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Original Article

Linc-POU3F3 is overexpressed in in-stent restenosis patients and induces VSMC phenotypic transformation via POU3F3/miR-449a/KLF4 signaling pathway

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Abstract: Background: With the extensive application of stent implantation in patients undergoing percutaneous coronary interventions (PCI), there are chances that in-stent restenosis (ISR)-a major vascular complication caused by vascular smooth muscle cell (VSMC) phenotypic transformation-might occur. Objectives: This study sought to evaluate the role of lincRNA-POU3F3 on VSMC phenotypic transformation and the underlying mechanism. Methods: VSMCs were used in our research. We first constructed a gene delivery system through an assembly of lipofectamine and a functional plasmid DNA (pDNA) encoding lincRNA-POU3F3 or MicroRNA-449a, and then, transfected it to VSMCs. Moreover, lentivirus-mediated KLF4 inhibitor (KLF4 siRNA) was also used in these cells. Expression of relevant proteins, such as smooth muscle myosin heavy chain (SM-MHC), alpha smooth muscle actin (α-SMA), osteopontin (OPN), and kruppel-like factor 4 (KLF4), was examined by western blot or immunofluorescence (IF) assay. CCK-8 and wound healing assays were performed to assess the growth and migration of VSMCs. qRT-PCR was used to assess linc-POU3F3 and miR-449a levels. Luciferase reporter assay was also performed. Results: POU3F3 levels were significantly higher in ISR patients compared to controls. We observed that linc-POU3F3 promoted VSMC proliferation and migration, and induced VSMC phenotypic transformation via POU3F3/miR-449a/KLF4 signaling pathway. Conclusion: Linc-POU3F3 promotes phenotypic transformation of VSMCs via POU3F3/miR-449a/KLF4 pathway. It may provide a theoretical basis to attenuate ISR via pharmacological inhibition of this biomarker or at least serve as a predictor of diagnosis or prognosis of patients with restenosis.

Keywords: In-stent restenosis, LincRNA-POU3F3, phenotypic transformation, POU3F3/miR-449a/KLF4 signaling pathway

Introduction

Percutaneous endovascular treatment has been successfully performed as an alternative to surgery since a long time, as it has low perioperative mortality and is associated with fewer postoperative complications in blood vessels, such as superficial femoral artery, mesenteric artery; this is especially true of coronary artery-percutaneous coronary interventions (PCI) [1-3]. Stent implantation plays an important role in patients adopting PCI for their coronary artery diseases; however, it might lead to a potentially difficult complication called in-stent restenosis (IRS) [4].

ISR is defined as a bane with evidence of myocardial ischemia, > 50% diameter stenosis in stent implanted previously via angiography [5, 6]. It mainly impacts patients undergoing Bare-metal stents (BMS) implantation due to its effect in increasing neointimal proliferation [7]. Furthermore, even with the advent of drug-eluting stents (DES) or new-generation DES that could reduce the incidence of ISR compared with conventional BMS, the risk of ISR still exists [8].

Although the process of ISR is complicated and its mechanism is not completely clear. Increasing evidence has revealed that intimal hyper-

Table 1. Associations between linc-POU3F3 expression and clinicopathological characteristics in ISR

Characteristics	Univariate analysis			Multivariate analysis		
	OR	95 CI %	P	OR	95 CI %	P
Male	0.84	0.51-1.37	0.466			
Age (y)	1.03	0.99-1.07	0.166			
BMI	0.94	0.83-1.07	0.343			
Systolic pressure	1.05	1.02-1.08	< 0.001			
Diastolic pressure	1.02	0.99-1.05	0.277			
Previous history						
Smoking	1.51	0.87-2.64	0.143			
Drinking	2.27	1.28-4.02	0.005	2.09	1.08-4.04	0.028
Hypertension	2.12	1.28-3.49	0.003	2.01	1.14-3.57	0.017
Diabetes	3.00	1.67-5.40	< 0.001	3.15	1.63-3.57	< 0.001
Stroke	1.63	0.81-3.29	0.174			
HDL	0.43	0.22-0.83	0.012	0.52	0.24-1.15	0.100
LDL	2.65	1.46-4.83	0.001	3.14	1.57-6.31	0.001
Creatinine	1.00	0.99-1.01	0.526			
LVEF (%)	1.01	0.97-1.05	0.631			
POU3F3	2.35	1.80-3.06	< 0.001	2.21	1.68-2.92	< 0.001

