• Basic Research •

Co-host ncRNA MIR503HG/miR-503-5p antagonistically interfere with the crosstalk between fibroblasts and microvascular endothelial cells by affecting the production of LMW FGF2 in pterygium

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Abstract

● **AIM:** To explore the effect of co-host non-coding RNA (ncRNA) MIR503HG/miR-503-5p on the angiogenesis of pterygium.

● **METHODS:** MIR503HG/miR-503-5p/fibroblast growth factor 2 (FGF2) expression levels in pterygium tissues, control conjunctival tissues, and human pterygium fibroblasts (HPF) were examined by reverse transcription-polymerase chain reaction (qRT-PCR) and immunohistochemical methods. Effects of MIR503HG/miR-503-5p on low molecular weight FGF2 (LWM FGF2), migration and angiogenesis of human retinal microvascular endothelial cells (HRMEC) were determined in an HPF and HRMEC co-culture model using Western blots, wound healing assay, Matrigel-based tube formation assay, and Transwell assay.

● **RESULTS:** MIR503HG/miR-503-5p/FGF2 pathway was actively increased in pterygium tissue and there was a negative correlation between the expression of the two ncRNAs. FGF2 expression level was positively correlated with MIR503HG and negatively correlated with miR-503-5p. Overexpressed MIR503HG/miR-503-5p did not affect the migration and angiogenesis of HRMECs cultured separately, but significantly affected migration and angiogenesis of HRMEC in HPF and HRMEC co-culture models. Western blotting revealed that MIR503HG/miR-503-5p overexpression significantly increased LMW FGF2 expression in HPF.

● **CONCLUSION:** MIR503HG/miR-503-5p inhibits HRMEC migration and angiogenic function by interfering with the interaction between HPF and endothelial cells *via* reducing LMW FGF2 in HPF.

● **KEYWORDS:** pterygium; MIR503HG; miR-503-5p; fibroblast growth factor 2; angiogenesis

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INTRODUCTION

Pterygium is a common ocular surface disease, with a triangular shape. It is created when the conjunctiva and the fibrous vascular tissue beneath it grow abnormally. In China right now, there are about 100 million pterygium patients[1]. Pterygium pulls the cornea, resulting in astigmatism or concealing the pupils, impairing vision. It also affects attractiveness. In extreme circumstances, it may even impair eye movement[2]. Surgical excision is the primary clinical therapy option. Surgery, on the other hand, is invasive, and pterygium is prone to recurrence after surgery. To prevent recurrence, several medications that inhibit angiogenesis and cell proliferation, such as bevacizumab, mitomycin C, 5-fluorouracil, and others, have been discovered and are used in conjunction with surgical treatments[3-5]. However, the impact is unsatisfactory, and even if anti-angiogenesis medications can prevent recurrence, the effect is only brief[6]. The study, creation, and therapeutic use of nucleic acid therapies have advanced significantly in recent years. Non-coding RNA (ncRNA)-based epigenetic mechanism research offers fresh opportunities for therapeutic development[7-9].

Although ncRNAs do not code for proteins, their non-coding transcripts can control how genes are expressed and how proteins work. The two primary subtypes of ncRNAs are

long ncRNAs and short ncRNAs[10]. MiRNA and lncRNA functioned as biomarkers[11-13] and potential therapeutic targets[14-15]. Previously, our research team has constructed a primary competitive endogenous RNA (ceRNA) network through bioinformatics analysis, which includes eight lncRNAs (FOXD2-AS1, MIR503HG, XLOC_002241, LINC00472, CCNT2-AS1, FAM155A-IT1, LINC00638, HOTTIP)[16]. Among them, MIR503HG has been widely studied in tumors and is suspected of having an anti-tumor effect[17-19], but may play a different role in non-tumorous tissue. By sponging the miR-143-3p, MIR503HG inhibited its function, increased the expression of its target gene Smad3, and accelerated the development of hypertrophic scars, according to Wei *et al*[20]; Monteiro *et al*[21] found that knocking down MIR503HG promoted endothelial-to-mesenchymal transition, decreased proliferation and migration of endothelial cells; Zhang *et al*[22] found the inhibition of MIR503HG alleviates HG-Induced cell apoptosis, inflammation, and fibrosis in human renal cortex proximal tubular epithelial cells, to reduce the high glucose (HG)-activated cytotoxicity in diabetes nephropathy. In conclusion, MIR503HG is related to fibrosis and angiogenesis in non-tumorous tissue. Pterygium is a benign tumor-like lesion that exhibits precancerous proliferation, inflammation, invasion, angiogenesis, and extracellular matrix alteration[23]. Pterygium development is intimately linked to fibrosis and neovascularization[24].

The host gene MIR503HG transcribing lncRNA MIR503HG is a conserved gene widely present in primates, locates at the distal end of the long arm of the X chromosome, with a length of 3524 bases and four exons (http://asia.ensembl.org/index.html). MIR503HG is also the host gene of miR-503-5p, and studies have revealed that MIR503HG and miR-503-5p exhibit synergistic transcription[25]. Liu *et al*[26] discovered that miR-503-5p inhibits the expression of the target gene FBXW7, thereby inhibiting high glucose-induced vascular endothelial cell function, and promoting apoptosis of pulmonary microvascular endothelial cells by reducing the expression of Bcl-2[27]. Furthermore, studies have found that miR-503-5p inhibits the migration function of vascular smooth muscle cells by inhibiting the expression of the target gene PAPPA[28]. In conclusion, miR-503-5p has been identified as a miRNA that reduces angiogenesis, but there is also evidence that miR-503-5p can enhance angiogenesis in lung cancer[29]. Based on the reported functions of MIR503HG/miR-503-5p in angiogenesis, and the differential expressions of MIR503HG observed in our previous study[16], we speculated that MIR503HG/miR-503-5p may play a role in neovascularization of pterygium.

