IGF-1 Promotes Epithelial-Mesenchymal Transition of Lens Epithelial Cells That is Conferred by miR-3666 Loss

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Research Article

Keywords: Posterior capsular opacification , EMT, IGF-1, miR-3666

Posted Date: June 2nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-488224/v1

Abstract

The abnormal proliferation, migration and epithelial-mesenchymal transformation (EMT) of lens epithelial cells (LECs) are the main reasons of vision loss caused by posterior capsular opacification (PCO) after cataract surgery. Insulin-like growth factor-1 (IGF-1) was found to be associated with the pathogenesis of cataract, but its biological role in PCO is poorly understood. In the present study, IGF-1 overexpression facilitated the proliferation, migration and EMT, whereas knockdown of IGF-1 markedly suppressed the proliferation, migration and TGF-β2-induced EMT of LECs. Additionally, To evaluate valuable microRNAs (miRNAs) which target IGF-1 to modulate LECs-EMT, we predicted miR-3666 might regulate IGF-1 by binding its 3'UTR according bioinformatics database. Furthermore, we verified that miR-3666 directly targeted to IGF-1 by luciferase reporter assay. By using miR-3666 mimics, cells proliferation, migration, and invasion were suppressed, while were enhanced by reduction of miR-3666. Knockout of IGF1 reverses the effect of miR-3666 inhibitor on the malignant behavior of LECs. These results indicate the role for miR-3666/IGF-1 in LECs-EMT that offer new strategies for the therapy and prevention of PCO.

1 Introduction

Cataract is the major cause of blindness in China, and it is also the main cause of blindness in most countries over the world[1, 2]. At present, surgery is the only effective treatment[3]. Posterior subcapsular opacification (PCO) or posterior cataract is a common complication after cataract surgery, and it is also the main cause of postoperative visual acuity decline[4]. Studies have reported that the residual lens epithelial cells (LECs) generated epithelial-mesenchymal transformation (EMT) plays an critical role in the development of PCO[5].

EMT refers to the biological process in which epithelial cells are transformed into cells with interstitial phenotype through some specific procedures. It plays an vital role in embryonic development, a variety of fibrotic diseases and cancer metastasis[6, 7]. PCO is one of the main reasons that affect visual quality after cataract surgery. The principle of formation is mainly caused by the surgical incision caused by cataract surgery, which leads to the release of various inflammatory factors in aqueous humor, stimulating the residual lens epithelial cells under the capsule[8, 9]. subsequently, LECs undergo a series of biological behaviors, such as proliferation, migration and transformation, which eventually lead to the opacity of the posterior capsule[10, 11]. Therefore, it is of great significance to explore the mechanism of EMT in LECs for cataract treatment.

Insulin-like growth factor-1 (IGF-1) has been reported involving in in many diseases and promoting EMT in tumor cells[12, 13]. Moreover, IGF-1 has been clearly reported to be related to the pathogenesis of diabetic ophthalmopathy. The level of IGF-1 in vitreous of patients with diabetic retinopathy are increased[14, 15]. IGF-1 overexpressing transgenic mice result in neovascular glaucoma[16], and IGF-1 has been found to affect the protein composition of LECs, leading to cataracts[17]. However, the effect of IGF-1 on lens epithelial cell EMT is not clear.

MicroRNAs (miRNAs) are a kind of non-coding small RNAs that cause gene degradation or translation inhibition by binding to the complementary sequence of the target mRNA 3'-untranslated region. Accumulating evidences shows that miRNAs plays an important role in PCO, such as miR-30a[18], miR-204-5p[19], miR-34a[20] and miR-486-5p [21]. The function of miR-3666 in the pathophysiological process of different types of cancer has been widely studied[22, 23, 24]. However, the function of miR-3666 in the pathogenesis of EMT in LECs has not been verified. In the present study, we found that the high expression of IGF-1 promotes the EMT process of HLECs, and miR-3666 bound to IGF-1, attenuated TGF-β2-induced EMT in HLECs.

2 Materials And Methods

2.1 Capsular injury model in rats.

1 month old Wistar rats were purchased from SLAC Laboratory Animal Co.,Ltd (Shanghai, China). Animal care and experimental protocols were complied with guidelines of Animal Ethics Committee of Harbin Medical University (number: sydwgzr2020-11). After anesthetized generally with pentobarbital sodium (70 mg/kg) and topically with dicaine eye drop, a 1 mm incision was made at the transparent cornea at 12 O'clock' and filled with hyaluronic acid to maintain the state of the anterior chamber. After cutting off the anterior capsule, BSS solution was injected into the capsule to completely separate the lens cortex from the capsule. Then sutured the corneal incision. The occurrence of PCO was observed by slit lamp microscope at 0, 3, 7, 14, 21 and 28 days after operation[25]. All the animals were intraperitoneally injected with 3% pentobarbital sodium and were euthanized by excessive anesthesia with a dose of 90 mL/kg. Then the eyes were enucleated and posterior capsular tissue of the lens were removed for the experiments.

2.2 Cell culture

Human lens epithelial cells SRA10/04 was purchased from ATCC (Manassas, VA, USA). The cells were cultured with complete medium including 89% DMEM and 10% FBS (Biological Industries, Beit-Haemek, Israel) and maintained in incubator with 37°C and 5% of CO_2 saturated humidity.

The final concentration of TGF-β2 (PeproTech, Rocky Hill, NJ, USA) was 10 ng/ml. Before being collected for further analysis, the cells were cultured in the medium containing TGF-β2 for 48 hours.

