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Original Article

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 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.

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* (*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.

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* increased by 7.3-fold. It was the top upregulated circRNA in injured arteries (Figure 1A; Supplemental Information). Quantitative real-time PCR confirmed the expression elevation (Figure 1B). Sanger sequencing verified the PCR products that contained the back-splicing junction area of *rno-circRNA_009723* (Figure 1C).

Rat VSMCs were treated with the transcriptional inhibitor actinomycin D. The expression of *circDcbld1* dropped slightly at 24 h (<20%). As a comparison, the expression of linear *Dcbld1* mRNA

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circRNA	Regulation	GeneSymbol	Sham (raw intensity)	Injured (raw intensity)	FC (abs)	P-value
circRNA_009723	up	Dcbld1	527	4987.333333	7.2972337	0.0059407
circRNA_014008	up	Nrxn1	113.333333	514.833333	4.6730374	0.009891107
circRNA_009358	up	Adamts6	120.166667	706.166667	4.4706212	0.023274042
circRNA_26904	up	Adamts6	114.833333	652.333333	4.3806285	0.026137402
circRNA_005717	up	Diaph3	85	563	4.2259602	0.048494742

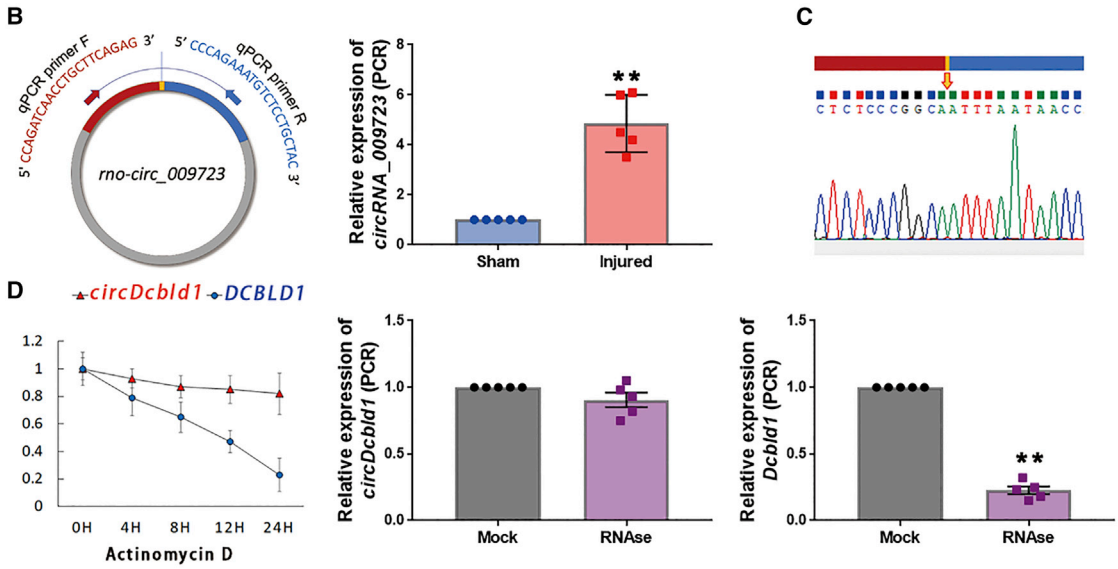

Figure 1. *rno-circRNA_009723* Was Highly Expressed in the Rat CCA after Balloon Injury

(A) Top five upregulated circRNAs in injured arteries. Among them, the change of the expression of *circRNA_009723* was the largest. (B) The quantitative real-time PCR primers were designed for targeting the back-splicing junction site of *rno-circRNA_009723*. PCR confirmed that *rno-circRNA_009723* was elevated in the injured CCA. (C) PCR product was subjected to Sanger Sequencing and was confirmed to contain the back-splicing junction sequence. (D) Actinomycin D was used to inhibit RNA synthesis of VSMCs. The half-life of *circDcbld1* was longer than that of *Dcbld1*. (E) RNase R was utilized to degrade the linear RNA in the total RNA extracted from rat VSMCs. *circDcbld1* was more stable to exonuclease than was *Dcbld1*. **p < 0.01.

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 *circDcbld1* was also lower than that of *Dcbld1* (Figure 1E). The results indicated that *circDcbld1* had a longer half-life and was more stable.

According to NCBI BLAST, the sequence of *rno-circRNA_009723* was matched entirely with the exon of the *Rattus norvegicus Dcbld1* gene. Therefore, we named *rno-circRNA_009723* as *circDcbld1*. The homology of the *Homo sapiens Dcbld1* gene and *Rattus norvegicus Dcbld1* gene is 98%.

The Expression and Localization of *circDcbld1* in Rat CCA

RNA fluorescent *in situ* hybridization (RNA-FISH) showed *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).