The miRDB database (https://mirdb.org/) predicted that fibroblast growth factor 2 (FGF2) is a target gene for miR-503-5p, which was verified by the dual-luciferase reporter assay[30] (miRDB, China, 2010. miRDB is an online database for miRNA target prediction and functional annotations. https://mirdb.org/cgi-bin/target_detail.cgi?targetID=1551782; accessed on 5 July 2022).

In this study, we analyzed the expressions of MIR503HG/miR-503-5p/FGF2 in pterygium and conjunctival tissues. We investigated their function in a co-culture model of human pterygium fibroblasts (HPF) and human retinal microvascular endothelial cells (HRMEC).

MATERIALS AND METHODS

Ethical Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Medical Ethical Committee of Zhongnan Hospital of Wuhan University (Date 2017.11.29/No.2017055). The experimental design was approved by the Medical Ethical Committee of Zhongnan Hospital of Wuhan University, and written informed consent was obtained according to the Declaration of the Helsinki World Medical Association.

Tissue Samples Pterygium tissues and adjacent control conjunctival tissues were resected from 37 patients (21 men and 16 women) who were diagnosed with pterygium at the Department of Ophthalmology, Zhongnan Hospital of Wuhan University, from October 2021 to July 2022. The ages of the subjects ranged from 39 to 74y (mean age 50.51±8.86y). RNA Keeper Tissue Stabilizer (Vazyme, Nanjing, China) was used to preserve all tissues during refrigerator storage at -20°C.

Cell Culture HPF is cultured from resected pterygium tissues in operation. HRMEC was purchased from Shanghai Fuyu Biotechnology Co., Ltd (Fuyu, Shanghai, China). The cells were all maintained at 37°C in a cell incubator with 5% CO_2 in DMEM (Hyclone, Logan, Utah, USA) with 10% fetal bovine serum and 1% penicillin-streptomycin.

Reverse Transcription Quantitative PCR The total RNA of the cells or tissues was extracted with HP Total RNA Kit (Omega, USA) according to the instruction and quantified by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription of miRNA was conducted by using miRNA 1st Strand cDNA Synthesis Kit (by stem-loop; MR101, Vazyme, Nanjing, China) based on the instruction. Reverse transcription of mRNA and lncRNA was conducted by using HiScript® III RT SuperMix for quantitative polymerase chain reaction (qPCR; +gDNA wiper; R323-01, Vazyme, Nanjing, China). Real-time qPCR was conducted by using the ChamQ Universal SYBR qPCR Master Mix (Q711-02/03, Vazyme, Nanjing, China) on CFX96 fluorescence qPCR instrument (Bio-Rad, USA). U6 was used as an internal reference for miR-503-5p, and GAPDH was used as an internal reference for FGF2 and MIR503HG. The $2^{-\Delta\Delta CT}$ method was used to detect the relative RNA expression. The primer sequence was shown in Table 1.

Table 1 Primers used for qRT-PCR

Gene	Primer sequence 5'-3'
MIR503HG	F: TCCCGCCAAATGAGTCAGTC
	R: CAGAGTTGTGACCACTGCCT
MiR-503-5p	RT: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCA CTGGATACGAC
	F: TAGCAGCGGGAACAGTTCTGCAG
	R: GTGCAGGGTCCGAGGT
FGF2	F: AGTGTGTGCTAACCGTTACCT
	R: ACTGCCCAGTTCGTTTCAGTG
GAPDH	F: GCACCGTCAAGGCTGAGAAC
	R: TGGTGAAGACGCCAGTGGA
U6	F: TGCTTCGGCAGCACATATAC
	R: TCACGAATTTGCGTGTCATC

qRT-PCR: Reverse transcription quantitative polymerase chain reaction.

Cell Transfection MIR503HG overexpression plasmid (pEXP-RB-Mam-MIR503HG), the mimics, and their negative control were synthesized by RiboBio (RiboBio, Guangzhou, China). Transfection of mimics into cells was performed using the riboFECT CP Transfection Kit (RiboBio, Guangzhou, China) according to the instructions. In contrast, plasmid transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the instructions. The cells were accordingly divided into the following groups: MIR503HG (transfected with pEXP-RB-Mam-MIR503HG), vector (transfected with the negative control of pEXP-RB-Mam-MIR503HG), miR-503-5p mimic (transfected with mimics of miR-503-5p), mimic NC (transfected with negative control mimics), MIR503HG+miR-503-5p (co-transfected with pEXP-RB-Mam-MIR503HG and miR-503-5p mimic), vector+mimic NC (co-transfected with the negative control of pEXP-RB-Mam-MIR503HG and negative control mimics).

Immunohistochemical Paraffin-embedded blocked 3 pterygium tissues and 3 adjacent control conjunctival tissues, then sections were prepared, dewaxed, and dehydrated. Sections were placed in citric acid antigen repair buffer (pH 6.0) using a microwave oven for antigen repair. Endogenous peroxidase was blocked with 3% H_2O_2 and non-specific binding was blocked with 3% bovine serum albumin. Sections were incubated with FGF2 rabbit monoclonal antibody (Abclonal, Wuhan, China) diluted at 1:100 at 4°C overnight, washed with phosphate buffered saline (PBS), and then incubated with goat anti-rabbit antibody labeled with horseradish peroxide (HRP) diluted at 1:500 at room temperature for 50min. Sections were rewashed with PBS again, then counterstained with hematoxylin, differentiated with 1% hydrochloric acid alcohol (about 1s), rinsed with tap water, returned to blue with ammonia, and washed with tap water, dehydrated, and mounted. The nucleus stained with hematoxylin in the optical microscope is blue, and the positive expression of FGF2 is brownish brown.