plastic lesions are strictly correlated to this restenosis and involve the participation of various cells, including endothelial cells (ECs), vascular smooth muscle cells (VSMCs) [4, 9, 10], with phenotypic transformation of VSMCs acting as a key event [11]. The principle function of VSMCs is to contract and relax in order to regulate the blood flow and blood pressure. However, VSMCs can modulate their phenotypes from a contractile to the synthetic one in response to the stresses/stimuli in microenvironment via a process known as phenotypic transformation, which involves a decrease in the levels of contractile markers and a concomitant increase in the levels of synthetic markers. Meanwhile, VSMCs exhibit increased proliferation, accelerated migration into the intimal space, and secrete high levels of extracellular matrix proteins, thereby leading to neointimal hyperplasia, ultimately initiating the progression of various cardiovascular diseases, including ISR [11-14].

Long non-coding RNAs (lncRNAs) are a type of non-coding RNA that are more than 200 nucleotides long; long intervening noncoding RNAs (lincRNAs) are a subtype of lncRNA [15, 16]. LincRNAs are known to be involved in the progression of a broad range of diseases, such as esophageal squamous cell carcinoma (ESCC) [17] and nasopharyngeal carcinoma [18]. They have been attracting a lot of attention from cardiovascular experts over last few years [19, 20].

In our previous study, lncRNA microarray analysis was conducted to screen for lncRNA that can be used as biomarkers of the circulating vascular system. We found increased levels of linc-POU3F3 conjugate of several candidate lincRNAs in ISR patients; this suggested a potential association between POU3F3 and ISR. Moreover, using logistic regression, we found that POU3F3 expression was an independent risk factor in ISR, in addition to alcohol consumption, hypertension, and diabetes (**Table 1**). Based on this strong association between cell phenotypic transformation and ISR, we inferred that POU3F3 may be crucial for VSMC phenotypic transformation. However, little is known about the effects of POU3F3 on VSMC biology. Meanwhile, we also found that miR-449a was significantly downregulated and KLF4 was upregulated in these serum components attracted our attention. In addition, using Targetscan 7.0, we found a potential binding site for miR-449a and KLF4. Hence, based on these findings, we proposed that miR-449a/KLF4 signaling pathway may be involved in mediating the biological effect of POU3F3 in VSMCs.

Thus, the aim of our study was to demonstrate whether POU3F3 functioned as a molecular promoter that induced the phenotypic transformation of VSMCs, and whether it acts via the POU3F3/miR-449a/KLF4 signaling pathway.

Materials and methods

Patients and specimen collection

The research was approved by the Ethics Committee of Shaoxing People's Hospital and informed written consent was obtained from participants. We collected a total of 59 serum samples from patients undergoing PCI (27 with

and 32 without ISR). POU3F3 levels were detected at Department of Cardiology of Shaoxing People's Hospital (zhejiang, China).

Cell culture

VSMCs were purchased from the Chinese Academy of Sciences. The cells were grown in RPMI 1640 medium (Sigma, USA), supplemented with 10% Fetal Bovine Serum (FBS, Gibco, USA), 1% streptomycin, and 1% penicillin, at 37°C in a humidified atmosphere under 5% CO_2 conditions.

Vector fabrication and cell transfection

Plasmid vectors expressing lncRNA POU3F3, miR-449a, lentivirus-mediated KLF4 inhibitor, and negative controls (blank plasmids) were designed and constructed by GenechemCo (Shanghai, China). Transfections were conducted using Lipofectamine 3000 reagent (Invitrogen, USA) with vectors at a suitable dose, according to the manufacturer's protocol. After the transfection, the cells were incubated for 48 h, and then, the transfection efficiency was examined by qRT-PCR.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. The cDNAs were subsequently synthesized from total RNA with PrimeScript RT reagent Kit (Takara, Japan). Primers for lincRNA POU3F3 and miR-449a were designed and synthesized by GenechemCo (Shanghai, China). qRT-PCR was conducted with TB Green Premix Ex Taq (Takara, Japan). The PCR was performed in triplicates.

