Suppression of circDcbld1 Alleviates Intimal Hyperplasia in Rat Carotid Artery by Targeting miR-145-3p/Neuropilin-1

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We replicated the rat common carotid artery (CCA) intima hyperplasia model and found the expression of a circular RNA, circRNA_009723 (circDcbld1), was markedly increased in the CCA with intimal hyperplasia. In vitro, the suppression of circDcbld1 in rat vascular smooth muscle cells (VSMCs) led to the increase of contractile smooth muscle cell markers and the decrease of cell migration. In vivo, the injection of chemically modified circDcbld1 small interfering RNA (siRNA) lessened the formation of neointima in rat CCA after balloon injury. Further experiments proved that circDcbld1, as a competing endogenous RNA, interacted with miR-145-3p and upregulated the level of neuropilin-1 (Nrp1), thereby regulating the migration of VSMCs. In this study, we demonstrated a new mechanism by which circular RNA promotes intimal hyperplasia. We deem that intervention in the circDcbld1-miR-145-3p/Nrp1 pathway might be a feasible approach to alleviate the post-injury intimal hyperplasia.

RESULTS
circDcbld1 Was Highly Expressed in Injured Artery
The rat CCA intimal hyperplasia model was made by balloon injury.11,12 14 days after the procedure, the animals were sacrificed and CCAs were harvested. Histological sections showed marked neointima formed in CCA. The circRNA microarray demonstrated the expression of rno-circRNA_009723 (circDcbld1) was the top upregulated circRNA in the injured artery. In this study, we investigated the function and mechanism of circDcbld1 in VSMCs. The findings may provide a new approach to alleviate intimal hyperplasia caused by vascular injury.

INTRODUCTION
Vascular surgery and endovascular procedure are the primary treatments for chronic obstructive vascular diseases. However, postoperative restenosis is an inevitable problem. The cause of restenosis is the abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) and the excessive production of the extracellular matrix. Drug-eluting balloons and drug-eluting stents are used to avoid intimal hyperplasia. These devices prevent cell overgrowth by locally releasing antiproliferation chemicals.1,2 Since the targets of these drugs are non-specific, vascular endothelial cells (ECs) are also affected by drugs, leading to a delay of reendothelialization and increasing the risk of thrombosis. Patients often require long-term antiplatelet therapy to avoid thrombosis. However, some patients are unable to use antiplatelet drugs due to drug contraindications, which creates a clinical paradox. Therefore, it is necessary to find other ways to reduce neointima hyperplasia by further exploring the mechanism of intimal hyperplasia.

In recent years, a variety of non-coding RNAs have been reported to be involved in vascular injury.3–5 Circular RNA (circRNA) is a long non-coding RNA that forms a closed loop through reversed splicing at the 3' and 5' ends. Without open ends, circRNA is resistant to exonuclease and is more stable than linear RNA. circRNAs modulate gene expression in a variety of ways, which include binding microRNAs (miRNAs).6,7 RNA-binding proteins,8 and mRNA.9 There is evidence that circRNAs also participate in VSMC proliferation.10 We have detected the circRNAs expression in healthy and balloon-injured rat common carotid arteries (CCAs). circRNA_009723 was the top upregulated circRNA in the injured artery. In this study, we investigated the function and mechanism of circDcbld1 in VSMCs. The findings may provide a new approach to alleviate intimal hyperplasia caused by vascular injury.
markedly declined with time (approximately 80% at 24 h) (Figure 1D).

The total RNA extract of VSMCs was treated with the exonuclease RNase R. The reduction of \( \text{circDcbld1} \) was also lower than that of \( \text{Dcbld1} \) (Figure 1E). The results indicated that \( \text{circDcbld1} \) had a longer half-life and was more stable.

According to NCBI BLAST, the sequence of \( \text{rno-circRNA}_009723 \) was matched entirely with the exon of the \( \text{Rattus norvegicus Dcbld1} \) gene. Therefore, we named \( \text{rno-circRNA}_009723 \) as \( \text{circDcbld1} \). The homology of the \( \text{Homo sapiens Dcbld1} \) gene and \( \text{Rattus norvegicus Dcbld1} \) gene is 98%.