Wound Healing Assay The wound healing assay was used to measure cell migratory capacity. At a density of 1.25×10^5 cells/mL, HRMECs were planted in 6-well plates and cultivated until the cells uniformly covered 80% of the plate area. At that moment, the HRMECs were transfected in triplicate by the experimental groups described above. After 48h of transfection. A straight scratch was made by a 200 μL sterile pipette tip in the monolayer. Using an inverted fluorescence microscope and the associated software, each well was photographed at 0, 12, and 24h after scratching in numerous views of the scratched area with one straight gap in the middle of the photo. Image J 1.8.0 software (National Institutes of Health, Bethesda, MD, USA) was used to analyze the migration rate.

Matrigel-based Tube Formation Assay The angiogenesis capacity of HRMEC was assessed by Matrigel-based tube formation assay. The 24-well plate and 200 μL pipette tips were pre-cooled, and the sub-packaged Matrigel (Corning, Tewksbury, MA, USA) was dissolved at 4°C. The bottom of the 24-well plate was covered with 30 μL Matrigel using the 200 μL pipette tips on ice. Then, the 24-well plate was placed into a cell incubator with 5% CO_2 at 37°C for 30min to solidify the Matrigel. The transfected HRMECs were seeded in a 24-well plate [2×10^5 cells/well; In HPF and HRMEC co-culture model, the suspended HPFs were added to the upper chamber of the transwell inserts (NY 14831, Costar, Corning Inc., USA) and the suspended HRMECs were added to the 24-well plate which was covered with 30 μL solidified Matrigel] and put in a cell incubator for 6h. In the end, an inverted fluorescent microscope was used to take pictures of the HRMECs. Image J 1.8.0 software (National Institutes of Health, Bethesda, MD, USA) was used to calculate the length and branch points of the vessels.

Western Blots The treated cells were lysed in RIPA lysate including protease inhibitor (Beyotime, Shanghai, China), centrifuged for 10min at 4°C at 12 000 rpm, and total protein was located in the supernatant. The BCA method was used to determine the protein concentration. The total protein was separated by 10% SDS-PAGE and then transferred to the PVDF membrane. The PVDF membrane was sealed at room temperature with 5% skim milk for 1h and then incubated with FGF2 rabbit monoclonal antibody (Abclonal, Wuhan, China) diluted at 1:2000 and GAPDH rabbit antibody (Huabio, Hangzhou, China) diluted at 1:5000 overnight at 4°C. Then, washed the PVDF membrane with Tris-buffered saline with Tween-20 (TBST), and then incubated with HRP-goat anti-rabbit (Jackson, USA) diluted at 1:5000 for 30min at room temperature. ECL-A and ECL-B (Biosharp, Guangzhou, China) were mixed and dropped onto the PVDF membrane. Then, the HB-980 exposure instrument (Protein Simple, USA)

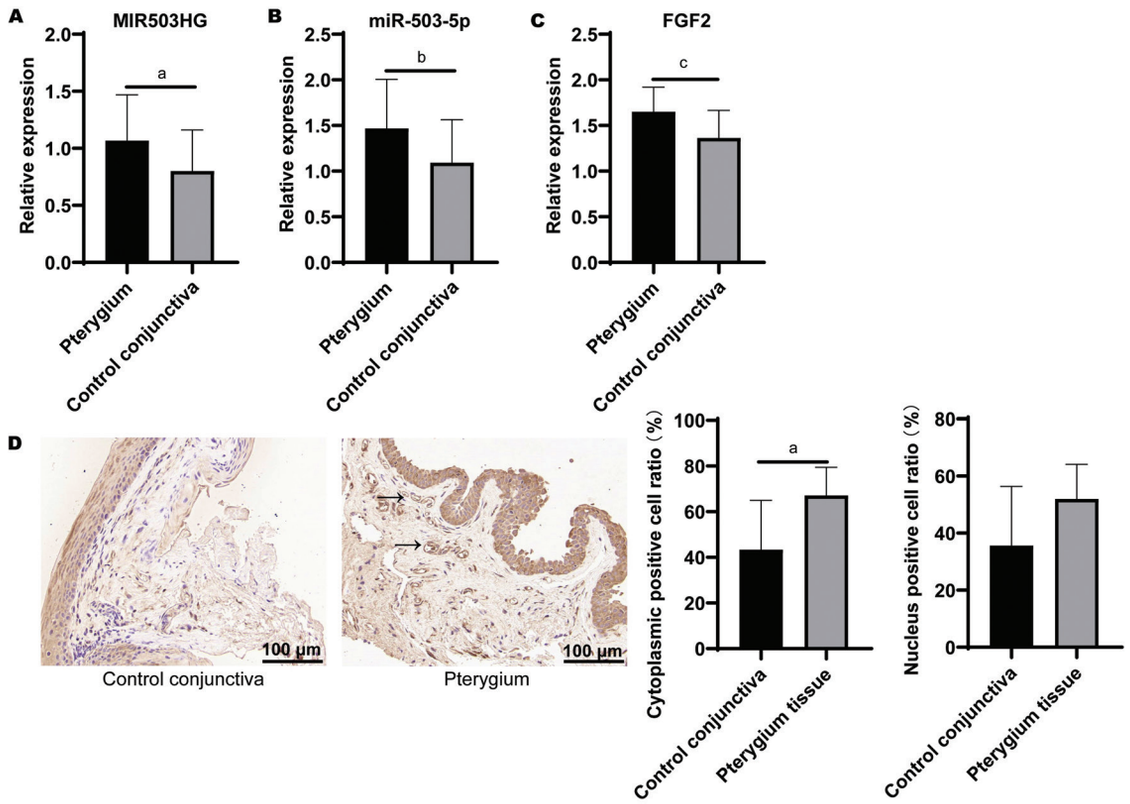

Figure 1 Differential expressions of MIR503HG/miR-503-5p/FGF2 in pterygium tissue and control conjunctival tissue A: The mRNA expression levels of MIR503HG in pterygium tissue and control conjunctival tissue; B: The mRNA expression levels of miR-503-5p in pterygium tissue and control conjunctival tissue; C: The mRNA expression levels of FGF2 in pterygium tissue and control conjunctival tissue; D: FGF2 immunohistochemical results of pterygium tissue and conjunctival tissue (400×, bar=100 μm). The arrows indicate microvessels. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001. FGF2: Fibroblast growth factor 2.