2.3 Western blot

Cells were lysed with RIPA buffer and 45 ug proteins were run on a 8% SDS-polyacrylamide gel then transferred to PVDF membrane. After 1 hour of blocking with 5% skim milk, the membranes were incubated with primary antibodies overnight at 4℃. The corresponding secondary antibodies were incubated at room temperature for 1 hour the next day. Primary antibodiy against IGF-1 (DF6096, 1:500), Vimentin (AF7013, 1:2000), E-cadherin (AF0131, 1:500) and β-actin (AF7018, 1:2000) were purchased

from Affinity Biosciences (Jiangsu, China), with β-actin as an internal control. Odyssey Infrared Imaging System (Odyssey CLx, Biosciences, USA) was used to detect immunoreactivity.

2.4 qRT-PCR.

Trizol reagent was used to extract RNA from cells or tissues. NanoDrop 8000(Thermo Scientific, Waltham, MA, USA) was used to detect the concentration and purity of RNA. The single-stranded cDNAs were synthesized from 1 μg of RNA. The expression of mRNAs and miRNAs were quantified by RT-PCR with SYBR Green I (Thermo Fisher Scientific, Inc). Primer sequence of human genes:

GAPDH:

F: 5’-ACCACAGTCCATGCCATCAC-3’

R: 5’-TCCACCACCCT GTTGCTGTA-3’;

miR-3666:

F: 5’-ACGAGACGACGACAGAC-3’

R: 5’-CAGTGCAAGTGTAGATGCCGA-3’;

U6:

F: 5’-GCUUCGGCAGCACAUAUACUAAAAU-3’

R: 5’-CGCUUCACGAAUUUGCGUGUCAU-3’ ;

IGF-1:

F: 5’-CAGCAGTCTTCCAACCCAAT-3’

R: 5’-GCTGACTTGGCAGGCTTGAG-3’ ;

CDH1:

F: 5’-CTGAGAACGAGGCTAACG-3’

R: 5’-CCACCATCATCATTCAATATG-3’ ;

N-cadherin:

F: 5’-ACACTGGTGGCACTACTAAG-3’ and R:5’-TACACAATACAGAGGCAAAG-3’;

Primer sequence of rat IGF-1,

F: 5’-CTTTACCAGCTCGGCCACA-3’

R: 5’-TTGGTCCACACACGAACTGAAG-3’.

2.5 miRNA and plasmid transfection.

The LECs were plated until the cell density reached 80% confluency of dishes to transfect. The miRNAs/inhibitors/plasmids were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). MiR-3666 mimics/inhibitor were bought from RiboBio (Guangzhou, China) and plasmid of IGF-1 or short hairpinRNA of IGF-1 (sh-IGF-1) were constructed by Genechem (Shanghai, China).

2.6 EdU assay

Using EdU Cell Proliferation Kit (RiboBio, Guangzhou, China) to test proliferation. LECs were seeded in 24-well plates. After miRNA or plasmid treansfection for 48h, cells were maintained with 200uL 50 uM EdU and for 2 h. Apollo Dye Solution (red) were used to stain proliferating cells, nucleic acids were stained with DAPI (blue) according to the protocols[26] and then photographed.

2.7 Wound-healing assay

To test the cell migration ability of LECs, wound healing assay was performed. LECs were plated and cultured with FBS-free medium in 6-well plates until the cells formed a confluent monolayer, then scratched using a 100 μL pipette tip. The scratch wounds were captured using microscopy at 0, 24h. The wound area was analyzed by image J.

2.8 Luciferase reporter assays

To verified if IGF-1 was a target of miR-3666, the 3'-UTR of IGF-1 including the predicted binding sites wild (wt) or mutated (mut) binding sites were inserted into pmirGLO vector. The reporter plasmids of IGF-1 were co-transfected with miR-3666 mimics or miR-NC. Luciferase activities were measured by dual luciferase reporter assay kit (Promega, USA) after 24 h.

2.9 Matrigel transwell assay

24-well matrigel transwell (Corning, USA) were used to investigated cell invasion. 2×10^5 LECs were seeded on the cell culture insert precoated with 1 μg/μL Matrigel (BD Biosciences, USA). Medium with FBS was used to stimulate invasion in the bottom of wells. After 48 h, the invasion cells were stained with a 0.1% crystal violet solution. The number of invading cells were counted under microscope.

2.10 Statistical analysis

Data were shown as mean ± SEM. Unpaired Student’s t-test or one-way ANOVA was used to compare the groups. $P < 0.05$ was considered significant

3 Results

3.1 IGF-1 was up-regulated in OPC and TGF-β2 induced HLECs-EMT.

We constructed injury-induced OPC model in rats. QRT-PCR confirmed that the level of IGF-1 was upregulated in lens posterior capsule tissue of OPC model compared to the control (Fig. 1A). We then tested the IGF-1 expression in TGF-β2-induced HLECs-EMT. We verified that the TGF-β2 exposure promoted IGF-1 mRNA level at 48 h (Fig. 1B). The result of Western blot revealed that IGF-1 protein was increased after treatment with TGF-β2 (Fig. 1C,D).

3.2 Over-expression of IGF-1 promotes the EMT of HLECs.

To analyze the potential effects of IGF-1 in EMT of LECs, we constructed IGF-1 plasmid and confirmed the overexpression efficiency using qRT-PCR (Fig. 2A). After transfected with IGF-1 plasmid, epithelial cells marker CDH1 mRNA level was decreased (Fig. 2B) and mesenchymal related marker vimentin mRNA level was increased (Fig. 2C). Overexpression of IGF-1 also downregulated protein level of E-cadherin and upregulated vimentin protein level(Fig. 2D,E). Subsequently, an in *vitro* wound healing assay demonstrated that IGF-1 overexpression promoted the ability of HLECs to close a wound, indicating that IGF-1 promotes a cell migratory phenotype (Fig. 2F,G). Furthermore, a matrigel invasion experiments displayed that upregulated IGF-1 promoted cell invasion (Fig. 2H,I). EdU staining was conducted to detect cell proliferation activity and we found that IGF-1 enhanced proliferation ability of HLECs (Fig. 2J,K). Collectively, the above results show that forced expression of IGF-1 promotes the EMT phenotype of HLECs.