**The Expression and Localization of \( \text{circDcbld1} \) in Rat CCA**

RNA fluorescent in situ hybridization (RNA-FISH) showed \( \text{circDcbld1} \) expressed in the media of the carotid artery (Figure 2A). It was highly expressed in the neointima and was located in the cytoplasm of cells (Figure 2B).

\( \text{circDcbld1} \) was located in the cytoplasm of cultured rat VSMCs (Figure 2D). As a comparison, it was lowly expressed in cultured ECs (Figure 2C).

**The Silence of \( \text{circDcbld1} \) Increased the Contractile Smooth Muscle Cell Markers and Decreased the Migration of VSMCs**

\( \text{circDcbld1} \) of VSMCs was knocked down by small interfering RNA (siRNA) (Figure 3A). The silence of \( \text{circDcbld1} \) led to an increase of typical contractile smooth muscle cell markers, including \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA), smooth muscle myosin heavy chain (SM-MHC), and calponin (Figures 3B–3D).

The knockdown of \( \text{circDcbld1} \) reduced VSMC migration in a Transwell assay (Figure 3E) and scratch wound healing assay (Figure 3F). Low expression of \( \text{circDcbld1} \) showed no marked effect on VSMC proliferation according to cyclin D1 detection (Figure 3G) and a 5-ethynyl-2'-deoxyuridine (EdU) assay (Figure 3H).

**\( \text{circDcbld1} \) Is the Competing Endogenous (ceRNA) of miR-145-3p**

By using TargetScan and miRanda, we predicted the possible target miRNAs of \( \text{circDcbld1} \). The most probable targets were miR-326-5p, miR-384-3p, miR-362-3p, miR-500-5p, and miR-145-3p (Figure 4A). According to the miRNA sequencing, miR-326-5p, miR-384-3p, miR-362-3p, and miR-500-5p had low expressions in CCA. Only
miR-145-3p was highly expressed in healthy CCA and decreased in injured CCA (Figure 4B). PCR verified the expression difference (Figure 4C). When circDcbld1-wild-type (WT)-Report and miR-145-3p mimics were co-transfected into HEK293 cells, the relative luciferase activity of the reporter was reduced. As a contrast, the co-transfection of circDcbld1-Mut-Report and miR-145-3p mimics showed no marked change in the relative luciferase activity (Figure 4D). RNA-FISH demonstrated that both circDcbld1 and miR-145-3p were located in the cytoplasm of cells in the neointima of injured CCA (Figure 4E).

miR-145-3p Inhibitors Reversed the Decrease in VSMC Migration Caused by circDcbld1 Knockdown

The expression of circDcbld1 was interfered with siRNA for 24 h. Then, miR-145-3p inhibitors were transfected into VSMCs. miR-145-3p inhibitors reversed the reduction of VSMC migration in both a Transwell assay (Figure 5A) and scratch wound healing assay (Figure 5B). It indicated that circDcbld1 regulates VSMC migration via miR-145-3p.

Nrp1 Is the Target of miR-145-3p in VSMCs

We predicted the possible targets of miR-145-3p with TargetScan and miRanda. Depending on gene sequence pairing and the potential effects in the vascular cell, we chose Nrp1 (neuropilin-1), Ccnd2 (cyclin D2), and Postn (periostin) as candidate genes (Figure 6A). They encode neuropilin-1, cyclin D2, and periostin, respectively. Western blot showed neuropilin-1 (Figure 6B), cyclin D2, and periostin were all increased in the injured CCA.

The silence of circDcbld1 reduced the level of neuropilin-1 (Figure 6C) but did not change the levels of cyclin D2 and periostin in VSMCs. Besides, miR-145-3p inhibitors elevated the level of neuropilin-1 (Figure 6D) and did not cause marked changes in cyclin D2 and periostin levels.

In the Dual-Luciferase Reporter Assay, the co-transfection of Nrp1-WT-Report and miR-145-3p mimics reduced the relative luciferase activity. As a contrast, the co-transfection of Nrp1-Mut-Report and miR-145-3p mimics showed no change in the relative luciferase activity (Figure 6E).

The Silence of circDcbld1 Reduced Intimal Hyperplasia

After balloon injury, the Stable siRNA of circDcbld1 was injected into the tissue surrounding the rat CCA (Figure 7A). PCR showed that the chemically modified siRNA was able to effectively inhibit the circDcbld1 expression in rat carotid arteries (Figure 7B). Western blot demonstrated that the low expression of circDcbld1 resulted in neuropilin-1 reduction (Figure 7C).