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 *circDcbld1* Increased the Contractile Smooth Muscle Cell Markers and Decreased the Migration of VSMCs

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

The knockdown of *circDcbld1* reduced VSMC migration in a Transwell assay (Figure 3E) and scratch wound healing assay (Figure 3F). Low expression of *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).

circDcbld1 Is the Competing Endogenous (ceRNA) of miR-145-3p

By using TargetScan and miRanda, we predicted the possible target miRNAs of *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

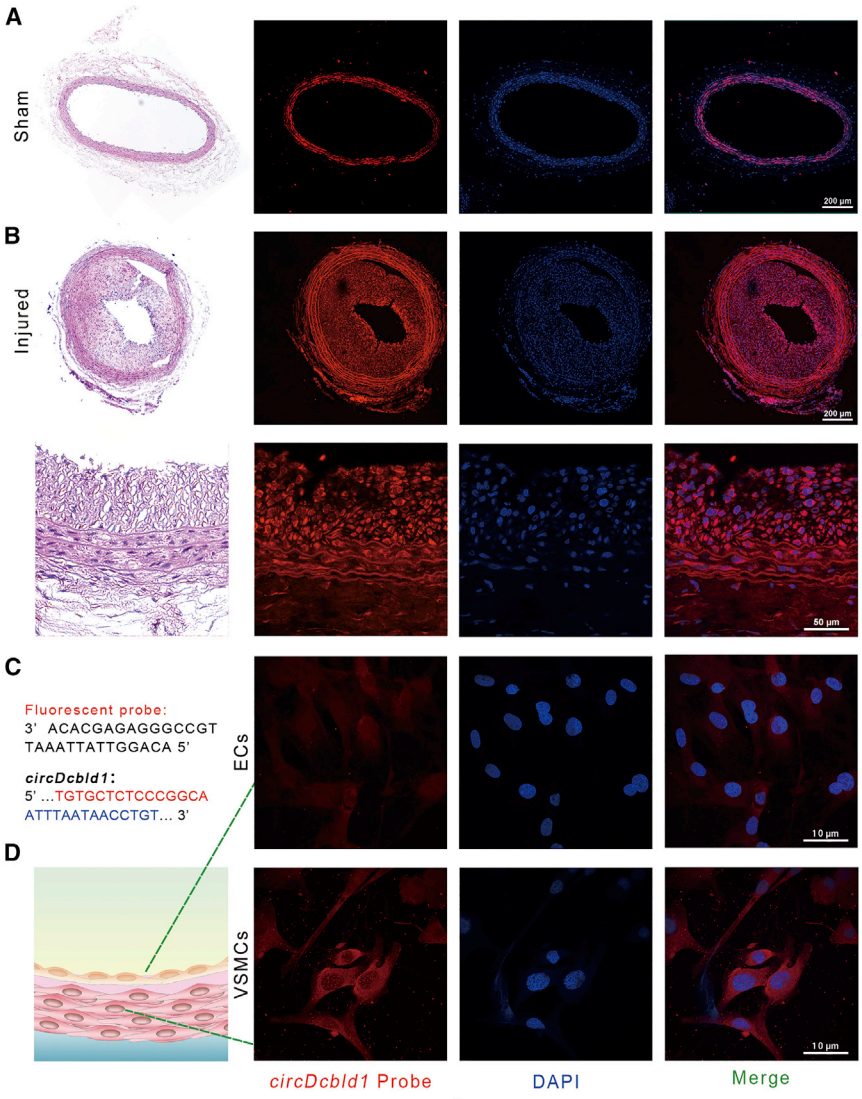

Figure 2. Localization of *circDcbld1* in the Rat CCA and Cultured Vascular Cells

(A) RNA-FISH showed that *circDcbld1* was localized in the media of the rat CCA, (B) especially in the neointima induced by balloon injury. (C) ECs had a low expression of *circDcbld1*. (D) *circDcbld1* was localized in the cytoplasm of cultured VSMCs.

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 *circDcld1* 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.