Cell proliferation and migration assays

Cell proliferation was assayed by Cell Counting Kit-8 (CCK-8) (Mce, China), according to the manufacturer's instructions. After 6 h of transfection, cells were harvested and diluted to a density of 2×10^4 cells/mL. Cell suspensions were cultivated in a 96-well plate with 100 μL cell suspension in each well; 10 μL of CCK-8 solution was added after 24, 48, 72, and 96 h and incubated for 2 h. Optical density values at 450 nm were measured by a microplate reader (Bio-Rad, Hercules, CA, USA). The CCK-8 assay was performed in triplicates.

Cell migration capability was measured by wound healing assays. When VSMCs grew to 90% confluency, cells were starved overnight for synchronization and growth inhibition. Next, VSMCs were scratched with a sterile 100 μL pipette tip. Then, phosphate-buffered solution (PBS) was used to remove the cell debris. After treatment with various test substances, cells were observed after every 24 h under a Nikon microscope (Nikon Corporation, Tokyo, Japan). The assay was performed in triplicates.

Immunofluorescence assay

VSMCs were washed three times with PBS and fixed in 4% paraformaldehyde for 15 min. Following permeabilization in 0.1% Triton and blocking in 10% goat serum for 15 and 30 min, respectively. VSMCs were incubated with α-SMA antibodies (1:250) at 4°C overnight, following which, they were incubated with anti-mouse or anti-rabbit fluorescein isothiocyanate conjugated secondary antibodies for 1 h at room temperature. Finally, cells were stained with DAPI for 5 min before visualizing the nuclei under an inverted fluorescence microscope. Immunofluorescence assay was performed in triplicates.

Western blot assay

The media was discarded 48 h after transfection. Transfected cells were lysed, and total proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer reagent (Beyotime, China), supplemented with protease inhibitors. The lysate was centrifuged and the resulting protein was quantified with the BCA protein assay kit (Beyotime, China). Equal amounts of protein extract were separated by 10% SDS-PAGE and blotted onto a PVDF membrane (Millipore, Billerica, USA). The membrane were blocked for 12 h in blocking solution, followed by incubation with the following primary antibodies: α-SMA (Abcam, USA), SM-MHC (Abcam, USA), OPN (Abcam, USA), KLF4 (Gene Tex, USA), and β-actin (1:1000, CST, USA) at 4°C overnight. The membrane was then incubated with HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Abcam, USA). Signals were observed by enhanced chemiluminescence reagents (Beyotime, China). The western blot assay was performed in triplicates.

