EXPERIMENTAL STUDY

LncRNA DIGIT Accelerates Tube Formation of Vascular Endothelial Cells by Sponging miR-134

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Summary
Atherosclerosis is one of the most prevalent and important cardiac diseases, involving the heart and brain. This study aimed to explore the impacts of lncRNA Divergent to GSC induced by TGF-b family signaling (DIGIT) on vascular endothelial cells tube-formation capacity so as to reveal the potentials of DIGIT in atherosclerosis therapy. DIGIT expression in human microvascular endothelial HMEC-1 cells was silenced by transfection with shRNAs-targeted DIGIT. The effects of DIGIT silence on cell viability, migration, apoptosis, and tube formation were then assessed. Additionally, the cross-regulation between DIGIT and miR-134, and between miR-134 and Bmi-1 was detected to further reveal through which mechanism(s) DIGIT mediated HMEC-1 cells. The results showed that DIGIT silence significantly reduced cell viability, migration, tube-like structures formation, and induced apoptosis in HMEC-1 cells. DIGIT worked as a sponge for miR-134, and the anti-growth, anti-migratory, and anti-tube-formation functions of DIGIT silence on HMEC-1 cells were abolished by miR-134 suppression. Bmi-1 was a target of miR-134, and Bmi-1 upregulation abolished miR-134 overexpression-diminished cell growth, migration, and tube formation of HMEC-1 cells. Furthermore, Bmi-1 upregulation activated PI3K/AKT and Notch signaling pathways. In conclusion, our study demonstrated that lncRNA DIGIT accelerated tube formation of vascular endothelial cells through sponging miR-134. Our findings suggest that DIGIT and miR-134 may be promising molecular targets for atherosclerosis therapy.

Key words: Divergent to GSC, Atherosclerosis

Atherosclerosis is one of the most prevalent and important cardiac diseases involving the heart and brain. Atherosclerosis is characterized by the development of complex atherosclerotic plaques, leading to hardening and narrowing of the arterial lumen. Its major clinical manifestations include coronary heart disease, ischemic stroke, and peripheral arterial disease. Currently, patients receive therapeutic cocktails containing statins, aspirin, adrenaline β-receptor inhibitors, and angiotensin-converting enzyme inhibitors; however, the risk of developing a major acute cardiovascular event still remains high.

Endothelium is one of the most important constituents of vascular homeostasis. Endothelial cells provide a selective and highly-responsive barrier that offers, under physiological conditions, an optimal ratio between vessel integrity and permeability. Efficient endothelial repair after endothelial damage or injury is a critical step for preventing atherosclerosis. Besides, angiogenesis is a critical feature of plaque development in atherosclerosis and might play a key role in both the initiation and later rupture of plaques that lead to myocardial infarction and stroke. It would seem that modulation of endothelial cells angiogenesis can be considered a promising approach for atherosclerosis treatment. In this regard, long non-coding RNAs (lncRNAs) appear to be an interesting tool for improving angiogenesis. For instance, in diabetes mellitus rat model overexpression of lncRNA, ANRIL upregulates vascular endothelial growth factor (VEGF) expression and promotes angiogenesis. Another in vitro study has reported that lncRNA SENCr induced proliferation, migration, and angiogenesis of human umbilical endothelial cells. These findings support the potential for targeted destruction for lncRNAs delivery, in particular for therapeutic angiogenesis.

Divergent to Goosecoid (GSC), a newly-discovered lncRNA is induced by TGF-b family signaling (DIGIT). DIGIT is divergently transcribed from the mesendoderm regulator GSC, and is required for productive endoderm differentiation of both human and mouse embryonic stem cells. Interestingly, endothelial cells originate from the endoderm. Based on these reasons, we speculated that...
DIGIT may play a regulatory role in endothelial cells angiogenesis. To test this hypothesis, we silenced DIGIT expression in human microvascular endothelial HMEC-1 cells, and the effects of DIGIT knockdown on cell viability, migration, apoptosis, and tube formation were assessed. Further, the cross-regulation between DIGIT and a recently reported angiogenic modulator (miR-134) was explored to reveal whether DIGIT modulated the tube-formation capacity of HMEC-1 cells in miR-134-dependent signaling. The findings of this study will add to the growing literature showing that targeting lncRNAs may represent a therapeutic strategy for the treatment of atherosclerosis.

