Modulation of the Proliferation/Apoptosis Balance of Vascular Smooth Muscle Cells in Atherosclerosis by IncRNA-MEG3 via Regulation of miR-26a/Smad1 Axis

Yang Bai, MD, Qiangnu Zhang, MD, Yijiang Su, MD, Zhenye Pu, MD and Kunsheng Li, MD

Summary
The balance between proliferation and apoptosis of vascular smooth muscle cells (VSMCs) plays a critical role in the initiation of atherosclerosis. IncRNA-MEG3 is involved in the pathophysiology of atherosclerosis through regulation of endothelial cell proliferation and migration. Its effect on the dysfunction of VSMCs and the corresponding mechanisms are actively researched. In this study, we observed that downregulated IncRNA-MEG3 expression was inversely correlated with the microRNA-26a level in coronary artery disease tissues. The overexpression of IncRNA-MEG3 could inhibit VSMCs proliferation while facilitating apoptosis. Moreover, alteration in the miR-26a/Smad1 axis could antagonize this effect. Bioinformatic analysis indicated that IncRNA-MEG3 could interact with miR-26a via complementary binding sites. The enforced expression of IncRNA-MEG3 could reduce the level of miR-26a in VSMCs, while the expression of Smad1 increases. Further, the direct binding between IncRNA-MEG3 and miR-26a was confirmed via dual-luciferase reporter assay, which indicated that Inc-MEG3 could sponge miR-26a as a competing endogenous RNA. In summary, we propose that IncRNA-MEG3 modulates the proliferation/apoptosis balance of VSMCs in atherosclerosis by regulating the miR-26a/Smad1 axis.

Key words: Coronary heart disease, Non-coding RNAs, ceRNA, Post-transcription

Atherosclerosis is a common and complex chronic inflammatory disease, in which fibrous and lipid elements are deposited in the wall of medium-sized arteries, which may eventually result in cardiovascular diseases such as coronary heart disease and stroke. Although the treatment for atherosclerosis has been recently developed, the exact cause and pathogenesis of atherosclerosis remain unclear. Emerging evidence suggests that vascular smooth muscle cells (VSMCs) dysfunction could partially mediate the onset of atherosclerosis. In addition to the phenotypic switching of VSMCs, the imbalance between proliferation and apoptosis of VSMCs also plays a crucial role during the early stage of atherosclerosis. Therefore, maintaining the balance between proliferation and apoptosis of VSMCs may be a potential strategy for the treatment of atherosclerosis.

Long non-coding RNAs (LncRNAs), which are a group of non-coding RNAs comprising > 200 nucleotides (nts), have no or negligible protein-coding capacity but remarkable regulatory effects on diverse biological cell functions such as cell proliferation and apoptosis because of their various functions in the regulation of gene transcription, regulation of post-transcriptional gene regulation, epigenetic regulation, and translation. LncRNAs are dysregulated in several diseases, including atherosclerosis. Several studies have suggested that LncRNAs play a role in atherosclerosis development. Among the investigated LncRNAs, LncRNA-MEG3 has been substantially researched. Wu, et al. reported that LncRNA-MEG3 plays a critical role in the proliferation of regulating endothelial cells in atherosclerosis. Zhang, et al. found that the LncRNA-MEG3/miR-223/NLRP3 axis mediates endothelial cell pyroptosis in atherosclerosis. We found that LncRNA-MEG3 expression was lower in the atherosclerotic arterial wall tissues of patients with coronary artery disease (CAD), and LncRNA-MEG3 also expresses endogenously in VSMCs according to the QRT-PCR analysis in the preliminary experiments. However, to our knowledge, no report has revealed whether LncRNA-MEG3 regulates the balance between proliferation and apoptosis of VSMCs in the pathological process of atherosclerosis. We conducted this study to examine the potential role of
lncRNA-MEG3 in the proliferation/apoptosis imbalance of VSMCs in atherosclerosis. Additionally, we investigated the underlying mechanisms based on the competing endogenous RNA (ceRNA) theory. As per this theory, lncRNAs regulate other RNA transcripts by competing for shared microRNAs. In the present study, we selected the microRNA-26a/Smad1 axis as the candidate pathway for regulation by lncRNA-MEG3. Bioinformatic evidence reveals the presence of binding sites between lncRNA-MEG3 and microRNA-26a. On the other hand, published study indicates that microRNA-26a regulates VSMCs function by targeting Smad1. Therefore, we propose that the lncRNA-MEG3/microRNA-26a/Smad1 axis may be involved in the proliferation/apoptosis imbalance of VSMCs in atherosclerosis.