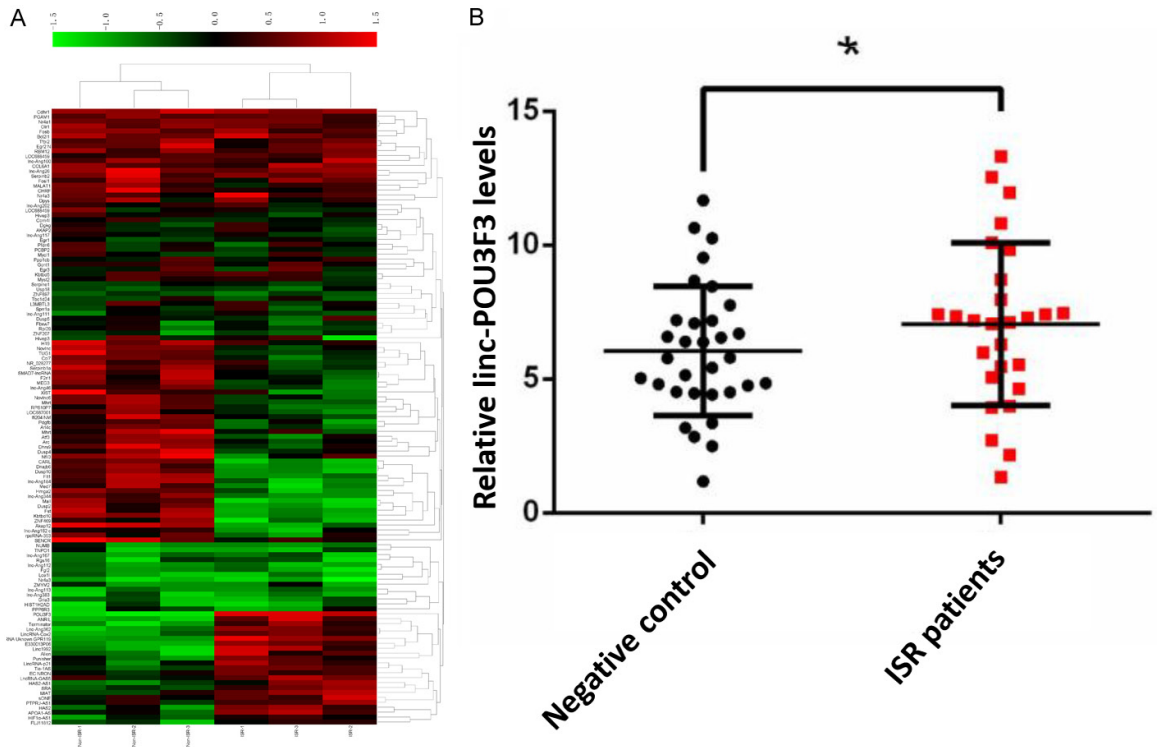

Figure 1. The linc-POU3F3 is selected as a crucial biological factor for ISR and its expression is upregulated in ISR patients. A. The novel lincRNA-POU3F3 was selected as a uniquely important biomarker through gene microarray analysis. B. Linc-POU3F3 was significantly upregulated in serum samples obtained from ISR patients compared with negative control (*P < 0.05 vs. control).

Luciferase reporter assay

Potential binding site for miR-449a and KLF4 was predicted using TargetScan 7.0. A fragment of KLF-4 3'-UTR and the mutated variants of the predicted miR-449a binding site on KLF-4 3'-UTR were PCR-amplified and fused to the luciferase reporter vector psi-CHECK-2 (Promega, Madison, WI, USA). VSMCs were transfected with luciferase plasmid and miR-449a mimic or control using Lipofectamine 3000 (Invitrogen, USA). After 48 h of transfection, the luciferase activity was evaluated using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical analysis

The data were expressed as the mean ± S.D. The Student's t-test and one-way ANOVA with Tukey's multiple comparison test was performed to evaluate statistical differences in our study. Statistical analysis was performed using SPSS 19.0 and presented with the GraphPad prism 6.0 software. In all cases, *P* value was set as less than 0.05.

Result

Aberrant increase in expression of lincRNA-POU3F3 in serums from ISR patients

LncRNA expressions were screened by microarray analysis (**Figure 1A**). The differential expression of linc-POU3F3 between clinical ISR patients and controls was further examined by RT-PCR. It was observed that there was a significant elevation of lincRNA-POU3F3 in ISR patients. The detailed values are presented in **Figure 1B** (P < 0.05). The result suggested that POU3F3 may play an important role in the progression of ISR.

Linc-POU3F3 induces VSMC transformation

VSMCs were cultivated in a 6-well plate for 24 h, following which they were starved for 6 h, and transfected with POU3F3. Transfection efficiency was determined by qRT-PCR (**Figure 2A**). After transfection, the cells were cultivated for another 48 h, following which they were washed with PBS three times. Proteins extracted from cells were analyzed by western blot assay. The