3.3 Silencing IGF-1 alleviated TGF-β2-induced EMT of HLECs.

Since Over-expression of IGF-1 has suggested a role for promoting EMT, we next validated whether silencing IGF-1 has an anti-EMT effect on HLECs. We constructed a IGF-1 short hairpinRNA (sh-IGF-1) and performed the functional assays (Fig. 3A). The expression of E-cadherin and vimentin were tested by qRT-PCR and Western blot. After IGF-1 silencing, we found that E-cadherin levels were increased (Fig. 3B,D,E), whereas levels of vimentin was decreased (Fig. 3C,D,E) in TGF-β2-induced HLECs-EMT. The wound healing assay showed sh-IGF-1 decreased cell motility induced by TGF-β2 (Fig. 3F,G). Strikingly, sh-IGF-1 inhibited HLECs invasion (Fig. 3H,I) and migration (Fig. 3J,K) that induced by TGF-β2 .

3.4 IGF-1 was a potential target of miR-3666.

It has been reported that microRNAs regulate the genes expression and function. To clarify the regulatory relationship between miRNAs and IGF-1, we used miRNA target identification tools TargetScan 7.2 (http://www.targetscan.org/vert_72/) to search for potential microRNAs binding with IGF-1. We identified miR-3666 which contained a potential-binding sites on 3’-UTR of IGF-1 (Fig. 4A). Luciferase reporter vector were constructed with the 3’-UTR sequence of IGF-1 containing the putative binding site for miR-3666. The luciferase activity was suppressed with wildtype IGF-1 (IGF-1-wt) but not with mutated IGF-1 vector (IGF-1-mut) (Fig. 4B). Contrary to the IGF-1, low miR-3666 expression were observed in cataract

tissues (Fig. 4C) and HLECs treated with TGF-β2 (Fig. 4D). Overexpression of miR-3666 with mimics suppressed the expression of IGF-1, which further proved the regulatory relationship between them.

3.5 Enhanced expression of miR-3666 attenuates EMT of LECs.

To further validate the role of miR-3666, HLECs were transfected with miR-3666 mimics and miR-NC with or without TGF-β2, respectively. The mRNA level of CDH1 was high expression (Fig. 5A) and vimentin was downregulated (Fig. 5B) treated with miR-3666 mimics in TGF-β2 induced cells. The protein levels that E-cadherin was upregulated and vimentin was downregulated in miR-3666 mimics group were further detected by Western blot (Fig. 5C,D). Wound healing (Fig. 5E,F) and invasion assays (Fig. 5G,H) demonstrated that the migratory capacities of and invasion ability of HLECs were markedly inhibited by miR-3666 mimics. EdU assay indicated that proliferating cells were decreased with the presence of miR-3666 mimics (Fig. 5I,J). AS has been reported to in human tumors, miR-3666 impeded the EMT process of HLECs.

3.6 Inhibition of miR-3666 promotes EMT by regulating IGF-1.

We further explored the effect of miR-3666 inhibitor and biological regulatory relationship between miR-3666 and IGF-1 in *vitro*. We constructed miR-3666 inhibitor and negative control inhibitor and verified their function (Fig. 6A). According to the results of qRT-PCR and Western blot, we found that treated with miR-3666 inhibitor, E-cadherin levels were decreased (Fig. 6C,E,F), whereas levels of IGF-1 and vimentin was increased (Fig. 6B,D,E,F) in TGF-β2-induced HLECs-EMT. Transfected sh-IGF-1 into HLECs we found that silencing IGF-1 partially reversed EMT promoting function of miR-3666 inhibitor. As expected, miR-3666 inhibitor promoted the migration (Fig. 6G,H), invasion (Fig. 6I,J) and proliferation (Fig. 6K,L) of HLECs, but silencing of IGF-1 partially restored cells function.

4 Discussion

This study shows that IGF-1 is directly related to the EMT process of HLECs. Overexpression of IGF-1 promoted the proliferation, migration, invasion and EMT of HLECs, while knockout of IGF-1 significantly inhibited the EMT phenotype induced by TGF-β2.

Previous studies have shown that IGF-1 receptors are widely distributed in eyes[27]. For example, there were IGF-1 receptors in lens epithelial cells, so that IGF-1 are capable of stimulating lens cell differentiation by binding it's receptors[28], which is one of the reasons leading to PCO. Crystallin aggregation is also the cause of cataract ,it is described that IGF1-1 affect the total ratio of β and γ crystallin to α crystallin in lens fibre cells, which may make the lens susceptible to cataracts. In humans, mutations of IGF-1 is able

to reduce retinal angiogenesis[29]. It is suggested that IGF-1 may play a critical role in the occurrence and development of ocular complications. Our study clearly defines the effect of IGF-1 on LECs.

In addition, we used bioinformatics website to screen valuable microRNAs, which bind to IGF-1 to regulate lens epithelial cell proliferation, and predicted that the miR-3666 may regulate IGF-1 by binding to the 3 'UTR of IGF-1. Luciferase reporter gene experiment verified the targeting relationship between them. Overexpression of miR-3666 can inhibit the proliferation, migration, invasion and interstitial phenotype of HLECs, while knockout of IGF-1 reverses the effect of miR-3666 inhibitor on the malignant behavior of HLECs. These results highlight the role of miR-3666/IGF-1 in lens EMT and provide a new strategy for the prevention and therapeutic method of PCO.

At present, most of the studies of EMT in PCO are focused on cell and animal experiments, and the mechanism is complex and needs to be further explored. Understanding the specific molecular mechanism of the occurrence and development of LECs-EMT is very important for treatment of PCO, and also provides ideas for the prevention of PCO from a new point of view.