The knockdown of circDcbld1 also led to a markedly decline in the neointima area (Figures 7D and 7E). That is, the silence of circDcbld1 alleviated intimal hyperplasia.
DISCUSSION

Intimal hyperplasia occurs after endovascular procedures and is also common in chronic cardiovascular diseases such as hypertension and atherosclerosis. It is characterized by the dysfunction of ECs and the excessive proliferation and migration of VSMCs, resulting in progressive stenosis and occlusion of the vascular lumen. A variety of cells, growth factors, and inflammatory mediators are involved in the process. Recently, many non-coding RNAs have been identified to participate in neointima formation. Nevertheless, the roles of many non-coding (ncRNAs) are not elucidated.

In this study, we found that circDcbld1 was highly expressed in rat CCA with intimal hyperplasia. Through bioinformatic prediction and biochemical experiments, we showed that circDcbld1 induces VSMC migration via miR-145-3p. It should be noted that the current understanding of miR-145 function is mainly from miR-145-5p. miR-145-3p and miR-145-5p are derived from the same miRNA precursor, but their sequences are quite different. In the biogenesis of miRNA, pre-miRNAs are processed by Dicer1 to generate miRNA duplexes. Typically, the guide strand (labeled as -5p) from the miRNA duplex is retained in cytoplasm and recruits RNA-induced silencing complex (RISC) to target mRNA. The passenger-strand (-3p) usually is inactive and degraded. However, studies have found that passenger strand also plays crucial roles.

It is well known that miR-145-5p is an essential vascular regulator that determines the fate of VSMCs. In this study, we found that miR-145-3p downregulated VSMC migration. Bioinformatics prediction pointed out several candidate genes of miR-145-3p. Nrp1, Ccnd2, and Postn are highly expressed in the injured CCA and have been reported to be involved in VSMC biology. Nrp1, also called vascular EC growth factor 165 receptor, is a membrane-bound coreceptor for vascular endothelial growth factor. It plays roles in angiogenesis, cell survival, migration, axon guidance, and invasion. In vascular biology, neuropilin-1 modulates EC growth and migration and also regulates VSMC migration. It was reported that neuropilin-1 and its isoform neuropilin-2 mediate neointimal hyperplasia and reendothelialization following arterial injury. Ccnd2 promotes cell proliferation. It is required for
cell cycle G1/S transition. Studies indicated that it modulates proliferation of ECs and VSMCs. Postn expression is elevated during vascular injury, and it modulates VSMC differentiation and migration. Dual-Luciferase Reporter Assay demonstrated that miR-145-3p targeted Nrp1. The transfection of miR-145-3p inhibitor also raised the level of neuropilin-1.

Taken together, miR-145-3p negatively regulates VSMC migration by targeting Nrp1 under physiological conditions. Induced by vascular injury, circDcbld1 was increased, and miR-145-3p was decreased, in VSMCs. As a ceRNA, circDcbld1 binds with cytoplasmic miR-145-3p, resulting in a further reduction of free miR-145-3p. As the effect of miR-145-3p is attenuated, the level of neuropilin-1 is increased, which promotes VSMC migration (Figure 7E). The migration of VSMCs is critical to intimal hyperplasia. Therefore, the regulation pathway of circDcbld1-miR-145-3p/neuropilin-1 should be essential. The knockdown of circDcbld1 or overexpression of miR-145-3p may be a promising therapeutic approach for intimal hyperplasia.
MATERIALS AND METHODS
The experimental animal protocol conformed to the recommendations in the 8th edition of the Guide for the Care and Use of Laboratory Animals of the NIH (NIH revised 2011) and the Animal Management Rules of China (documentation 55, 2201, Ministry of Health, China).

Intima Hyperplasia Model of Rat CCA
12-week-old male Sprague-Dawley rats were anesthetized by intra-peritoneal injection of 10% chloral hydrate (300 mg/kg). A 2F Fogarty arterial embolectomy catheter (Edwards Lifesciences, Irvine, CA, USA) was inserted into the left CCA of rats, pulled and rotated three times to injure the intima. The right CCA was used as the sham control. 14 days after the procedure, the rats were sacrificed, and the bilateral CCAs were harvested.