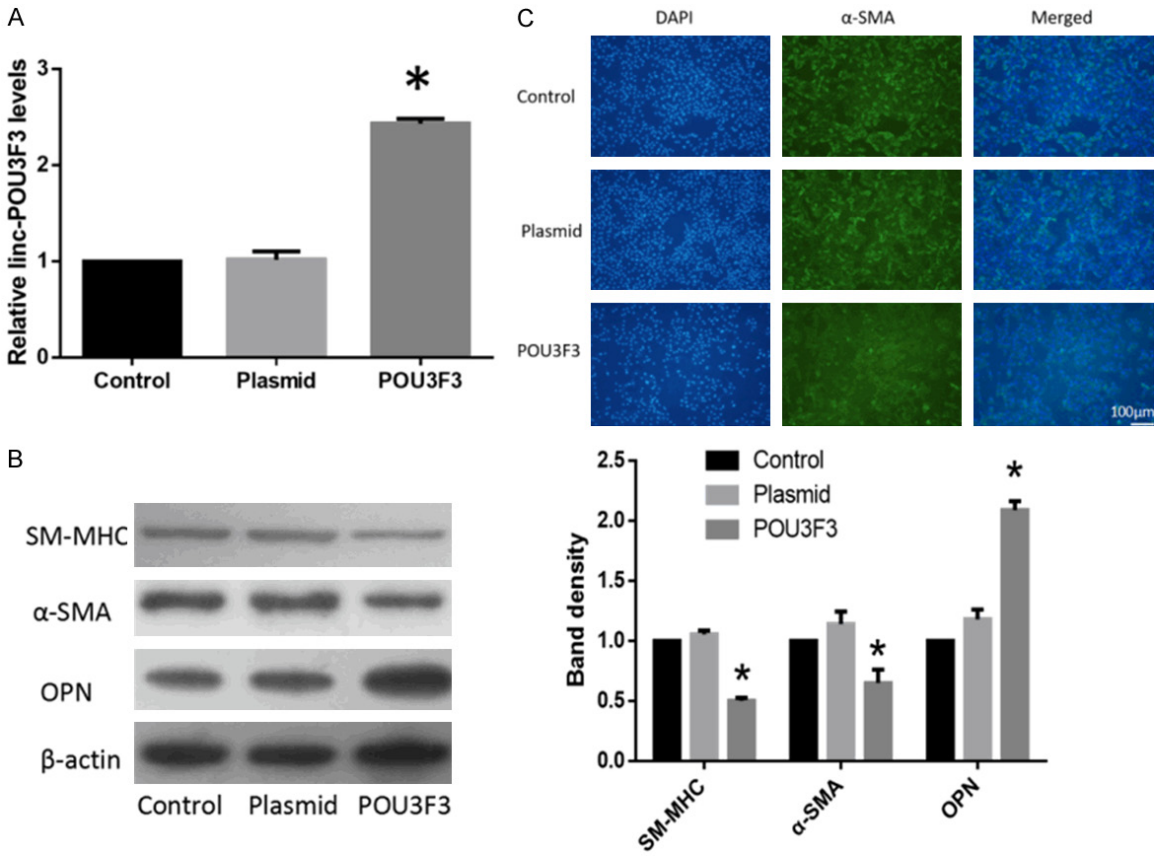

Figure 2. The linc-POU3F3 induces VSMC phenotypic transformation from a contractile phenotype to a synthetic state. Control, VSMCs were cultured without any treatment for 48 h; Plasmid, VSMCs were transfected with blank plasmid and cultivated for another 48 h; POU3F3, VSMCs were transfected with linc-POU3F3 and cultivated for another 48 h. Then, PCR, protein extraction, and immunofluorescence assays were performed. A. Linc-POU3F3 was overexpressed in the VSMCs following the transfection of POU3F3 compared with blank control and negative control (blank plasmid) (*P < 0.05 vs. control). B. POU3F3 downregulates the expression of SM-MHC, α-SMA while it upregulates OPN levels (*P < 0.05 vs. control). C. POU3F3 not only reduces the expression of α-SMA, but also changes myonemes and cell morphology of VSMCs. The scale bar = 100 μm.

expression levels of SM-MHC, α-SMA, and OPN (all of them are biomarkers of phenotypic transformation) were markedly altered after the transfection with POU3F3. As shown in **Figure 2B**, SM-MHC and α-SMA expression were significantly lower while OPN expression was markedly higher than those in control VSMCs. In addition, immunofluorescence assay revealed that transfection with POU3F3 not only reduced the fluorescence intensity of α-SMA, but also disturbed the myonemes and the morphology of VSMCs (**Figure 2C**). These results indicated that linc-POU3F3 induces phenotypic transformation of VSMCs.

Linc-POU3F3 promotes VSMC proliferation and migration

Subsequently, we examined the effect of linc-POU3F3 transfection on VSMC proliferation and migration. Three groups of blank control, negative control, and linc-POU3F3 were examined using CCK-8 assays. The CCK-8 assays showed that POU3F3 promoted the proliferation of VSMCs (**Figure 3A**). Meanwhile, we evaluated cell migration using wound healing assays, which revealed that the migratory ability of VSMCs was increased in the POU3F3 group (**Figure 3B**, **3C**). Overall, the results indicated that overexpression of POU3F3 promotes proliferation and migration of VSMCs.