Methods
Bioinformatic analysis: The lncRNA-MEG3-miR-26a interaction was predicted using the prediction module by the DIANA LncBase2 tools. The target relationship between miR-26a and Smad1 was predicted using miRanda/miRSVR and Target Scan.

Samples collection and cell culture: CAD tissues \( (n = 40) \) and control tissues \( (n = 35) \) were collected from Tongji Hospital. Briefly, the CAD tissues (atherosclerotic arterial wall) were collected from the coronary artery of patients with CAD during coronary artery bypass grafting surgery. The control tissues (normal arterial wall) were collected from the coronary artery of subjects without CAD during autopsy. The protocol of this study was approved by the ethics committee of Tongji Hospital, and all patients provided informed consent. Human VSMC line was purchased from American type culture collection (ATCC) and cultured in vascular cell basal medium supplemented with the components in the ATCC VSMC growth kit. The cultures were maintained in a humidified incubator at 37°C and in 5% CO₂.

Transfection: To induce overexpression of lncRNA-MEG3 in VSMCs, pcDNA3.1-MEG3 vectors were transfected into the cells. To enhance the miR-26a level, miR-26a mimics were transfected. siRNA of Smad1 (siRNA-Smad1) was used to knockdown the expression of Smad1 in VSMCs. pcDNA3.1-MEG3 was transfected with Lipofectamine 2000 DNA Transfection Reagent. The miR-26a mimics and siRNA-Smad1 were delivered by Lipofectamine RNAiMAX Reagent. The pcDNA3.1 empty vectors and all stars negative control sequences were used as a negative transfection control. All transfections were performed in accordance with the manufacturer’s instructions.

Cell proliferation: Cell proliferation assay was performed using the CellTiter-Blue™ Cell Viability Assay Kit according to the manufacturer’s instructions. Briefly, 5,000 cells were seeded into 96-well plates, and 50 μL of 1:10 diluted reagent was added into each well. After an incubation period of 1 hour, the fluorescence \( (560_{\text{nm}}/590_{\text{nm}}) \) was measured at 24 hours and 96 hours using a fluorometer. The proliferation rate of VSMCs was determined using the following formula: proliferation rate = (96-hour cell viability − 24-hour cell viability)/24-hour cell viability * 100%; the relative proliferation rate was determined using the following formula: relative proliferation rate = proliferation rate (treatment)/proliferation rate (control) * 100%.

Flow cytometry assay for apoptosis: After 48 hours of transfection, the cells were collected and stained with Annexin V-FITC and propidium iodide. The apoptosis rates of the treated cells were detected by the Guava® easyCyte Flow Cytometers and analyzed using built-in software.

Real-time quantitative PCR (qRT-PCR): Total RNA was isolated from cells using TRizol reagent. To detect lncRNA-MEG3 and Smad1 mRNA levels, reverse-transcription PCR was performed using the PrimeScript RT Master Mix kit. Next, the cDNA was amplified and quantified using the SYBR Premix Ex Taq II. Data were normalized using GAPDH. cDNA was synthesized by reverse-transcription PCR using the miScript II RT Kit to measure miR-26a, and the subsequent quantitative PCR was conducted using Mir-X™ miRNA qRT-PCR SYBR® Kit. U6 snRNA was used as a reference gene. The relative expression of target genes was calculated using the 2⁻ΔΔCT method. All primers are listed in the Supplemental Table.

Western blot: VSMCs were lysed using RIPA buffer, and total protein was collected. Proteins were separated by SDS-PAGE and transferred onto NC membranes using routine protocol. The membranes were blocked with 5% nonfat milk at room temperature for 2 hours and incubated with Smad1 and GAPDH primary antibodies at 4°C for 12 hours. The membranes were washed and incubated with HRP secondary antibody at room temperature for 2 hours. The blots were visualized using electrochemiluminescence Western Blotting kit. The band intensity was quantified using ImageJ software.