**Methods**

**Cell culture:** Human microvascular endothelial HMEC-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured in Dulbecco’s Modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

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**Tube formation assay:** Cell tube formation was measured, as previously described. In brief, HMEC-1 cells were seeded in a matrigel (BD Biosciences, San Jose, CA, USA) extracellular matrix (ECM)-coated 6-well plate at a density of 5\times10^4 cell/well. After culture for 24 hours at 37°C, the formation of tube-like structures was photographed and tubulogenesis was determined by tube-like cells/total cells ratio.

**Cell viability assay:** For cell viability assay, a Trypan Blue Staining Cell Viability Assay Kit which was purchased from Beyotime (Shanghai, China) was used in the present work. In brief, 5\times10^5 cells were collected and suspended in 1 mL cell resuspension solution, and then 1 mL Trypan Blue Solution was added. After 5 minutes incubation at room temperature, cells were counted under hemocytometer. Viability was determined as the ratio of stained cells/total cells.

**Apoptosis assay:** Cell apoptosis was performed using an Annexin V-FITC Apoptosis Detection Kit (Beyotime). Cells (1\times10^6) were collected and washed twice with PBS. Cells were then suspended in 200 μL Annexin V-FITC Binding Buffer containing 5 μL Annexin V-FITC, and 10 μL PI was added. After incubation at room temperature in the dark for 30 minutes, 300 μL PBS was added, and the samples were immediately analyzed under flow cytometry (FACS can; Beckman Coulter, Fullerton, CA). Data were analyzed using FlowJo software (Treestar, San Carlos, CA).

**Migration assay:** Cell migration was determined using a modified two-chamber migration assay with a pore size of 8 μm. Cells (5\times10^5) suspended in 200 mL of serum-free medium were added to the upper chamber of the Transwell inserts, and 600 mL complete medium with 10% fetal bovine serum (FBS) was added to the lower compartment. After incubation at 37°C for 24 hours, non-transferred cells were removed from the upper surface of the filter with a cotton swab. Migrated cells were fixed with 100% methanol for 15 minutes and stained with 0.1% crystal violet (Sigma-Aldrich) for 30 minutes. Stained cells were viewed and photographed under an inverted microscope (Olympus IX51).

**Luciferase reporter assay:** The fragment from DIGIT containing the predicted miR-134 binding site was amplified by polymerase chain reaction (PCR) and was then cloned into a pmirGlo Dual-luciferase miRNA Target Expression Vector (Promega) to form the reporter vector DIGIT-wild-type (DIGIT-WT). To mutate the putative binding site of miR-134 in the DIGIT, the sequence of the putative binding site was replaced and named as DIGIT-mutated-type (DIGIT-mt). Similarly, the vector Bmi-1-WT which contained the predicted miR-134 binding site, and the vector Bmi-1-nt which contained the mutated binding site were constructed. These vectors were co-transfected with miR-134 mimic or mimic NC into cells, and the luciferase activity was tested by using a Dual-Luciferase Reporter Assay System (Promega).