Linc-POU3F3 induces VSMC phenotypic transformation via POU3F3/miR-449a/KLF4 pathway

To investigate the cellular mechanisms by which POU3F3 induces VSMC phenotypic transformation, VSMCs were treated with miR-449a mimic at different concentrations (50 and 100

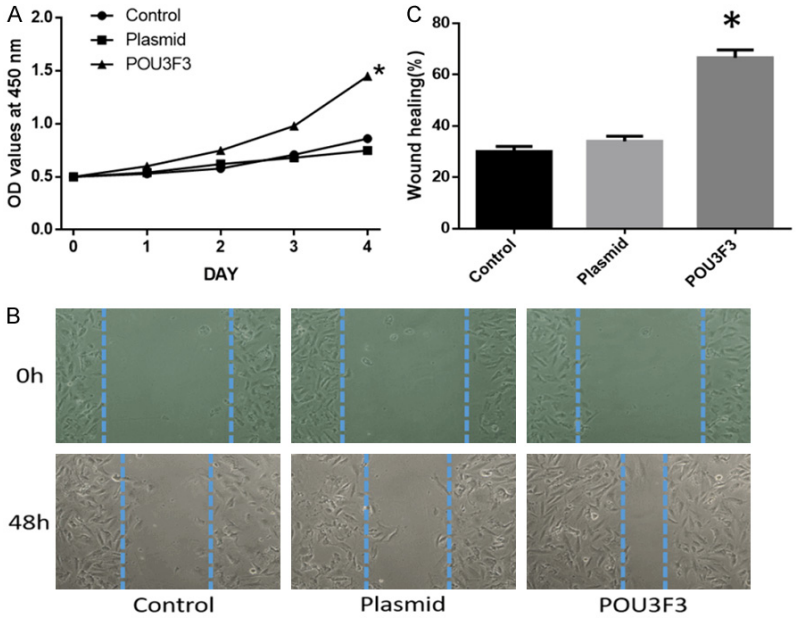

Figure 3. Linc-POU3F3 promotes VSMC proliferation and migration. Control, VSMCs were cultured without any treatment; Plasmid, VSMCs were transfected with blank plasmid; POU3F3, VSMCs were transfected with linc-POU3F3. All of these VSMCs were cultivated and examined at different time points with CCK-8 and wound healing assay. A. CCK-8 assay was used to assess the proliferation of VSMCs. B, C. Wound-healing assay was employed to evaluate migration of VSMCs (*P < 0.05 vs. control group).

nM) after transfection with POU3F3, for 48 h (**Figure 4A**). As shown in **Figure 4B**, following the restoration of the levels of miR-449a, expression of SM-MHC and α-SMA were positively correlated while those of KLF4 and OPN were negatively correlated with miR-449a levels. This indicated that miR-449a could downregulate the expression of KLF4 and promote the VSMC phenotypic transformation. In addition, VSMCs were still transfected with POU3F3 for another 48 h followed by treatment with KLF4 inhibitor at different concentrations (low dose and high dose) for 24 h (**Figure 4B**). With the declining trend of KLF4 expression, SM-MHC and α-SMA protein expression upregulated while OPN protein expression downregulated, demonstrating that KLF4 expression positively correlated with phenotypic transformation of VSMCs. Meanwhile, association between miR-449a and KLF4 was predicted by TargetScan 7.0. (**Figure 4D**, **4E**) and verified by luciferase reporter assay (**Figure 4F**). Hence, we concluded that linc-POU3F3 induces VSMC phenotypic transformation via POU3F3/miR-449a/KLF4 pathway signaling.

Discussion

With the increasing proportion of population suffering from coronary heart disease, PCI represents the default strategy in most of these people, while both BMS and DES are typically used in this process. However, neither BMS nor DES could bypass a significant problem, that is, ISR [8]. The incidence rate of ISR is increasing every year even with constant technological advancement due to larger proportion of population undergoing angioplasty and stent implantation [21].