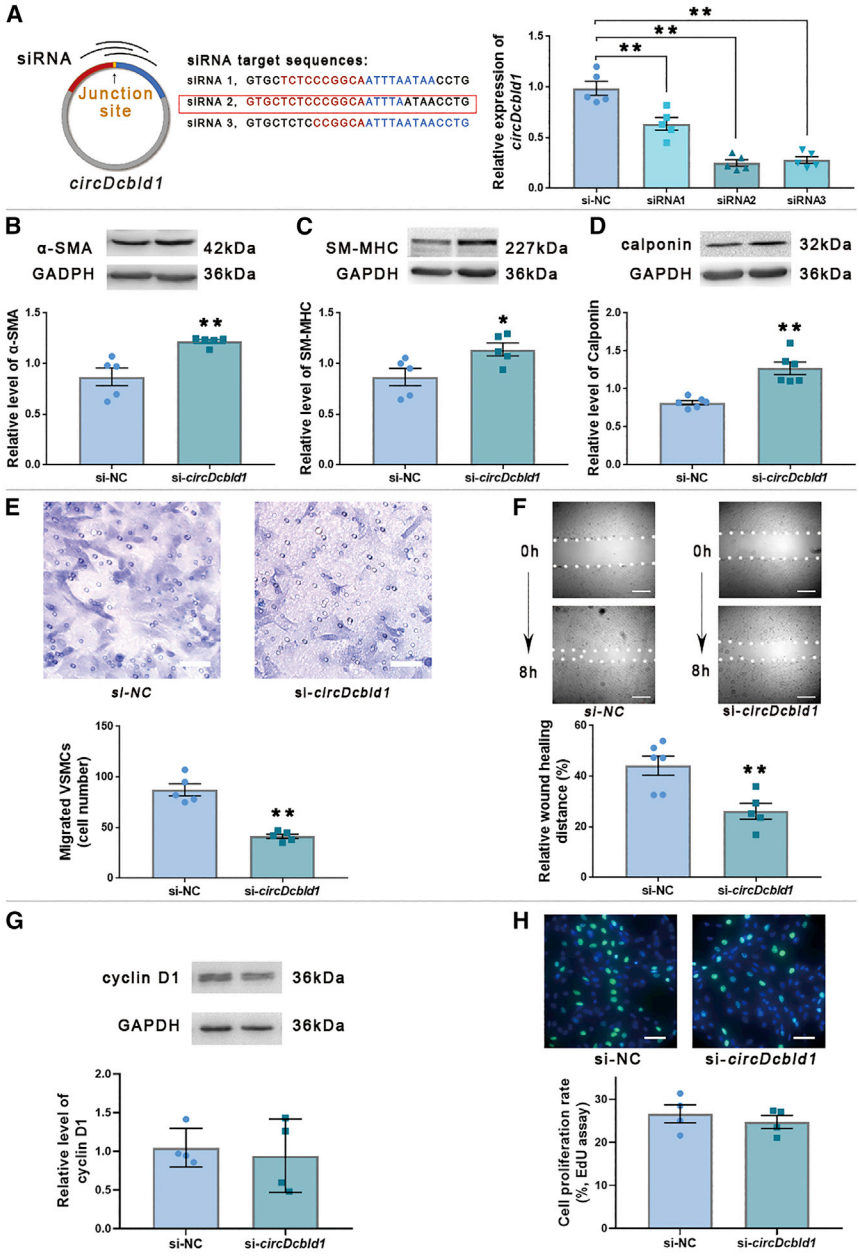

Figure 3. The Knockdown of *circDcbld1* Affected VSMC Differentiation and Migration

(A) siRNAs were designed for targeting the back-splicing junction of *circDcbld1* and transfected into VSMCs. The interference efficiency of the siRNAs was detected by quantitative real-time PCR, and the most effective siRNA was picked out for the subsequent experiments. (B–D) The knockdown of *circDcbld1* elevated the levels of the contractile smooth muscle cell markers, including α-SMA (B), SM-MHC (C), and calponin (D). (E and F) The knockdown of *circDcbld1* resulted in a decrease of VSMC migration in both a Transwell assay (E) (scale bars, 100 μm) and wound healing assay (F) (scale bars, 500 μm). (G and H) According to the cyclin D1 level (G) and EdU incorporation assay (H) (scale bars, 50 μm), the inhibition of *circDcbld1* did not significantly affect VSMC proliferation. *p < 0.05, **p < 0.01.

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.[13] 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.[14–16] However, studies have found that passenger strand also plays crucial roles.[17,18]

It is well known that *miR-145-5p* is an essential vascular regulator that determines the fate of VSMCs.[19–21] 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[22–26] and also regulates VSMC migration.[27–29] It was reported that neuropilin-1 and its isoform neuropilin-2 mediate neointimal hyperplasia and reendothelialization following arterial injury.[30] Ccnd2 promotes cell proliferation.[31,32] It is required for