**Western blot:** Cellular protein was isolated by using RIPA lysis buffer (Beyotime), according to the manufacturer’s instructions. Quantitation of the whole-cell extracts...
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**Figure 1.** Formation of tube-like structures. A: HMEC-1 cells were cultured in a matrigel-coated plate for 24 hours, and the formation of tube-like structures was monitored. HMEC-1 cells were cultured for 24 hours, the mRNA (B), and protein level expressions of angiogenesis associated factors (C), were measured by qRT-PCR and western blot analysis. Data are represented as mean ± SD from three independent experiments. ***P < 0.001 versus 0 hours group.

was performed by the BCA Protein Assay Kit (Solarbio Science and Technology, Beijing, China). Protein (0.1 mg) from each sample was resolved by 10%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk and then incubated with primary antibodies for the detection of VEGF (1:500 dilution, #710151, Invitrogen), VEGFR2 (1:1000 dilution, ab11939, Abcam, Cambridge, MA), CD144 (1:250 dilution, #14-1442-82, Invitrogen), eNOS (1:1000 dilution, ab76198, Abcam), Bcl-2 (1:1000 dilution, ab32124, Abcam), Bax (1:1000 dilution, ab53154, Abcam), caspase-3 (1:1000 dilution, ab 13586, Abcam), caspase-9 (1:1000 dilution, ab32539, Abcam), p-PI3K (1:1000 dilution, #4228, Cell Signaling Technology, Danvers, MA), PI3K (1:1000 dilution, ab 86714, Abcam), p-AKT (1:2000 dilution, #4060, Cell Signaling Technology), AKT (1:1000 dilution, #4981, Cell Signaling Technology), Notch1 (1:200 dilution, #41-3500, Invitrogen), Notch2 (1:2000 dilution, #PA5-47091, Invitrogen), and Notch3 (1:1000 dilution, #PA5-13203, Invitrogen). GAPDH (1:5000, ab8245, Abcam) was detected as an internal control. Then, the membranes were incubated with anti-mouse (#7076) and anti-rabbit (#7074) secondary antibodies (1:2000 dilution, Cell Signaling Technology). The bands were visualized using the enhanced chemiluminescence reagent (GE Healthcare, Little Chalfont, UK).

**Statistical analysis:** Data were presented from three independent experiments as mean ± standard deviation (SD). The data from cell viability assay were analyzed by two-way analysis of variance (ANOVA), and all other data were analyzed by one-way ANOVA. GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA) was used for these analyses. A P-value < 0.05 was considered to indicate a statistically significant result.

**Results**

**Formation of tube-like structures:** HMEC-1 cells were cultured in a matrigel-coated plate for 24 hours, and the formation of tube-like structures was monitored. As shown in Figure 1A, the ratio of tubes/nucleus was increased with the increased time elapsed, and the highest ratio was observed at 24 hours with a 1.2 ± 0.12-fold increase. Additionally, we measured the expression changes of angiogenesis-associated factors in HMEC-1 cells. qRT-PCR analysis data showed that the mRNA levels of VEGF, VEGFR2, CD144, and eNOS were all significantly upregulated after 24 hours of culture (P < 0.001, Figure 1B). Western blotting results showed that all these four angiogenesis-associated proteins accumulated with the increase of time elapsed (Figure 1C). Also, the most remarkable accumulations were observed at 24 hours. These
DIGIT silence inhibited cell growth, migration, and tube formation of HMEC-1 cells: To explore the functional impacts of DIGIT on vascular endothelial cells, DIGIT expression was silenced in HMEC-1 cells by transfection with two different sequences of shRNA-targeted DIGIT (sh-DIGIT#1 and sh-DIGIT#2), and then the expression of DIGIT was detected by qRT-PCR analysis. HMEC-1 cells were transfected with sh-DIGIT#1 or with its negative control (sh-NC), and then cell viability (B), migration (C), tubules/nucleus ratio (D), the expression of apoptosis related proteins (E), and the expression of angiogenesis-related factors (F) were assessed, respectively. Data represented as mean ± SD from three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 versus sh-NC group. h indicates hours.