was used for exposure. Adobe Photoshop software (Adobe, USA) was used to organize the exposed photos, and Image J 1.8.0 software (National Institutes of Health, Bethesda, MD, USA) was used to analyze the images.

Transwell The transfected HPFs were seeded in a 24-well plate (5×10^5 cells/well) with 600 μL of DMEM/high glucose (SH30022.01, Hyclone, Logan, Utah, USA) containing 20% fetal bovine serum. After the HPFs adhered to the wall, the 24-well format transwell inserts with 8.0 μm pore size (NY 14831, Costar, Corning Inc., USA), were put on the 24-well plate. At the same time, HRMEC was digested and re-suspended with serum-free DMEM/high glucose (SH30022.01, Cyclone, Logan, Utah, USA). Added the suspended HRMEC to the upper chamber of the transwell inserts (1×10^5 cells per insert), and the 24-well plate was placed in the cell incubator with 5% CO_2 at 37°C for 24h. After 24h, take out the transwell insert and clean it several times with PBS. Fixed the transwell insert in methanol for 15-20min, washed it with PBS, and stained it in crystal violet dye solution with a final concentration of 0.1% for 20min. After staining, we cleaned it with PBS and wiped off any cells that had not penetrated the PET track-etched membrane of the transwell insert with a cotton swab. After drying slightly, we placed the transwell insert under an inverted fluorescence microscope for observation and photography. Image J 1.8.0 software (National Institutes of Health, Bethesda, MD, USA) was used for image analysis.

Statistical Analysis Every experiment was carried out at least three times. The statistics were analyzed using GraphPad 8.0 software (GraphPad Software, San Diego, CA, USA). Before analysis, statistics that did not conform to the normal distribution or had uneven variance were logarithmically converted using SPSS 23.0 software (IBM, Armonk, NY, USA). The *t*-test was used to evaluate whether there were significant differences between the two groups, and a one-way analysis of variance was employed to compare more than two groups. $P<0.05$ was considered statistical significance.

RESULTS

Expression of MIR503HG, miR-503-5p, and FGF2 The expression levels of MIR503HG, miR-503-5p, and FGF2 were significantly increased in pterygium tissue compared to control conjunctival tissue (Figure 1A-1C), indicating that the three may be related to the formation of pterygium. The high molecular weight (22, 22.5, 24, 34 kDa) FGF2 (HMW FGF2) isoform generated by the translation of the CUG initiator codon is located in the nucleus. In comparison, the low molecular weight (18 kDa) FGF2 (LMW FGF2) generated

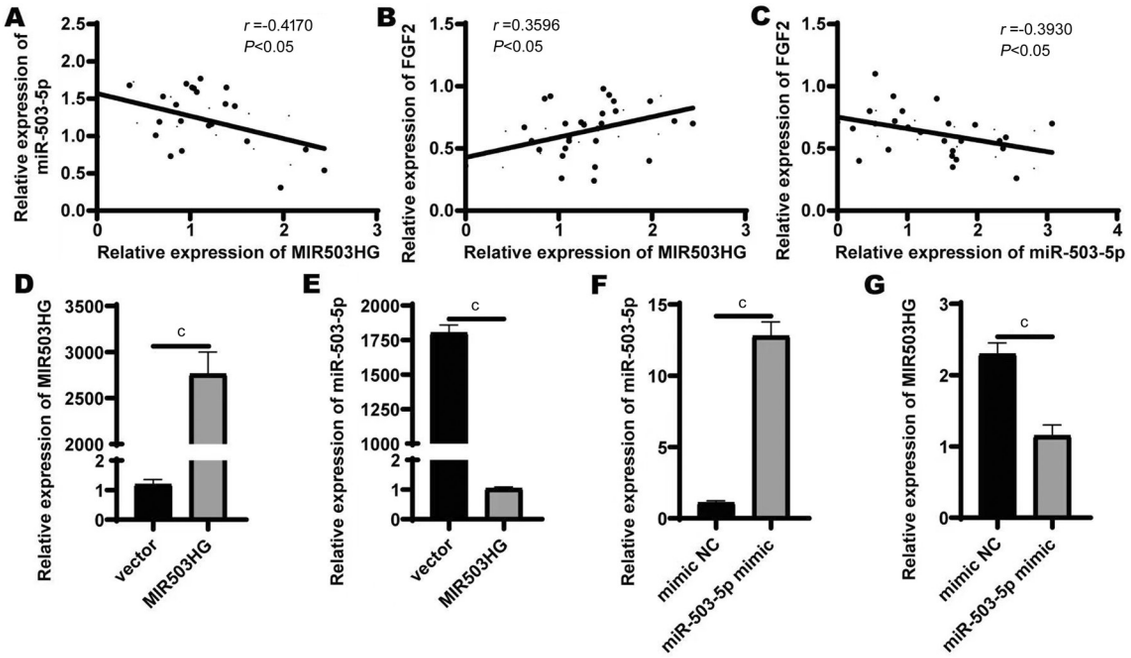

Figure 2 Correlations among expressions of MIR503HG, miR-503-5p, and FGF2 in pterygium tissue, and the mutual inhibition of MIR503HG/miR-503-5p expressions in HPF cells A: In pterygium tissue, MIR503HG is negatively correlated with the expression of miR-503-5p; B: In pterygium tissue, there is a positive correlation between MIR503HG and FGF2 expression; C: In pterygium tissue, miR-503-5p is negatively correlated with the expression of FGF2; D: Overexpression of MIR503HG in HPF; E: After overexpression of MIR503HG in HPF, the expression of miR-503-5p significantly decreased; F: Overexpression of miR-503-5p in HPF; G: After transfection with miR-503-5p mimics in HPF, the expression of MIR503HG is significantly decreased. ^{c}P<0.001. FGF2: Fibroblast growth factor 2; HPF: Human pterygium fibroblasts.