In summary, Our present study provide evidence for the miR-3666/IGF-1 pathway involved in TGF-β2-induced HLEC-EMT. miR-3666 acts as an endogenous sponge of IGF-1 and regulate EMT phenotype and cell activity. These findings provide novel insight into understanding the mechanisms HLEC-EMT provide in *vitro* support for the specific pathogenesis of PCO. Our results indicate the potential therapeutic target to heal the damaging process after cataract surgery.

Declarations

Conflicts of interest

The authors declare no conflicts of interest.

Funding

This study was supported by the Heilongjiang Province Postdoctoral Funds（LBH-Z18194）.

Author contribution

LP and HYG conceived and designed the experiments; SZ, ZXM, SY, WC and LP performed the experiments; SZ analyzed the data; SZ, LP and HYG wrote the paper. All authors read and approved the final manuscript.

Consent to Participate

All participants provided written informed consent to participate in this research.

Consent to Publish

We have obtained consent to publish from all the participants.

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Figures

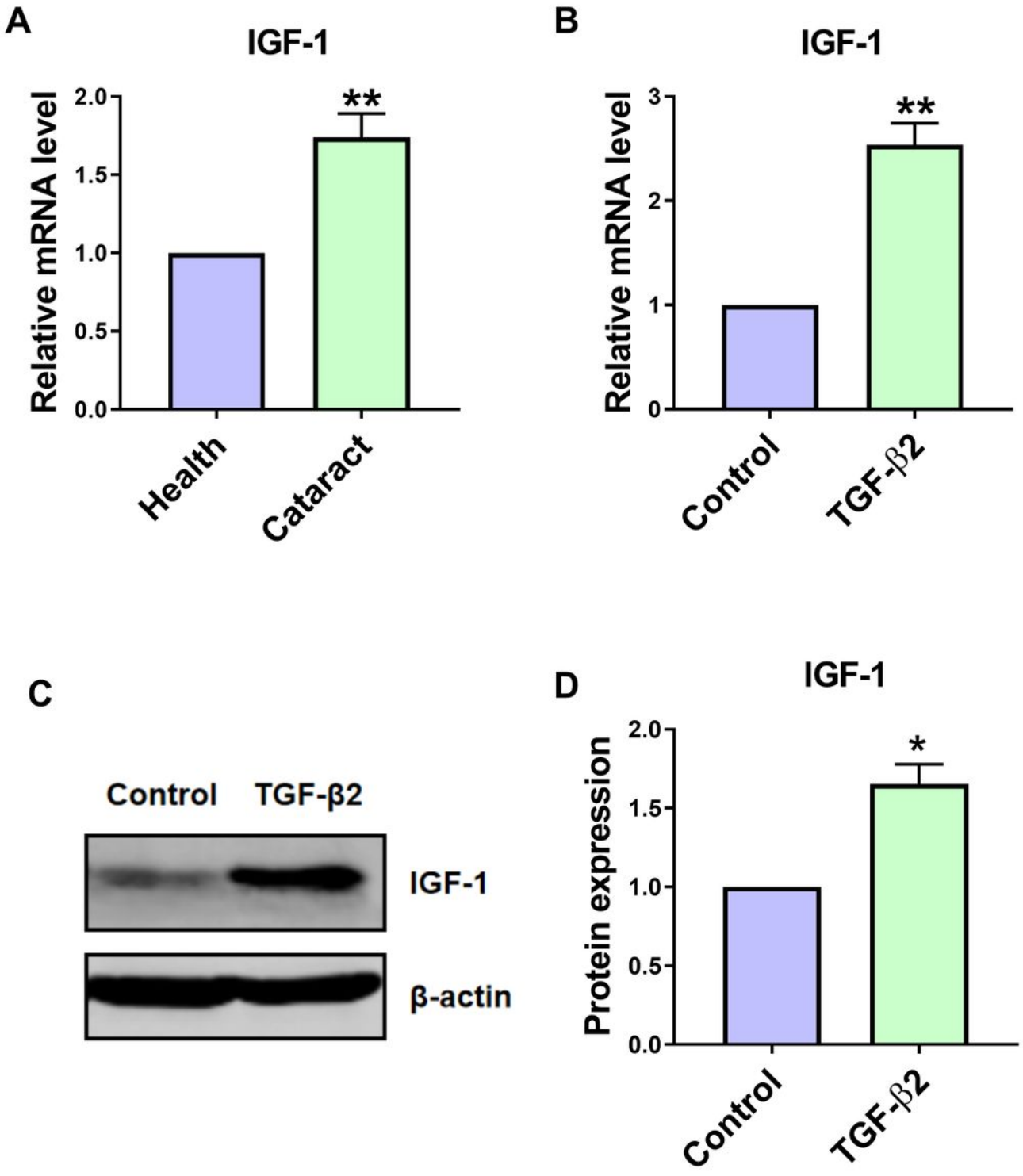

Figure 1

The expression of IGF-1 in PCO and TGF-β2 induced HLECs. (A) The mRNA expression of IGF-1 in posterior capsule tissue of PCO model and healthy control were detected by qRT-PCR, n=6 . (B) The expression level of IGF-1 mRNA in TGF-β2 induced HLECs was determined by qRT-PCR, n=6. (C) The protein level of IGF-1 confirmed in TGF-β2 induced HLECs and Control group by Western blot. (D) Statistical data of Western blot, n=4. *$p < 0.05$; **$p < 0.01$.