Dual-luciferase reporter assay: The sequence of lncRNA-MEG3 containing the miR-26a binding site was amplified and cloned into PsiCHECK2 luciferase reporter vector (PsiCHECK2-MEG3-wt). A PsiCHECK2 luciferase reporter vector that contained a matching mutant sequence of lncRNA-MEG3 was also constructed (PsiCHECK2-MEG3-mut). VSMCs were co-transfected with PsiCHECK2-MEG3-wt/PsiCHECK2-MEG3-mut and miR-26a mimic/mimic negative controls (Allstar). Luciferase and Renilla signals were measured at 48 hours after transfection using Promega Dual Luciferase® Reporter Assay System. The wild-type and mutant miR-26a-binding sites in Smad1 were inserted into PsiCHECK2 luciferase reporter vectors (named PsiCHECK2-Smad1-wt and PsiCHECK2-Smad1-mut), respectively, to confirm direct binding between miR-26a and Smad1. A dual-luciferase reporter assay was performed using the above-mentioned protocol.

Statistical analysis: Statistical analysis was performed using R Statistical Software (version 3.3.3). Statistical significance between ≥ 2 groups was determined using the t-test or one-way analysis of variance. \( P < 0.05 \) was considered to be statistically significant.

Results
LncRNA-MEG3 expression was downregulated in CAD tissues and was associated with miR-26a/Smad1 level: We determined the expression levels of lncRNA-
MEG3 and miR-26a in CAD tissues using qRT-PCR. As shown in Figure 1A, lncRNA-MEG3 expression was reduced in CAD tissues as compared with that in normal tissues \( (P < 0.05) \). On the other hand, as shown in Figure 1B, the expression level of miR-26a increased in CAD tissues as compared with that in normal arterial tissues \( (P < 0.05) \). The mRNA Smad1 level was lower in CAD tissues than in normal tissues \( (P < 0.05) \). Correlation analysis revealed (Figure 1D and E) that the lncRNA-MEG3 level was negatively correlated with the miR-26 level but was positively correlated with Smad1 in CAD tissues \( (r^2 = 0.403 \text{ and } 0.372, P < 0.05) \).

**Enforced expression of lncRNA-MEG3 inhibits proliferation but promotes apoptosis of VSMCs in a miR-26/Smad1 axis-dependent manner:** To confirm the functions of lncRNA-MEG3 on the proliferation and apoptosis of VSMCs, the expression of lncRNA-MEG3 in cells was elevated using transfection pcDNA3.1-MEG3. As shown in Figure 2A, compared with the negative transfection control (NG), the overexpression of lncRNA-MEG3 significantly inhibited the proliferation of VSMCs \( (P < 0.05) \). However, the apoptosis of VSMCs was induced after pcDNA3.1-MEG3 transfection \( (P < 0.05) \). Importantlly, these effects of lncRNA-MEG3 overexpression could be alleviated to a certain extent by co-transfection with either miR-26a mimic or Smad1-siRNA. On the other hand, lncRNA-MEG3 could inhibit miR-26-induced or Smad1-siRNA induced proliferation. Collectively, this evidence suggests that lncRNA-MEG3 modulates the proliferation/apoptosis balance of VSMCs in a miR-26/Smad1 axis-dependent manner.

**LncRNA-MEG3 could regulate miR-26a/Smad1 axis in VSMCs as a ceRNA:** We assessed the modifications in miR-26a and Smad1 after lncRNA-MEG3 overexpression in VSMCs by qPCR (Figure 3A), which indicates that the overexpression of lncRNA-MEG3 decreased the level of miR-26a \( (P < 0.05) \). However, the mRNA level of Smad1 increased as a consequence of lncRNA-MEG3 upregu-
ROLE OF lncRNA-MEG3 IN CAD

The expression of LncRNA-MEG3 in VSMCs was upregulated by transfection of PcDNA3.1-MEG3. The function of enforced expression of LncRNA-MEG3 on proliferation/apoptosis balance was assessed. Overexpression of LncRNA-MEG3 could inhibit VSMCs proliferation (A), but promote apoptosis (B and C). Either miR-26a upregulation or Smad1 knockdown could alleviate these effects. n = 5, *P < 0.05 compared with NG control, #P < 0.05 compared with pcDNA3.1-MEG3 transfected cells.