Figure 2. Effects of DIGIT silence on cell growth, migration, and tube formation of HMEC-1 cells. A: HMEC-1 cells were transfected with two different sequences of shRNA-targeted DIGIT (sh-DIGIT#1 and sh-DIGIT#2), and then the expression of DIGIT was detected by qRT-PCR analysis. HMEC-1 cells were transfected with sh-DIGIT#1 or with its negative control (sh-NC), and then cell viability (B), migration (C), tubules/nucleus ratio (D), the expression of apoptosis related proteins (E), and the expression of angiogenesis-related factors (F) were assessed, respectively. Data represented as mean ± SD from three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 versus sh-NC group. h indicates hours.

data suggested that after 24 hours of culture, HMEC-1 cells could form tuber-like structures significantly. Considering that sh-DIGIT#1 group possessed a lower DIGIT level than the sh-DIGIT#2 group, sh-DIGIT#1 was selected for use in the forthcoming study.

Next, we identified the growth, migration, and tube formation capacity of HMEC-1 cells which were detected by Trypan Blue Staining, Transwell, and tube formation assays after silencing DIGIT. As results show in Figure 2 B-D, cell viability, migration capacity, and tubes/nucleus ratio were all significantly reduced in sh-DIGIT group when compared with sh-NC group (P < 0.01, or P < 0.001). Further, the effects of DIGIT silence on HMEC-1
cells growth and tube formation were analyzed by detection of expressions of apoptosis and angiogenesis-associated proteins. Western blotting results showed that DIGIT silence downregulated the levels of Bcl-2, VEGF, VEGFR2, CD144, and eNOS, upregulated Bax, and activated caspase-3 and caspase-9 expressions (Figure 2E, F). The data collectively suggested that DIGIT plays a crucial role in the modulation of HMEC-1 cells growth, migration, and tube formation.

**DIGIT worked as a sponge for miR-134:** In a recent study, miR-134 was identified as an angiogenic modulator that decreases endothelial colony forming cells migration and microvascular formation.15) Thus, herein we detected the cross-regulations between DIGIT and miR-134 in order to explore the underlying mechanism(s) via which DIGIT impacts the tube-formation capacity of HMEC-1 cells. qRT-PCR analysis results showed that miR-134 level was significantly downregulated in sh-DIGIT transfected cells when compared with the sh-NC group of cells \( P < 0.01 \), indicating that miR-134 was negatively regulated by DIGIT. Then luciferase reporter assay was performed and results showed that the luciferase activity was significantly reduced after cells were co-transfected with DIGIT-WT and miR-134 mimic \( P < 0.05 \), Figure 3 B). These data suggest that DIGIT could target miR-134.

**miR-134 suppression abolished DIGIT silence-diminished cell growth, migration, and tube formation of HMEC-1 cells:** To ask if miR-134 was involved in DIGIT-mediated cell growth, migration, and tube formation of HMEC-1 cells, sh-DIGIT and miR-134 inhibitors were co-transfected into HMEC-1 cells. We found that DIGIT silence-diminished cell viability, migration, and tubes/nucleus ratio, as well as DIGIT silence-induced increase in apoptotic cells rate, were all abolished after the addition of miR-134 inhibitor \( P < 0.05 \), \( P < 0.01 \), or \( P < 0.001 \), Figure 4A-D). Besides, results from western blot analysis showed that DIGIT silence induced the down-regulations of Bcl-2, VEGF, VEGFR2, CD144, eNOS, upregulation of Bax, and activations of caspase-3 and caspase-9 were all recovered by miR-134 suppression. Altogether, these data suggest that DIGIT silence inhibited HMEC-1 cells growth, migration, and tube formation via negative regulation of miR-134.

**Bmi-1 was a target of miR-134:** Previous studies have reported the angiogenesis-promoting effects of Bmi-1 on hepatic cells,10) glioma cells,20) and lung adenocarcinoma cells.21) Thus, we detected whether miR-134 could crosstalk with Bmi-1, which may be helpful for us to understand the role of miR-134 in tube formation. As shown in Figure 5A, the mRNA level of Bmi-1 was downregulated in miR-134 overexpressing-cells \( P < 0.05 \), while it was upregulated in miR-134 suppressing-cells \( P < 0.05 \), which indicated Bmi-1 was negatively regulated by miR-134. Luciferase reporter assay results then showed that the luciferase activity was significantly reduced after cotransfection with Bmi-1-WT and miR-134 mimic \( P < 0.05 \), Figure 5B), suggesting that Bmi-1 was a target of miR-134.