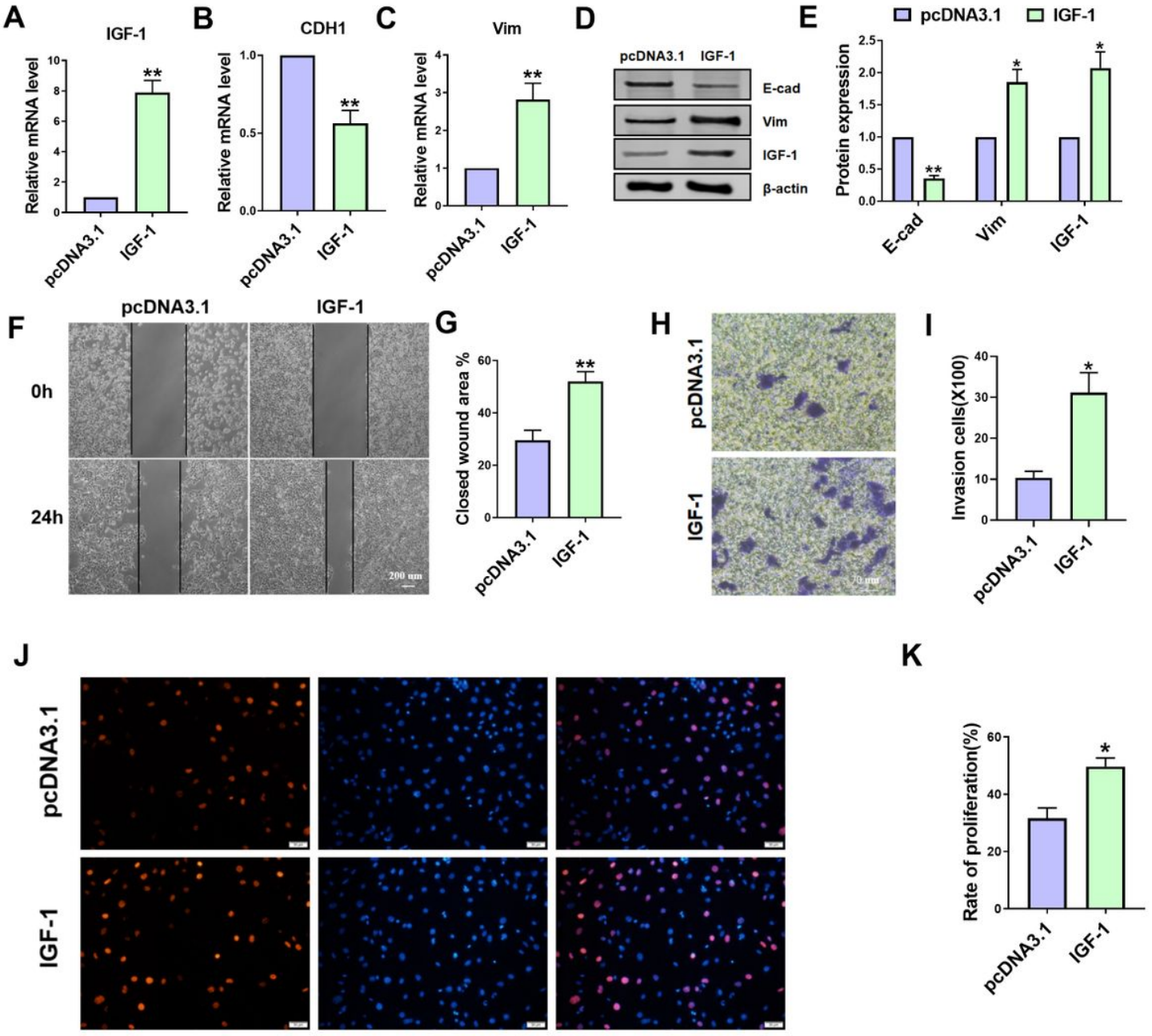

Figure 2

Overexpression of IGF-1 results in EMT in HLECs. (A-C) qRT-PCR was used to confirmed mRNA expression of IGF-1, CDH1, vimentin to detected the effect of IGF-1 overexpression in HLECs, n=6. (D) The protein level of IGF-1, E-cadherin, vimentin determined by Western blot to detected the effect of IGF-1 overexpression in HLECs. (E) analysis of Western blot, n=4 . (F) Wound healing assay revealed that overexpression of IGF-1 increased cell migration in HLECs. (G) Analysis of Wound healing assay, n=6 . (H) Transwell assay showed invasion ability of HLECs was promoted by IGF-1. (I) Analysis of Transwell assay, n=6. (J) EdU staining illustrated that forced expression of IGF-1 promoted cell proliferation. (K) Analysis of EdU staining, n=6. *p < 0.05; **p < 0.01.