by the translation of the AUG initiator codon is involved in autocrine and paracrine processes, so it is mainly located in the cytoplasm. Immunohistochemistry revealed that the epithelial layer and stroma of pterygium had significantly higher positive levels of FGF2 than did the conjunctival tissue. Compared with the control conjunctival tissue, the ratio of cytoplasmic positive cells in the stroma of pterygium was significantly increased (P<0.05; Figure 1D). In contrast, the ratio of nuclear positive cells did not increase substantially, despite the higher average value. This showed that compared to controls, the pterygium tissue most likely had an increase in LMW FGF2 rather than HMW FGF2. Additionally, in pterygium tissue, it was observed that FGF2 was strongly positive in neovascular endothelial cells. The arrangement pattern of epithelial cells in control conjunctival tissue can be observed, while in pterygium tissue, the epithelial cells are incredibly disorganized and many more microvessels are visible (Figure 1D).

Correlation Between Expressions of MIR503HG, miR-503-5p, and FGF2 In pterygium tissue, MIR503HG, miR-503-5p, and FGF2 expressions were correlated. MIR503HG was negatively correlated with miR-503-5p expressions (P<0.05; Figure 2A), while FGF2 was positively correlated with MIR503HG expressions (P<0.05; Figure 2B) and negatively correlated with miR-503-5p expressions (P<0.05; Figure 2C). MiR-503-5p expression was significantly reduced when MIR503HG was overexpressed in HPF (P<0.001; Figure 2E), while MIR503HG expression was decreased when miR-503-5p mimics were transfected into HPF (P<0.001; Figure 2G). MIR503HG and miR-503-5p are co-host ncRNAs, and the crossover between splicing and miRNA processing factors influences both the lncRNA and the miRNA, which may be a "collaborative/cooperative model" or a "competitive model"[31]. As a result, the negative connection between MIR503HG and miR-503-5p expressions in HPF cells and pterygium tissue is speculated to be owing to this "competitive model".

MIR503HG and miR-503-5p Overexpression on Migration and Angiogenic Function of HRMEC The effects of MIR503HG and miR-503-5p overexpression in HRMECs on their migration and angiogenic function were detected. MIR503HG overexpression plasmid, empty plasmid, miR-503-5p mimic, and mimic NC were transfected into HRMECs, respectively. The migration function of HRMEC was detected through scratch experiments. There was no significant difference in the migration rate of HRMEC among the groups at 12 and 24h after treatments (Figure 3A). The angiogenic activity of HRMEC was tested through tube formation assay. There was no significant difference in vascular length among the groups (Figure 3B). The results showed that whether the MIR503HG or miR-503-5p were overexpressed in HRMEC, there was no significant effect on migration and angiogenic functions. This indicates that the target cells of MIR503HG and miR-503-5p regulation on angiogenesis were not HRMEC.

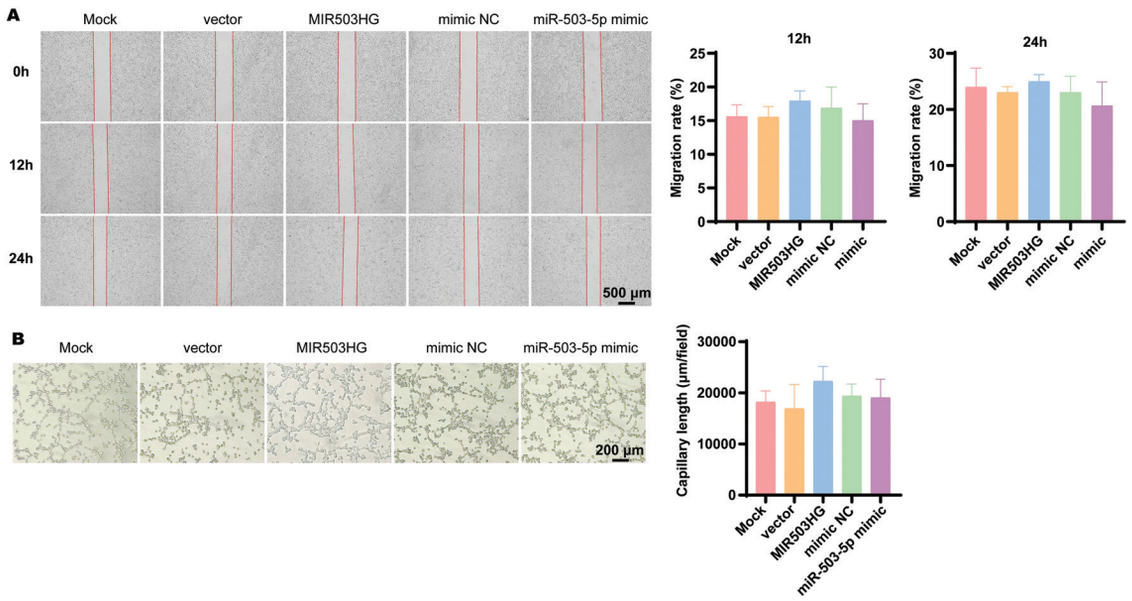

Figure 3 MIR503HG/miR-503-5p overexpression in HRMEC on its migration and angiogenic function A: Overexpression of MIR503HG or miR-503-5p mimics in HRMEC did not significantly affect the migration function of HRMEC; B: Overexpression of MIR503HG or miR-503-5p mimics in HRMEC did not considerably affect the angiogenic function of HRMEC. HRMEC: Human retinal microvascular endothelial cells.