The alterations of miR-26a and Smad1 after LncRNA-MEG3 overexpression in VSMCs were estimated by qRT-PCR and western blot. Overexpression of LncRNA-MEG3 decreased the level of miR-26a in VSMCs (A) but induced the Smad1 mRNA (B) and protein level (C). n = 5, *P < 0.05 compared with NG control.

The DIANA LncBase2 tools revealed putative miR-26a binding sites in lncRNA-MEG3, which was confirmed via a dual-luciferase reporter assay (Figure 4A). The relative fluorescence intensity in cells co-transfected with Psi-CHECK2-MEG3-wt and miR-26a was lower (P < 0.05), thus indicating that miR-26a could directly bind to lncRNA-MEG3. Although a target relationship between miR-26a and Smad1 has been previously reported, we still elucidated the association of miR-26a with Smad1 in VSMCs (Figure 4B). In the dual-luciferase reporter assay, transfection of miR-26a mimics significantly reduced the relative luciferase activity of the luciferase reporter vectors containing Smad1-wt but not the reporter containing Smad1-mut. The sequence of mutant sites in MEG3 3’UTR-mut and Smad1 3’UTR-vectors were showed in Figure 4C. As revealed by qRT-PCR and western blotting (Figure 5A and B), cells transfected with miR-26a mimic had lower Smad1 expression. Further, miR-26a upregulation reduced the level of lncRNA-MEG3 in VSMCs. Collectively, these findings corroborate our hypothesis that lncRNA-MEG3 may be a degradable sponge for miR-26a and mediated miR-26a/Smad1 axis in VSMCs.

Discussion

In this study, we evaluated the lncRNA-MEG3 level in arterial tissues samples. A lower expression of lncRNA-MEG3 was observed in samples from patients with CAD compared with normal arterial tissues. Furthermore, we detected higher microRNA-26a expression and lower Smad1 expression in CAD samples. In vitro, elevated lncRNA-MEG3 inhibited proliferation but induced apoptosis in VSMCs. In addition, upregulation of lncRNA-MEG3 could induce alteration of the microRNA-26a/Smad1 axis. Combining the results of the bioinformatic analysis and dual-luciferase reporter assay, we suggest that lncRNA-MEG3 has the potential for sponging miR-26a. The microRNA-26a/Smad1 axis reportedly participates in the regulation of VSMCs function in atherosclerosis, which enabled the confirmation of the target relationship between miR-26a and Smad1. These results indicated that a lncRNA-MEG3/microRNA-26a/Smad1 regulatory pattern may exist in the dysfunction of VSMCs in atherosclerosis.
Figure 4. A dual-luciferase reporter assay was performed to prove the potential LncRNA-MEG3-miR-26a and miR-26a-Smad1 binding sites. Co-transfecting VSMCs with PsiCHECK2-MEG3-wt and miR-26a mimic could reduce the relative fluorescence intensity (A). Transfection of miR-26a mimic significantly reduced the relative luciferase activity of the luciferase reporter vectors containing Smad1-wt (B). The sequence of mutant sites in MEG3-3’UTR-mut and Smad1-3’UTR-mut vectors (C). n = 5, *P < 0.05 compared with NG control.