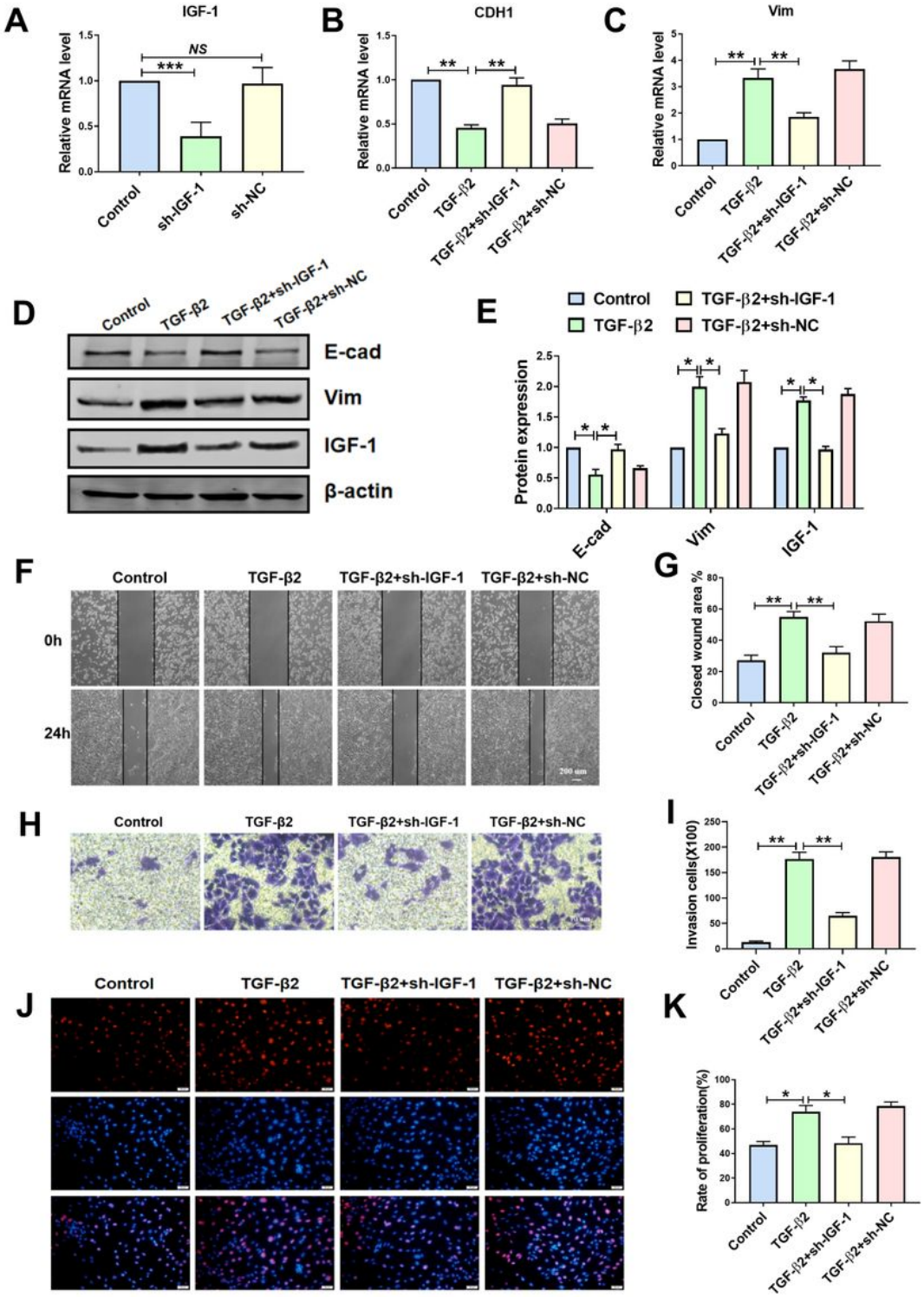

Figure 3

Inhibition of IGF-1 ameliorated HLECs-EMT after TGF-β2 treated. (A) qRT-PCR was used to verify the efficiency of sh-IGF-1, n=6. (B-C) Silencing IGF-1 reversed TGF-β2 induced mRNA expression of CDH1, vimentin, which was measured by qRT-PCR assay. (D) Using Western blot to investigate the effect of sh-IGF-1 with or without TGF-β2 on the level of IGF-1, E-cadherin, vimentin proteins. (E) Analysis of Western blot, n=4. (F) Wound healing revealed that inhibiting of IGF-1 ablated the cell migration induced by TGF-

β2. (G) Analysis of Wound healing assay, n=6 . (H) Transwell assay showed invasion ability of HLECs was inhibited by sh-IGF-1. (I) Analysis of Transwell assay, n=6. (J) EdU staining revealed that knockdown of IGF-1 inhibited cell proliferation in induced by TGF-β2. (K)Analysis of EdU staining, n=6. *p < 0.05; **p < 0.01.