About 14.7% ISR patients develop restenosis or even blood loss in interventional vessel within one year while more than 50% ISR patients develop acute coronary syndromes (ACS), such as acute myocardial infarction, and thus, the importance of this issue needs to be highlighted [22, 23]. The collected clinical samples showed a noticeable elevation of linc-POU3F3 in ISR patients in comparison to negative control. Furthermore, we discovered that POU3F3 expression is an independent risk factor for ISR as well as hypertension, diabetes, etc. Thus, the main aim of our study was to explore the association between ISR and lincRNA-POU3F3 and the specific underlying mechanism.

LncRNAs influence gene expression under diverse conditions and play important roles in various diseases [24]. LincRNAs is a subtype of lncRNAs; a novel type of lincRNA, Linc-POU3F3 has attracted a lot of attention in recent years. Linc-POU3F3, also known as LINC01158, is located on the chromosome 2q12 and encodes a 747-bp long transcript with four exons [25].

microRNAs are a class of short (~22 nucleotides), non-coding RNA molecules which mainly act through binding to complementary sites on the 3'-UTR of the target mRNA and negatively Mregulate gene expression [26, 27]. Various

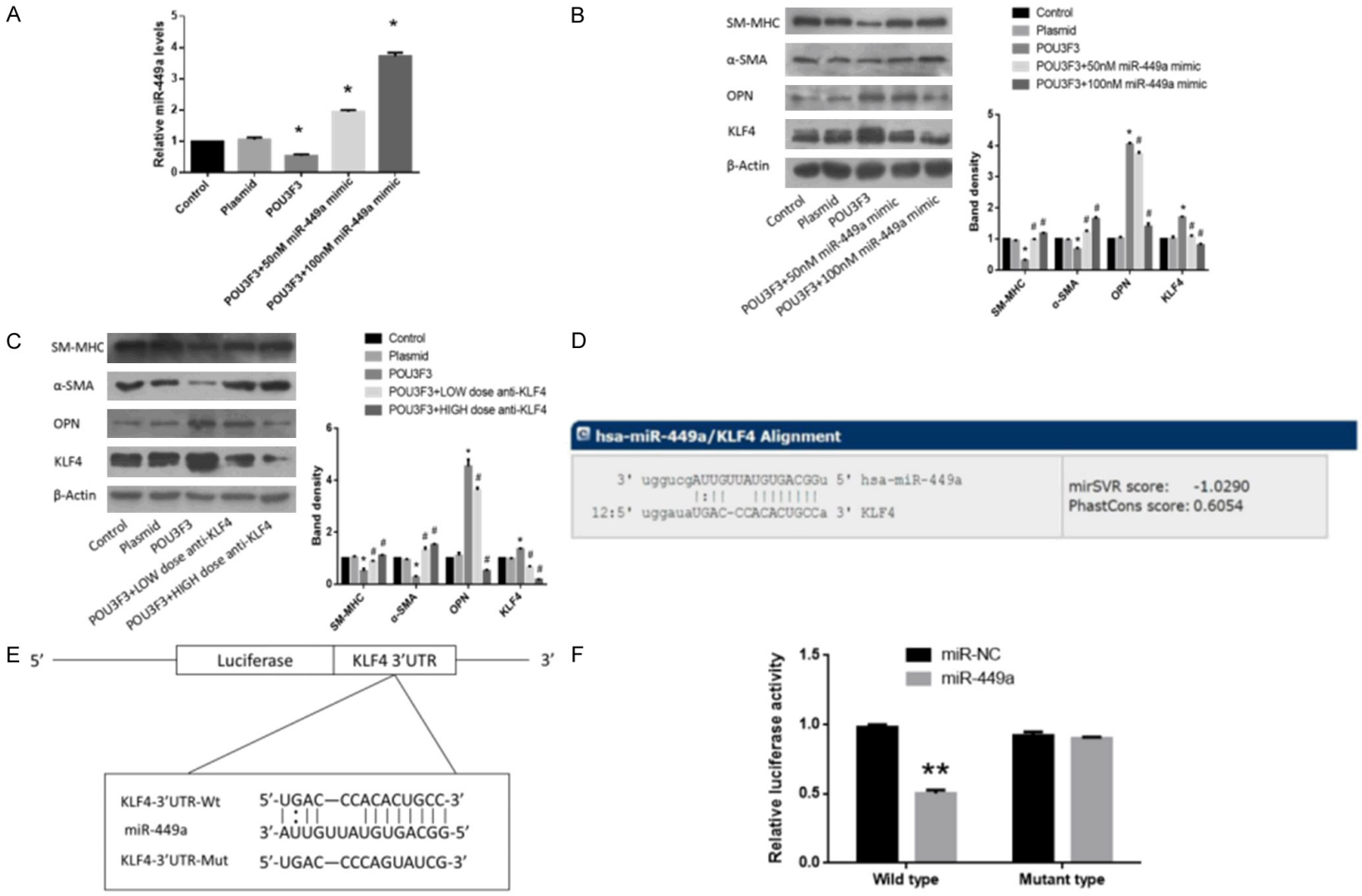

Figure 4. Linc-POU3F3 induces VSMC phenotypic transformation via POU3F3/miR-449a/KLF4 signaling pathway. VSMCs were treated either with plasmid, linc-POU3F3, linc-POU3F3 with different dose miR-449a mimics, or linc-POU3F3 with different dose KLF4 inhibitors to explore the molecular mechanism of VSMC phenotypic transformation. A. miR-449a is overexpressed in the VSMCs transfected with miR-449a mimic compared with controls (*P < 0.05 vs. control, n = 3). B. SM-MHC, α-SMA levels upregulated while KLF4 and OPN levels downregulated following the transfection of different concentrations miR-449a mimic compared with POU3F3 alone (*P < 0.05 vs. control; #P < 0.05 vs. treatment with POU3F3 alone, n = 3). C. SM-MHC, α-SMA levels upregulated but KLF4 and OPN levels downregulated following the application of different concentrations KLF4 siRNA compared with POU3F3 alone (*P < 0.05 vs. control; #P < 0.05 vs. treatment with POU3F3 alone, n = 3). D, E. The potential binding site for miR-449a and KLF4 was predicted by bioinformatics tools. F. Luciferase reporter assay validated the existence of the potential binding site between miR-449a and KLF4 (**P < 0.01 vs. control).