The Detection of circRNA
As previously described, the total RNA of CCA was extracted, and the linear RNAs were degraded with exonuclease (Thermo Fisher Scientific, Waltham, MA, USA). circRNAs in samples were detected using Arraystar rat circRNA array (8K/215K; Kangchen Biotech, Shanghai, China).

For verifying the expression of circRNA, PCR primers were designed for the back-splicing region of the circRNA. The region was amplified according to standard quantitative real-time PCR protocols. The PCR product was subjected to Sanger sequencing to verify circRNA containing the back-splicing junction.

The stability and half-life of circRNA were tested by RNase R and actinomycin D treatment assays. 1 μg of total RNA was incubated with 1 U RNase R (Epicenter, Madison, WI, USA) at 37°C for 10 min. Then, circRNA and the corresponding linear mRNA were detected by quantitative real-time PCR. 2 μM/mL actinomycin D (MilliporeSigma, Burlington, MA, USA) or DMSO was added to the medium of VSMCs. At different intervals, circRNA and the corresponding linear mRNA were detected by quantitative real-time PCR.

The Culture of Rat VSMCs and ECs
The thoracic aorta was harvested from a healthy male 8-week-old Sprague-Dawley rat. The adventitia of the vessel was removed. The intima was digested with 0.1% type I collagenase to isolate ECs. Cells were collected and cultured in EC medium (ScienCell, Carlsbad, CA, USA). The purity of ECs was identified using the von Willebrand factor (vWF) antibody (Abcam, Cambridge, UK).

The vascular media was cut into small pieces and placed in DMEM (Thermo Fisher Scientific) containing 10% fetal bovine serum (Gibco) for growing VSMCs. The purity of VSMCs was identified using the α-SMA antibody (Cell Signaling Technology, Danvers, MA, USA).

RNA-FISH
The specific fluorescent probe was designed for the back-splicing junction of circDcbld1. Following the instruction of the manufacturer (GenePharma, Shanghai, China), the paraffin sections and cell slides were hybridized with probe and observed with a laser confocal microscope (Nikon A1R Plus).
Bioinformatics Analysis

Database: TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org) were used to predict the target miRNAs of circDcbld1. The miRNAs are ranked based on the potential binding force with circDcbld1. The target genes of the miRNA were also predicted and annotated by TargetScan and miRanda.

The Detection of MiRNAs

The miRNA sequencing was performed on the Illumina platform (Kangchen Biotech). The expressions of miRNAs were verified by quantitative real-time PCR. Reverse transcription of miRNAs was performed by using a miScript II RT Kit (QIAGEN, Hilden, Germany). The miScript SYBR Green PCR Kit (QIAGEN) and the miR-145-3p primer (forward primer, 5'—GCTCCGTAGTGTCTTCATCTT-3'; reverse primer, 5'—GTGCAGGTCCGAGGT-3') were used for PCR amplification. Small nuclear RNA (snRNA) RNU6B (U6) was used as the internal reference (forward primer, 5'—CTGGTCGCCAGCA-3'; reverse primer, 5'—AACGCTTCAGA ATTTGCCG-3').

Knockdown circDcbld1 in VSMCs

The siRNAs were designed for targeting the back-splicing junction of circDcbld1. siRNAs were transfected into VSMCs respectively with Lipofectamine 3000 (Thermo Fisher Scientific) and incubated for 48 h.

Figure 6. Nrp1 is the Target of miR-145-3p in VSMCs

(A) The potential targets of miR-145-3p were predicted by TargetScan and miRanda. (B) The level of neuropilin-1 was elevated in the injured CCA. (C) The inhibition of circDcbld1 in VSMCs resulted in the reduction of neuropilin-1 (n = 25). (D) miR-145-3p inhibitors raised the level of neuropilin-1 in VSMCs (n = 25). (E) The co-transfection of the luciferase reporter plasmid containing wild-type Nrp1 and miR-145-3p mimics into HEK293 cells reduced the relative luciferase fluorescence intensity. As a comparison, the co-transfection of the luciferase reporter plasmid containing mutated Nrp1 and miR-145-3p mimics did not change the fluorescence activity. **p < 0.01.
circDcbld1 expression was detected by quantitative real-time PCR to determine the most effective siRNA for the subsequent experiments.

miR-145-3p Overexpression and Inhibition
The mimics (5'-GGAUUCCUGGAAAUAUGUUACGU-3') and inhibitors (5'-GAACAGTATTTCCAGGAATCC-3') of miR-145-3p were synthesized (Ribobio, Guangzhou, China). 50 nmol of mimics or inhibitors was transfected into VSMCs with Lipofectamine 3000. The regulation of the mimics or inhibitors of miR-145-3p on the target proteins was detected by western blot.