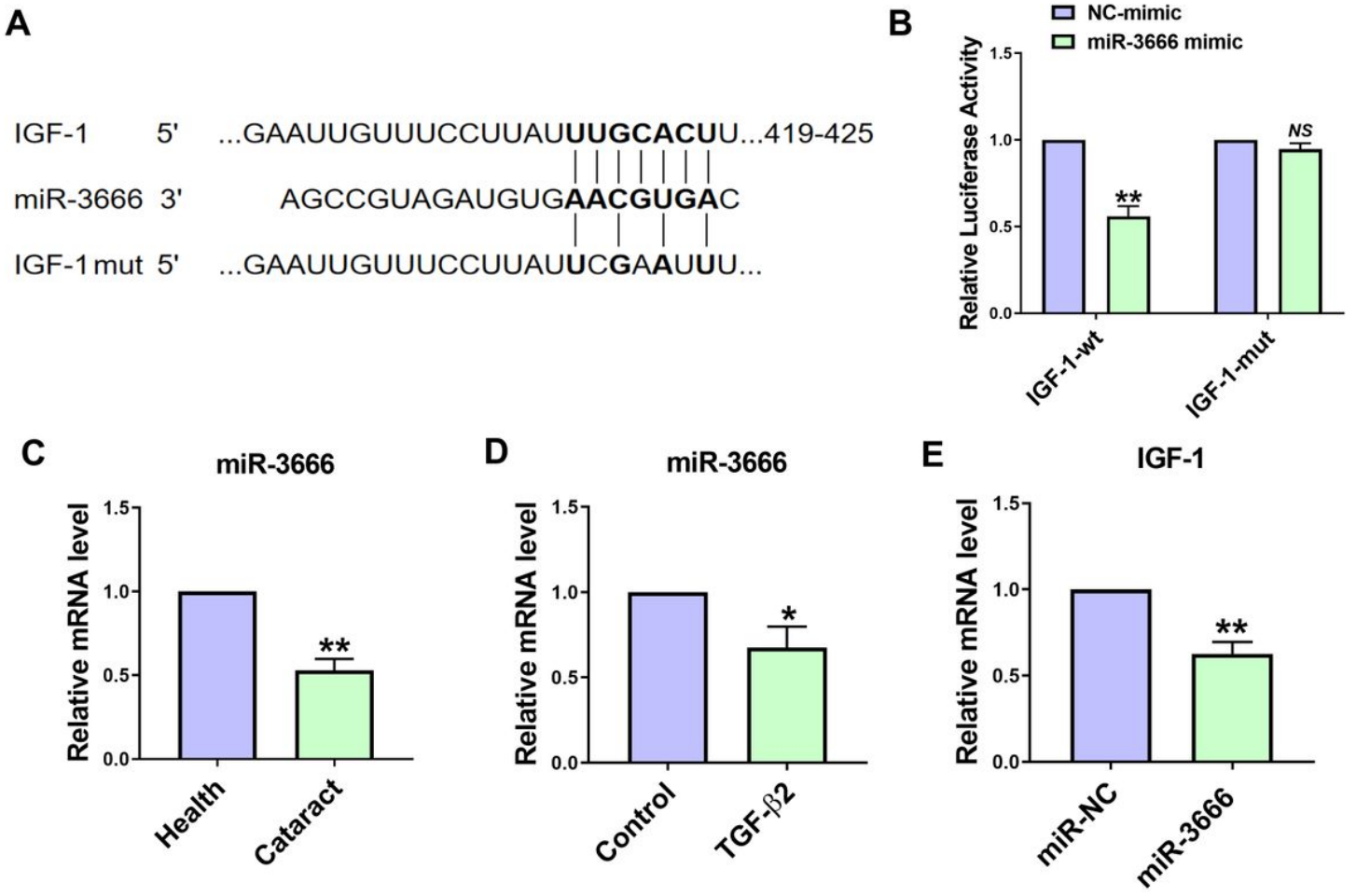

Figure 4

Identification of miR-3666 as a master miRNA regulating the IGF-1. (A) Predicted binding sites of IGF-1 and miR-3666. IGF-1-Mut, mutated binding site. (B) Luciferase activities of chimeric vectors with IGF-1 containing wild-type or mutated. (C) The expression of miR-3666 in posterior capsule tissue of PCO model and healthy control were detected by qRT-PCR, n=6. (D) The expression of miR-3666 in HLECs with or without TGF-β2 was determined by qRT-PCR, n=6. (E) The effect of miR-3666 on IGF-1 mRNA expression, n=6. *p < 0.05; **p < 0.01; NS, not significant.

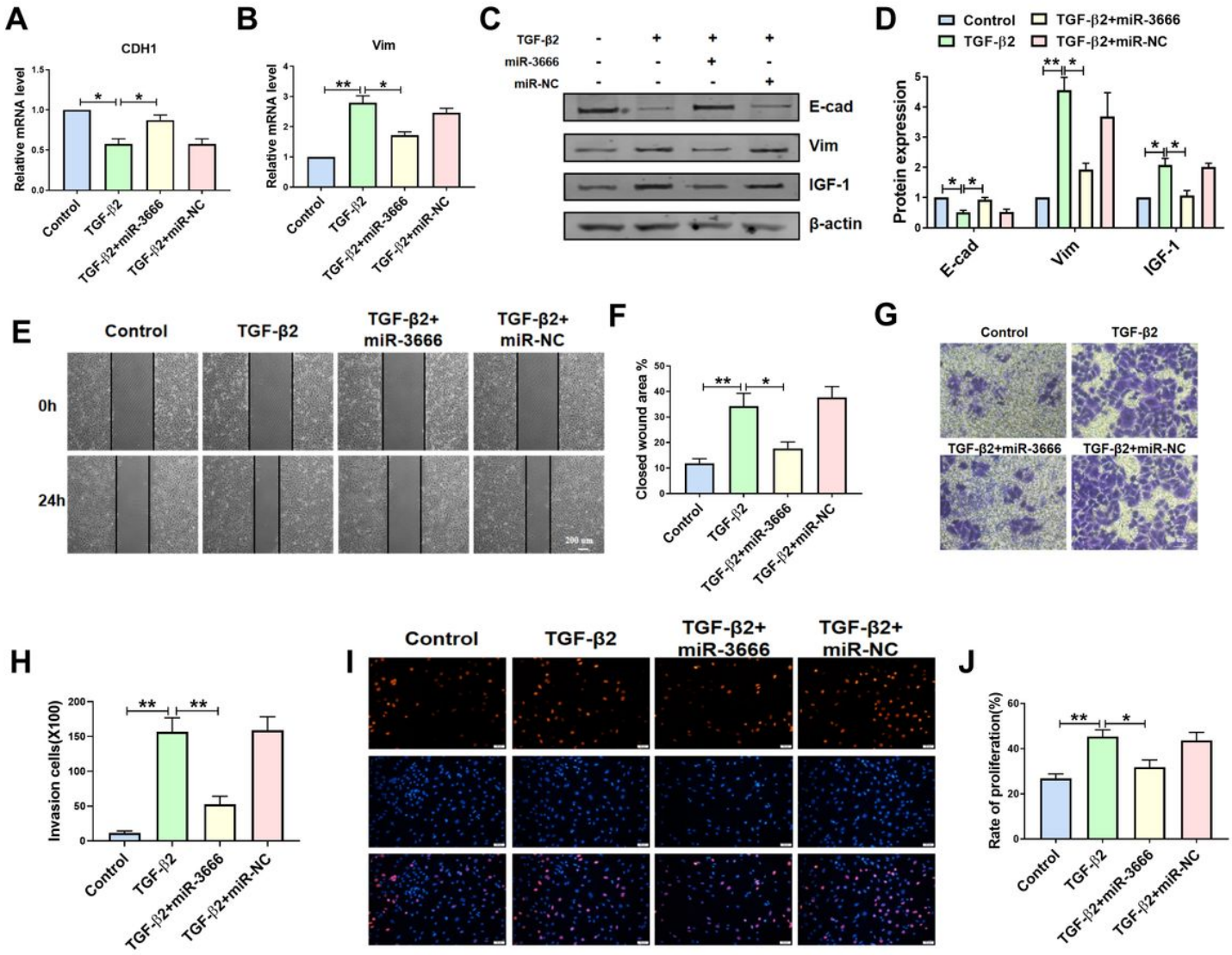

Figure 5

Overexpression of miR-3666 attenuated TGF-β2-induced EMT in HLECs. (A-B) miR-3666 mimics reversed TGF-β2 induced mRNA expression of CDH1, vimentin, which was measured by qRT-PCR. (D) Using Western blot to investigate the effect of miR-3666 mimics with or without TGF-β2 on the level of IGF-1, E-cadherin, vimentin proteins. (E) Analysis of Western blot, n=4. (F) Wound healing revealed that miR-3666 mimics ablated the increased cell migration induced TGF-β2. (G) Analysis of Wound healing assay, n=6. (H) Transwell assay showed invasion ability of HLECs was inhibited by miR-3666 mimics. (I) Analysis of Transwell assay, n=6 . (J) EdU staining illustrated that miR-3666 mimics inhibited cell proliferation in induced by TGF-β2. (K) Analysis of EdU staining, n=6 . *$p < 0.05$; **$p < 0.01$.