**Bmi-1 upregulation abolished miR-134 overexpression-diminished cell growth, migration, and tube formation of HMEC-1 cells:** Next, we detected the effects of Bmi-1 on miR-134-mediated cell growth, migration, and tube formation of HMEC-1 cells. The data in Figure 6A shows that miR-134 mimic transfection-induced downregulation of Bmi-1 was recovered by addition of Bmi-1 expressing vector (pEX-Bmi-1) \( P < 0.001 \), indicating the success of transfection. Data in Figure 6B-E showed that miR-134 overexpression-diminished cell viability, migration, tubes/nucleus ration, and miR-134 overexpression-induced apoptosis were all abolished by addition of pEX-Bmi-1 \( P < 0.05 \), or \( P < 0.001 \). Additionally, western blotting analysis results showed that miR-134 overexpression-induced downregulations of Bcl-2, VEGF, VEGFR2, CD144, eNOS, upregulation of Bax, and activations of caspase-3 and caspase-9 were all recovered by Bmi-1 upregulation (Figure 6F, G). These data suggest that miR-134 overexpression inhibited HMEC-1 cells growth, migration, and tube formation might be via negative regulation of Bmi-1.

**Bmi-1 upregulation activated PI3K/AKT and Notch**
Figure 4. Effects of miR-134 suppression on DIGIT-mediated cell growth, migration, and tube formation of HMEC-1 cells. HMEC-1 cells were co-transfected with sh-DIGIT#1 and miR-134 inhibitor, and then cell viability (A), migration (B), tubes/nucleus ratio (C), apoptotic cells rate (D), the expression of apoptosis related proteins (E), and the expression of angiogenesis-related factors (F) were assessed, respectively. Data represented as mean ± SD from three independent experiments. ** P < 0.01, *** P < 0.001 versus sh-NC + inhibitor NC group. *P < 0.05, **P < 0.01, ***P < 0.001 versus sh-DIGIT + inhibitor NC group.

signaling pathways: Given that PI3K/AKT and Notch signaling pathways are known to play key roles in numerous cellular functions including proliferation, migration, survival, and angiogenesis,22-24 we focused on these two signaling pathways to further reveal the underlying mechanism(s) via which Bmi-1 influenced the tube-formation capacity of HMEC-1 cells. Western blot analysis results showed that p-PI3K, p-AKT, Notch1, Notch2, and Notch3 were all downregulated in Bmi-1 suppressing-cells, while the same p-PI3K, p-AKT, Notch1, Notch2, and Notch3 were upregulated in Bmi-1 overexpressing-cells (Figure 7 A, B). These data implied that Bmi-1 could activate both PI3K/AKT and Notch signaling pathways in HMEC-1 cells.

Discussion
Endothelial dysfunction represents an early step in
LncRNA DIGIT is a newly-discovered lncRNA, and to date little was known about its functions in organisms. To the best of our knowledge, only one literature to date has reported about DIGIT, in which DIGIT was identified as a key regulator of human and mouse definitive endoderm differentiation. In this study, we demonstrated that DIGIT silence significantly reduced cell viability, migration, tube-like structures formation, and also induced apoptosis of HMEC-1 cells, implying the pro-growth, pro-migratory, and pro-angiogenic roles of DIGIT in endothelial cells.