MIR503HG and miR-503-5p Overexpression in HPF on Migration and Angiogenic Function of HRMEC in Co-culture Model Then we used a co-culture model of HPF and HRMEC to test whether the migration and angiogenic function of HRMEC could be indirectly regulated by HPF with overexpression of MIR503HG and miR-503-5p. At first, MIR503HG overexpression plasmid, empty vector, miR-503-5p mimic, mimic NC, MIR503HG overexpression plasmid+miR-503-5p mimic or empty vector+mimic NC were transfected into HPF, respectively. The transfected HPF cells were co-cultured with HRMEC before the migration function of HRMEC was tested through transwell. The migration function of HRMEC in the MIR503HG overexpression group was significantly enhanced (P<0.001; Figure 4A), while the migration function of HRMEC in the miR-503-5p overexpression group was significantly weaker than that in the mimic NC group (P<0.01; Figure 4A). The migration function of HRMEC in the MIR503HG+miR-503-5p group was stronger than that in the vector+mimic NC group (P<0.001; Figure 4A).

In tube formation experiments, HRMEC were classified as MIR503HG overexpression, empty vector, miR-503-5p mimic, mimic NC, MIR503HG overexpression+miR-503-5p mimic, or empty vector+mimic NC based on the transfected HPF. Figure 4B showed the experimental results, which represent the angiogenic function of HRMEC by assessing the total length of blood vessels produced. HRMEC angiogenic function was significantly greater in the MIR503HG over-expression group than in the empty plasmid group (P<0.001; Figure 4B). In contrast, HRMEC angiogenic function was significantly weaker in the miR-503-5p mimic group than in the mimic NC group (P<0.01; Figure 4B). HRMEC angiogenic function was substantially stronger in the MIR503HG+miR-503-5p group than in the vector+mimic NC group (P<0.01; Figure 4B). Indicating that miR-503-5p can reduce endothelial cell migration function and angiogenic function by regulating HPF. However, MIR503HG has the opposite effect and MIR503HG can reverse the inhibitory effect of miR-503-5p on the angiogenic function of HRMEC.

MIR503HG and miR-503-5p Overexpression on LMW FGF2 in HPF Activated fibroblasts can induce angiogenesis *via* paracrine FGF2[32], and LMW FGF2, with a molecular weight of 18 kDa, is the only secretory transcript of FGF2. In our experiment, the effects of MIR503HG and miR-503-5p overexpression on the FGF2 produced by HPF were studied. FGF2 expression was considerably higher in HPF cells overexpressing MIR503HG than in controls (P<0.01; Figure 5A), but significantly lower in HPF cells overexpressing miR-503-5p (P<0.001; Figure 5B). Western blots was used to detect the protein expression of LMW FGF2 in each group of HPF (Figure 5C). Expression level of LMW FGF2 protein in the MIR503HG group was significantly higher than that in the vector group (P<0.05; Figure 5C), indicating that MIR503HG can promote the HPF expression of LMW FGF2. The expression level of LMW FGF2 protein was significantly lower in the miR-503-5p mimic group than in the mimic NC group (P<0.05; Figure 5C), indicating that miR-503-5p suppressed HPF expression of LMW FGF2. The expression of LMW FGF2 protein was slightly higher in the MIR503HG+miR-503-5p mimic group than in the vector+mimic NC group, indicating that MIR503HG overexpression can reverse the inhibitory effect of miR-503-5p on LMW FGF2 expression.