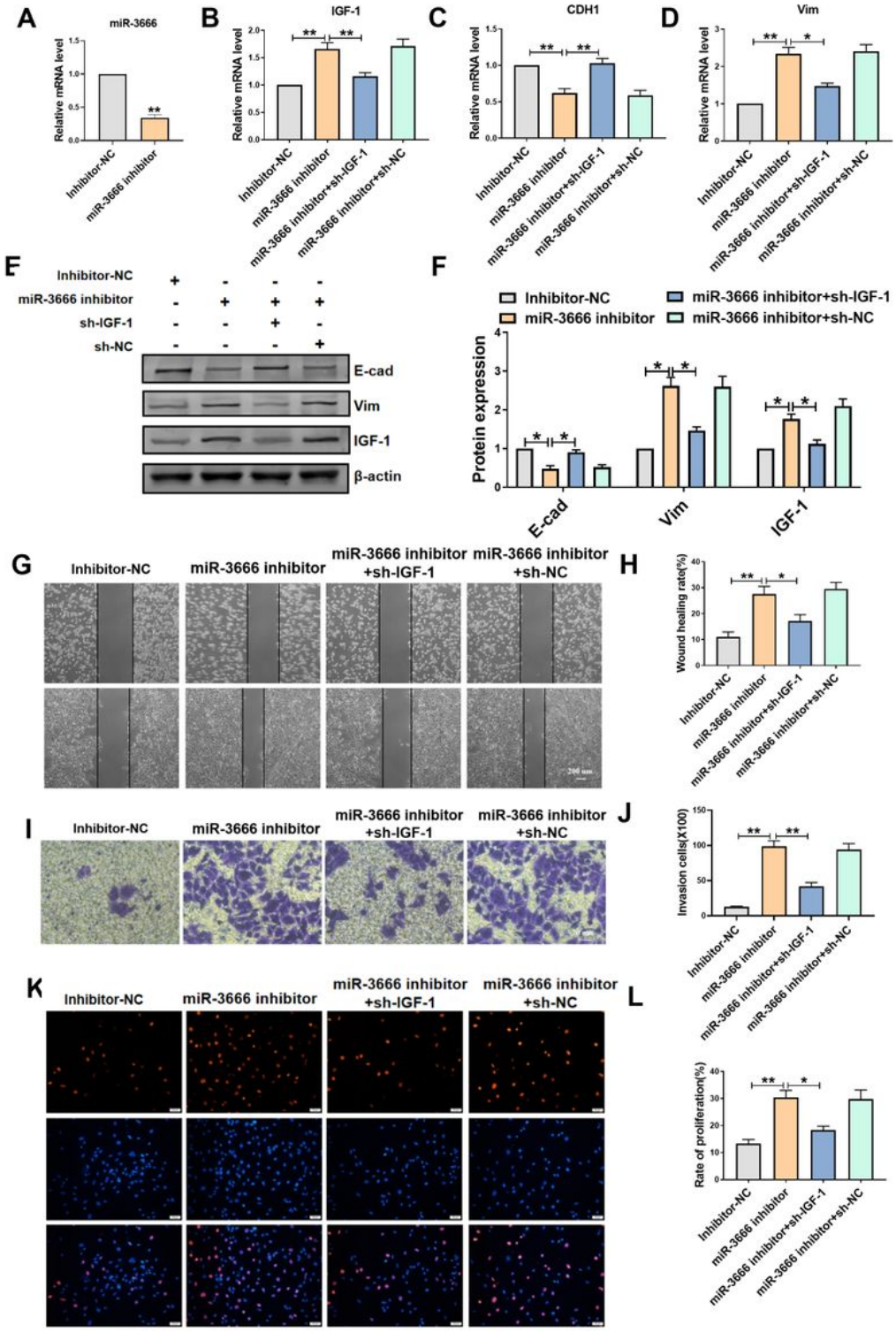

Figure 6

IGF-1 was necessary for the promoting EMT effects of miR-3666 inhibitor. (A) qRT-PCR was used to detected the silencing efficiency of miR-3666 inhibitor. n=6 . (B-D) Silencing IGF-1 reversed miR-3666 inhibitor induced mRNA expression of IGF-1, CDH1, vimentin, which was measured by qRT-PCR. n=6. (E) Using Western blot to investigate the effect of sh-IGF-1 with or without miR-3666 inhibitor on the level of IGF-1, E-cadherin, vimentin protein. (F) Analysis of Western blot, n=4. (G) Wound healing tested showed

that inhibition of IGF-1 ablated the cell migration induced by miR-3666 inhibitor. (H) Analysis of Wound healing assay, n=6. (I) Transwell assay showed miR-3666 inhibitor induced invasion ability of HLECs was inhibited by sh-IGF-1. (J) Analysis of Transwell assay, n=6. (K) EdU staining showed that silencing of IGF-1 inhibited cell proliferation in induced by miR-3666 inhibitor (L) Analysis of EdU staining, n=6 . *$p < 0.05$; **$p < 0.01$.