metabolic diseases, including diabetes mellitus [28] and cancer [29] are known to involve miRNA alterations. KLF4, a member of the KLF sub-family of zinc-finger proteins, is one of the regulators of VSMC phenotype transformation [30, 31]. A number of studies have reported that KLF4 can act as a downstream target of miRNAs [32, 33]. In addition, TargetScan 7.0 was employed to predict the association between the two genes mentioned above and successfully found the relevant binding site.

We collected serum samples from 27 patients with ISR and 32 patients without ISR, and investigated whether some biomarkers or treatments for ISR can be identified. Through this analysis we found that POU3F3 is significantly upregulated in serum samples from patients with ISR. Therefore, elucidating the association between POU3F3 and ISR and uncovering the underlying mechanism may serve as a novel strategy for preventing or predicting ISR. Moreover, VSMC phenotypic transformation plays a pivotal role in ISR development [11]. According to our results, SM-MHC and α-SMA were downregulated and OPN was upregulated, respectively, after the transfection with POU3F3, indicating that phenotypic transformation occurred in smooth muscle cells. Furthermore, upon transfection with miR-449a mimic and KLF4 siRNA, the levels of SM-MHC, α-SMA and OPN gradually returned back to normal, which showed the roles of miR-449a and KLF4. Finally, by combining the results of the luciferase reporter assay with these findings, we conclude that POU3F3/miR-449a/KLF4 pathway participates in VSMC phenotypic transformation. Thus, it can be said that linc-POU3F3 induces VSMC phenotypic transformation via the POU3F3/miR-449a/KLF4 signaling pathway; this indicates that this lncRNA in serum might be used as novel diagnostic biomarkers or a potential therapeutic targets for patients with ISR.

Taken together, the present data suggest that a novel lincRNA-POU3F3 is overexpressed in ISR patients and induces phenotypic transformation of VSMCs via POU3F3/miR-449a/KLF4 signaling pathway, which may be a promising approach for diagnosis and treatment of ISR.

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Disclosure of conflict of interest

None.

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