Western Blot
The total protein was extracted with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) for SDS-PAGE. The target proteins were identified with the anti-α-SMA (Cell Signaling Technology, Danvers, MA, USA), anti-SM-MHC (Cell Signaling Technology), anti-calponin (Absin, Shanghai, China), anti-neuropilin-1 (Cell Signaling Technology), anti-cyclin D2 (Cell Signaling Technology), and anti-periostin (Absin) antibodies. Images were acquired by an optical scanner and analyzed by Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

Cell Proliferation Assay
Cells were seeded into 96-well plates and cultured to 50%–70% confluence. Following the manufacturer’s instructions, 50 μM EdU (Ribobio, Guangzhou, China) was added to the culture media for 2 h. Proliferating cells were labeled as green and counted under a fluorescence microscope (Olympus TH-4-200).
Cell Migration Assay
In the Transwell assay, cell were seeded into the Transwell chamber (pore size 8 μm) (Corning Life Sciences, Tewksbury, MA, USA) with serum-free DMEM. DMEM containing 20% newborn calf serum (Thermo Fisher Scientific) was placed below the chamber. 8 h later, the un-migrated VSMCs were wiped off with a cotton bar. The migrated cells were counted under a microscope (Olympus CKX41).

In the wound healing assay, cells were seeded onto the 24-well culture plate with DMEM containing 10% serum. 24 h later, cells reached a 70%–80% confluence. A 200-μL pipette tip was used to gently and stably scratch the monolayer of cells to create straight lines. The gap of the cell monolayer was observed and imaged under an optical microscope at 0, 4, 8, 16, and 24 h.

Dual-Luciferase Reporter Assay
The WT circDcbld1 or the 3' UTR of Nrp1 containing the miR-145-3p binding site was cloned into the pmir-RB-Report vector (Ribio, Guangzhou, China), designated as circDcbld1-WT-Report or Nrp1-WT-Report. Site-directed mutation of circDcbld1 or the 3' UTR of Nrp1 was performed by the QuickMutation site-directed mutagenesis kit (Beyotime Biotechnology, Shanghai, China) (Figures 4D and 6E). The mutated sequences were also cloned into the pmir-RB-Report vector and named as circDcbld1-Mut-Report or Nrp1-Mut-Report. HEK293 cells were seeded onto the 24-well plates and cultured overnight to 80% confluence. 100 ng of reporter and 50 nM of miR-145-3p were co-transfected into cells. The relative fluorescence intensity of the samples was detected with a Dual-Luciferase reporter assay system (Promega, Fitchburg, WI, USA).

Knockdown circDcbld1 in Rat CCA
After balloon injury to rat CCA, 7.5 nmol of chemically-modified Stable siRNA (GenePharma, Shanghai, China) was injected into the subcutaneous tissue around CCA by local injection (Figure 7A). Thereafter, 5 nmol of siRNA was injected every 3 days until 14 days. Diethyl pyrocarbonate (DEPC)-treated H2O was used as a mock. The scrambled siRNA was used as a negative control. The silencing efficiency was detected by quantitative real-time PCR. The level of neuropilin-1 in the artery was detected by western blot. The degree of intimal hyperplasia was assessed by measuring the ratio of intima/media area.

Statistical Analysis
In all experiments, at least 5 rats were used per group unless stated differently in the figure legends. The statistical analysis was processed with Prism 7 (GraphPad, San Diego, CA, USA). The comparisons between two groups were performed by a two-tailed Student’s t test, and the comparisons between multiple groups were performed by one-way ANOVA. Bonferroni corrected p < 0.05 was considered statistically significant. The results were expressed as the mean ± SEM.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.10.023.

AUTHOR CONTRIBUTIONS
J.J. and R.J. contributed to the design of research; Q.-P.Y. performed the data analysis; Z.-H.R., T.L., X.-L.Z., and Y.C. guided the cellular experiments; N.-B.C., M.-J.J., and Y.-S.C. conducted animal experiments; and J.J. wrote and edited the manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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