Figure 5. miR-26a could regulate the level of Smad1. Overexpression of miR-26a inhibits the expression of Smad1 at the mRNA (A) and protein level (B). The LncRNA-MEG3 level was also reduced by miR-26a mimic transfection (C). n = 5, *P < 0.05 compared with NG control.
**MEG3** is defined as a maternally imprinted gene located on chromosome 14q32.3. This gene is widely expressed in normal arterial tissues but is often lost in a variety of diseases.\(^{15}\) The ectopic expression of the functions of lncRNA-MEG3 were initially reported in studies on tumors, including hepatocellular carcinoma, non-small cell lung cancer, gastric cancer, and gliomas.\(^{16-18}\) In general, evidence supports lncRNA-MEG3 as a cancer suppressor gene. The inhibitory effect of lncRNA-MEG3 on cell proliferation has been reported in several tumors. Kruer, et al.\(^{19}\) reported that lncRNA-MEG3 expression decreased in human lung tumors and lncRNA-MEG3 contributed to the regulation of lung cancer cell proliferation via the Rb pathway. In hepatocellular carcinoma, Zhuo, et al. demonstrated that lncRNA-MEG3 partially regulates hepatocellular carcinoma cell proliferation and apoptosis via the accumulation of p53.\(^{20}\) Downregulated lncRNA-MEG3 in breast cancer was also observed. The overexpression of lncRNA-MEG3 resulted in the proliferation of breast cancer cells being inhibited.\(^{21}\) The aberrant expression of lncRNA-MEG3 has also been reported in diseases of the cardiovascular system, including atherosclerosis. The role of lncRNA-MEG3 in endothelial cells was well investigated in atherosclerosis pathophysiology. Zhang et al. found that lncRNA-MEG3 enhanced pyroptosis of human aortic endothelial cells, which play a vital role in the development of atherosclerosis. Furthermore, the authors suggested that lncRNA-MEG3 as an endogenous sponge suppressed the miR-223 level and increased the NLRP3 expression.\(^{22}\) This regulatory axis could be used to explain how lncRNA-MEG3 functions in endothelial cell pyroptosis. Another study\(^{23}\) suggested that lncRNA-MEG3 suppresses the endothelial cell proliferation in atherosclerosis by mediating microRNA-21. However, to our knowledge, there is no published evidence proving the association of lncRNA-MEG3 with the dysfunction of VSMCs in atherosclerosis. We detected lncRNA-MEG3 expression in patients and analyzed its function in vitro. As expected, downregulated lncRNA-MEG3 was observed in patients with CAD, and lncRNA-MEG3 could inhibit the proliferation but induce apoptosis of VSMCs. These results indicated that lncRNA-MEG3 plays a role in atherosclerosis, which could be applied as a potential target for therapy. The mechanisms by which lncRNA-MEG3 regulates proliferation/apoptosis warrants further study. However, the molecular functions that lncRNAs execute and the corresponding mechanisms are substantially complicated.\(^{24}\) To simplify the question, we only attempted a popular theory known as the ceRNA theory, to partially explain how lncRNA-MEG3 executes a function. Briefly, we established a lncRNA-microRNA-mRNA regulatory model based on the ceRNA concept and designed experiments to confirm whether some mechanisms could fit this model. Additionally, we performed bioinformatic analysis to obtain the potential interaction between lncRNA-MEG3 and microRNAs. Using the DIANA LncBase2 tools, we presumed that > 200 microRNAs may have interacted with lncRNA-MEG3. Among these candidate microRNAs, we selected miR-26a because it was completely investigated in the regulation of VSMCs function. Leeper, et al. reported that miR-26a is a novel regulator of VSMCs function. In summary, their data showed that miR-26a promotes VSMCs proliferation while inhibiting cellular apoptosis by targeting Smad1. Yang, et al.\(^{25}\) concluded that miR-26a mediated the VSMCs phenotypic transition by targeting Smad1. Based on this evidence, we detected the change in the levels of miR-26 and Smad1 after overexpressing lncRNA-MEG3; our results showed that lncRNA-MEG3 could reduce miR-26a while increasing Smad1. In addition, an increasing p-Smad1 level was observed after lncRNA-MEG3 overexpression, which indicated that the activity of Smad1 was influenced by the total Smad1 changes. However, the downstream gene or pathway alteration induced by p-Smad1 level changes remains to be investigated.

To further confirm our findings, dual-luciferase reporter assay results indicated that lncRNA-MEG3 could sponge miR-26a directly. Hence, we believe that an lncRNA-MEG3/miR-26a/Smad1 regulatory model reasonably exists in the dysfunction of VSMCs in atherosclerosis.

**Conclusion**

In conclusion, we identified lncRNA-MEG3 as a crucial regulator in the balance between proliferation and apoptosis of VSMCs in the development of atherosclerosis. LncRNA-MEG3 may execute this function by regulating the miR-26a/Smad1 axis as a ceRNA. Recovering the expression of lncRNA-MEG3 in VSMCs may serve as a potential target for atherosclerosis therapies.

**Disclosures**

**Conflicts of interest:** None.

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Supplemental Files

Supplemental Table
Please see supplemental files: https://doi.org/10.1536/ihj.18-195