.

## Acknowledgements

None.

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