VEGF and its receptors (VEGFRs) are key modulators of angiogenesis. Recent study has shown that VEGF may also be implicated in the survival, proliferation, and migration of endothelial cells. CD144 is a strictly endothelial-specific adhesion molecule and is of vital importance in maintaining and controlling the endothelial phenotype through appropriate cell-cell contacts. CD144 is essential during embryonic angiogenesis, cell proliferation, apoptosis, and modulation of VEGFR. eNOS is a constitutively expressed gene in endothelial cells that plays a central role in preventing thrombosis and atherogenic processes by maintaining endothelial cells functional integrity, regulating hemodynamics, and establishing collateral circulation. In this study, we found that DIGIT downregulated VEGF, VEGFR2, CD144, and eNOS protein expressions in HMEC-1 cells. These findings further confirm our hypothesis that DIGIT could promote endothelial cells growth, migration, and tube formation. However, whether VEGF, VEGFR2, CD144, and eNOS also have a regulatory role in endogenous DIGIT expression still needs to be revealed.

miR-134 belongs to chromosome 14q32 miRNAs clusters, and it has been reported that miR-134 is essential for human carcinoma and participates in tumor cell proliferation, apoptosis, invasion, and metastasis. In this study, we found that miR-134 can be targeted by DIGIT, and thus we speculated that it might be implicated in DIGIT-modulated cell growth, migration, and tube formation of HMEC-1 cells. Recently, Wang et al. demonstrated that miR-134 reduced endothelial colony forming cells migration and tube formation ability, but had no impact on cell proliferation and cell cycle arrest. Our study was partially consistent with their study, which provided evidence that miR-134 may possess anti-migration and anti-angiogenic functions in endothelial cells as miR-134 suppression abolished DIGIT silence-diminished migratory and angiogenic capacities. However, inconsistent with this study of Wang et al., we demonstrated that miR-134 also exerted anti-growth role as miR-134 suppression abolished DIGIT silence-diminished cell viability and DIGIT silence-induced apoptosis. A possible explanation for this contradiction is that the functions of miR-134 depend on different cell types. More importantly, our findings have evidenced that DIGIT promotes endothelial cells growth, migration, and tube formation via sponging miR-134.

Bmi-1 regulates a myriad of cellular processes criti-
Effects of Bmi-1 upregulation on miR-134-mediated cell growth, migration, and tube formation of HMEC-1 cells. HMEC-1 cells were co-transfected with miR-134 mimic and a Bmi-1 expressing vector (pEX-Bmi-1), and then the expression levels of Bmi-1 (A), cell viability (B), migration (C), tube/nuclei ratio (D), apoptotic cells rate (E), the expression of apoptosis related proteins (F), and the expression of angiogenesis-related factors (G) were assessed respectively. Data represented as mean ± SD from three independent experiments. **P < 0.01, ***P < 0.001 versus mimic NC + pEX group. #P < 0.05, ###P < 0.001 versus miR-134 mimic + pEX group.

Bmi-1 is known to play important roles in regulating numerous cellular functions including proliferation, migration, survival, and angiogenesis in both normal tissues and cancers. In their study, Wang et al. demonstrated that Bmi-1 activating PI3K/AKT signaling pathway is the main mechanism for promoting metastasis ability of pancreatic cancer stem cells. Besides, Suh and Han in their study have reported that Bmi-1-activating Notch is one of the signaling pathways which modulates embryonic stem cells self-renewal. Our findings were consistent with those findings of previous studies, which suggest that Bmi-1 could activate PI3K/AKT and Notch signaling pathways in HMEC-1 cells, and these findings provide the evidence that Bmi-1 promotes endothelial cells growth, migration,
and tube formation via regulation of PI3K/AKT and Notch signaling pathways.

In conclusion, we have demonstrated that lncRNA DIGIT silence inhibits endothelial cells growth, migration, and tube formation. DIGIT functions as a pro-growth, pro-migratory, and pro-tube-formation factor in endothelial cells through sponging miR-134. Our findings suggest that DIGIT and miR-134 may be promising molecular targets for atherosclerosis therapy. Further in vivo investigations are required to confirm these hypotheses.

Disclosures

Conflicts of interest: Authors report no conflict of interest in this work.

References

26. Bai Y, Wang X, Shen L, et al. Mechanical stress regulates endothelial progenitor cell angiogenesis through VEGF receptor en-