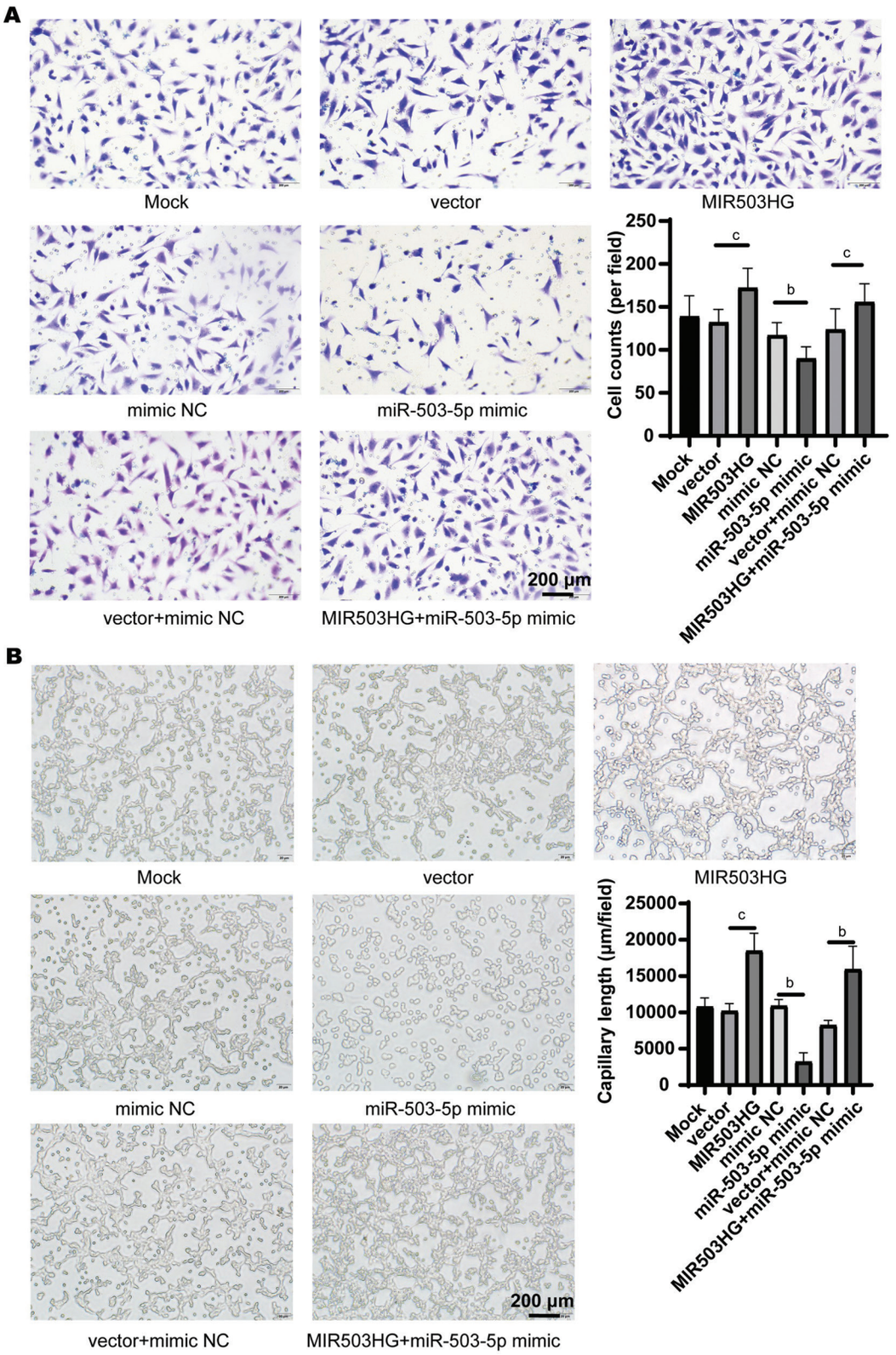

Figure 4 MIR503HG/miR-503-5p overexpression in HPF impacted the migration and angiogenic function of HRMEC in a co-culture model
A: In co-culture model, HRMEC migration function was significantly enhanced when co-cultured HPF with MIR503HG overexpression; HRMEC migration function was significantly weakened when co-cultured HPF transfected with miR-503-5p mimics; the effect of miR-503-5p could be rescued when co-cultured HPF co-transfected with MIR503HG overexpression plasmid and miR-503-5p mimics; B: In co-culture model, HRMEC angiogenic function was significantly enhanced when co-cultured HPF with MIR503HG overexpression. However, when HPF transfected miR-503-5p mimic, the results were the opposite; when HPF was co-transfected with MIR503HG overexpression plasmid and miR-503-5p mimics, the effect of miR-503-5p could be rescued. $^{b}P<0.01$, $^{c}P<0.001$. HRMEC: Human retinal microvascular endothelial cells; HPF: Human pterygium fibroblasts.

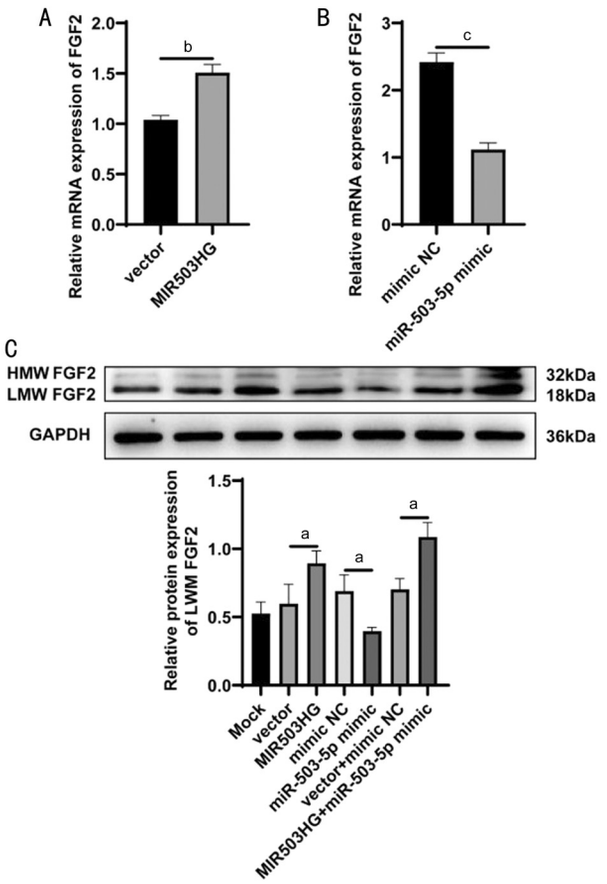

Figure 5 Expression of FGF2 was regulated by MIR503HG/miR-503-5p in HPF A: Expression level of FGF2 significantly increased in HPF with the MIR503HG overexpression plasmid; B: After HPF transfection with miR-503-5p mimics, the expression of FGF2 significantly decreased; C: MIR503HG overexpression resulted in a significant increase of LMW FGF2 in HPF; Transfection with miR-503-5p mimics resulted in a significant decrease in HPF expressing LMW FGF2; Transfection of the MIR503HG overexpression plasmid reversed the inhibitory effect of miR-503-5p on LMW FGF2 expressing in HPF. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. LMW: Low molecular weight; FGF2: Fibroblast growth factor 2; HPF: Human pterygium fibroblasts.

When combined with the results of the HPF and HRMEC co-culture experiment, the expression of LMW FGF2 in the HPF transfection group was significantly lower in the miR-503-5p mimic group compared to the control group, and HRMEC migration and angiogenic function were also considerably lower. The MIR503HG group produced the opposite results. As a result, we speculate that MIR503HG and miR-503-5p indirectly regulate HRMEC migration and angiogenic function by influencing the expression level of LMW FGF2 in HPF.

DISCUSSION

Pterygium, a frequent ocular surface condition, impairs aesthetics and eyesight in severe cases; yet, the particular pathophysiology of pterygium remains unknown. Pterygium has two significant characteristics: matrix fibrosis and angiogenesis, and decreasing angiogenesis is also a treatment target[33]. The primary treatment for pterygium at the moment is surgical resection, although the problem of postoperative recurrence is still challenging to tackle. The anti-recurrence effect of multiple anti- vascular endothelial growth factor (VEGF) drugs is also not ideal, which probably is due to the hostile microenvironment involved in angiogenesis in pterygium. For example, activated fibroblasts in the pterygium microenvironment may play a key role in angiogenesis *via* interaction with endothelial cells. As a result, there is still room for improvement in the therapy of pterygium, and research on ncRNA may offer fresh perspectives.

ncRNA has been linked to the emergence and growth of pterygium in certain research. Gao *et al*[34] discovered that FOXD2-AS1 stimulates cell proliferation and prevents apoptosis in recurrent pterygium cell lines *via* the miR-205-VEGF pathway. Wu *et al*[35] found that miR-221 negatively regulates downstream p27Kip1 gene expression and is involved in the pathogenesis of pterygium. Additionally, our group has revealed that miR-199a-3p/5p targets DUSP5/MAP3K11 in the pterygium epithelial-mesenchymal transition produced by epidermal growth factor (EGF), contributing to transforming growth factor (TGF)-β[36]. These ncRNA are involved in the occurrence and development of pterygium and play a role in the pathological process of pterygium. As therapeutic medicines, ncRNAs have the benefit of targeting many targets. Suppose we find a ncRNA that can regulate angiogenesis and activated fibroblasts. In that case, we may have found a new target for a more effective anti-recurrence medication for pterygium.

In our study, we hypothesized that MIR503HG and miR-503-5p, two co-host non-coding RNAs, could influence angiogenesis in pterygium *via* regulating the production of LMW FGF2 in pterygium fibroblasts. We discovered that: 1) there were significant differential expressions of MIR503HG, miR-503-5p, and FGF2 in pterygium tissue compared to control conjunctival tissue; 2) there was a negative correlation between expressions of MIR503HG and miR-503-5p in pterygium tissue, and FGF2 expression is negatively correlated with miR-503-5p expression but positively correlated with MIR503HG expression; 3) MIR503HG and miR-503-5p overexpression in HRMEC cannot directly affect migration and angiogenic function; however, in a co-culture model, HPF with MIR503HG/miR-503-5p overexpression could; 4) LMW FGF2 expressions were induced by MIR503HG overexpression but inhibited by miR-503-5p mimic transfection in HPF. These findings imply that MIR503HG and miR-503-5p may influence angiogenesis in pterygium by affecting fibroblast-vascular endothelial cell communication. FGF2, particularly LMW FGF2, is essential for interacting between fibroblasts and vascular endothelial cells.

FGF2 is a peptide synthesized by fibroblasts that can stimulate rapid proliferation of fibroblasts at low doses[37]. FGF2 is involved in cell proliferation, wound healing, and various endocrine signaling pathways, and has a strong ability to promote angiogenesis[37-40], while FGF2 downregulation results in angiostasis and endothelial cell apoptosis[41]. Five subtypes of FGF2 proteins in humans are divided into HMW FGF2 and LMW FGF2 according to their molecular weight[39]. LMW FGF2 is translated from the traditional Kozak AUG initiator codon and contains 155 amino acids, representing the core sequence common to all FGF2 subtypes. HMW FGF2 is translated from the upstream CUG initiator codon and is an amino-terminal extension of LMW FGF2[42]. LMW FGF2 and HMW FGF2 not only have different molecular weights but also have different expression localization and functions[43]. LMW FGF2 is mainly expressed in the cytoplasm and stored in the cytoplasm, while HMW FGF2 is often located in the nucleus[39]. LMW FGF2, as the only secretory FGF2, can participate in direct intercellular interactions[39,42]. Efstathios found high expression of FGF2 in mast cells, epithelial cells, and vascular endothelial cells of pterygium[44]. Mastronikolis *et al*[45] found that the expression level of FGF2 in pterygium was significantly higher than that in the control conjunctiva, with the highest level in recurrent pterygium. However, these studies did not provide direct evidence for FGF2 involvement in the crosstalk between fibroblasts and endothelial cells.

Our experiment has some limitations, such as the relatively small number of tissue samples. However, the paired pterygium tissue and control conjunctival tissue might provide reliable results. Moreover, the specific mechanism of interaction between MIR503HG and miR-503-5p on co-host ncRNA needs to be further studied. Since no binding site has been found between MIR503HG and miR-503-5p according to the prediction of the miRDB website (miRDB, China, 2010. miRDB is an online database for miRNA target prediction and functional annotations. https://mirdb.org/cgi-bin/target_detail.cgi?targetID=1551782; accessed on 5 July 2022), it may be necessary to pay attention to the effects related to splicing factors, miRNA biological components, and translation factor regulation[31,46]. Finally, the mechanism for the angiogenic function of LMW FGF2 in pterygium needs further studies. It is attributed to the direct role of secreted LMW FGF2 or indirect role with downstream protein signaling, which has not been clarified. Park *et al*[47] found that cyclooxygenase 2 (COX2), as a key enzyme in inflammatory cytokine-induced angiogenesis, is not present in normal conjunctiva but in pterygium. The downstream pathways regulated by FGF2, such as COX2 can also be further explored.

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