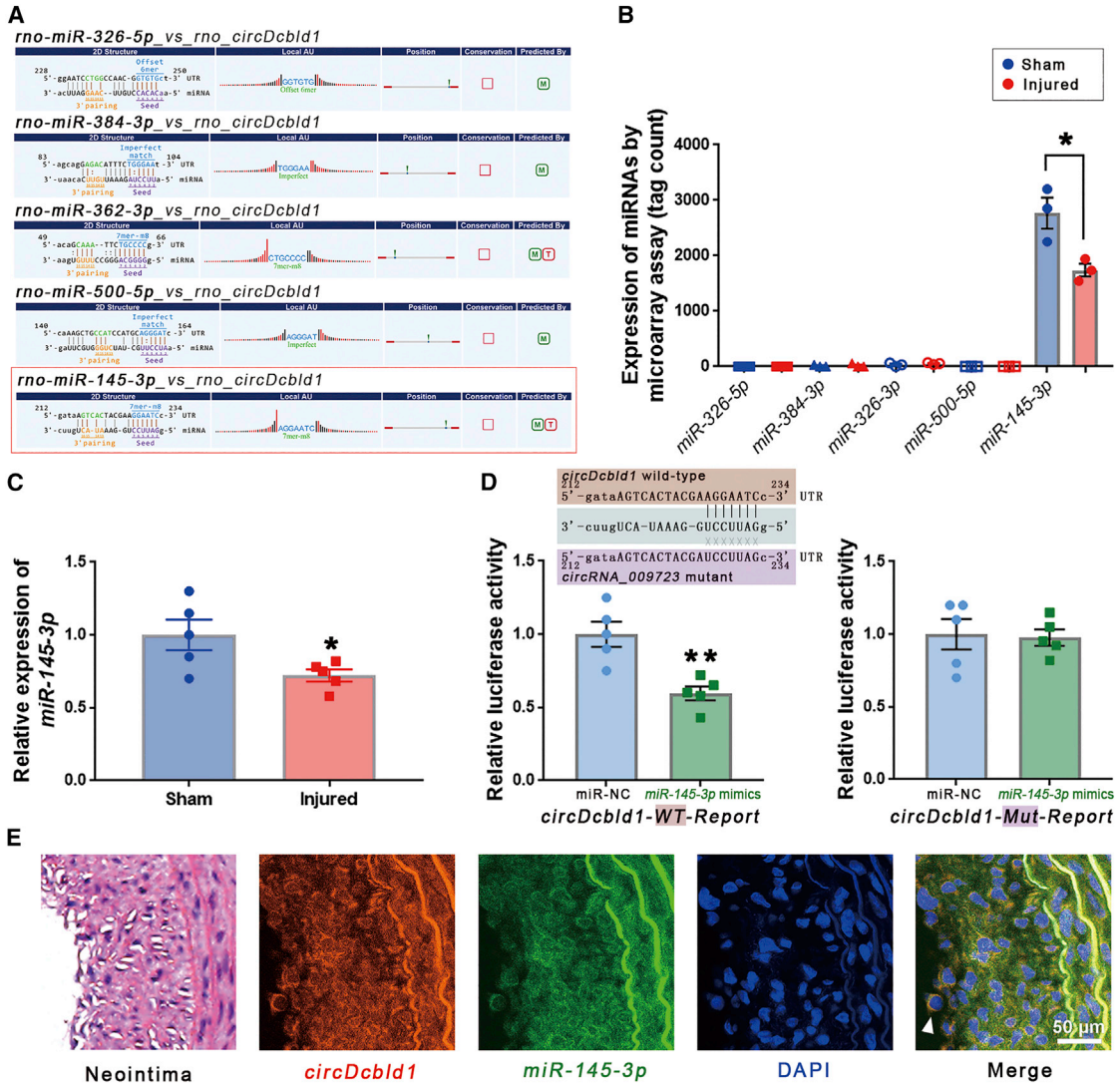

Figure 4. *circDcbld1* Is the ceRNA of *miR-145-3p* in VSMCs

(A) The possible target miRNAs of *circDcbld1* were predicted by TargetScan and miRanda. (B) The miRNA sequencing revealed that *miR-326-5p*, *miR-384-3p*, *miR-362-3p*, and *miR-500-5p* were expressed at low levels in the CCA. *miR-145-3p* was abundantly expressed in the CCA, and its expression decreased in the CCA with intimal hyperplasia (n = 3). (C) Quantitative real-time PCR verified the reduction of *miR-145-3p* in the injured CCA. (D) The co-transfection of luciferase plasmid containing wild-type *circDcbld1* and *miR-145-3p* mimics reduced the relative luciferase activity. As a comparison, the co-transfection of luciferase plasmid containing mutated *circDcbld1* and *miR-145-3p* mimics did not change the relative luciferase activity. (E) RNA-FISH showed that *circDcbld1* and *miR-145-3p* were co-localized in the cytoplasm of cells in neointima. *p < 0.05, **p < 0.01.

cell cycle G1/S transition. Studies indicated that it modulates proliferation of ECs[33,34] and VSMCs.[35] *Postn* expression is elevated during vascular injury, and it modulates VSMC differentiation and migration.[36–38] 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.

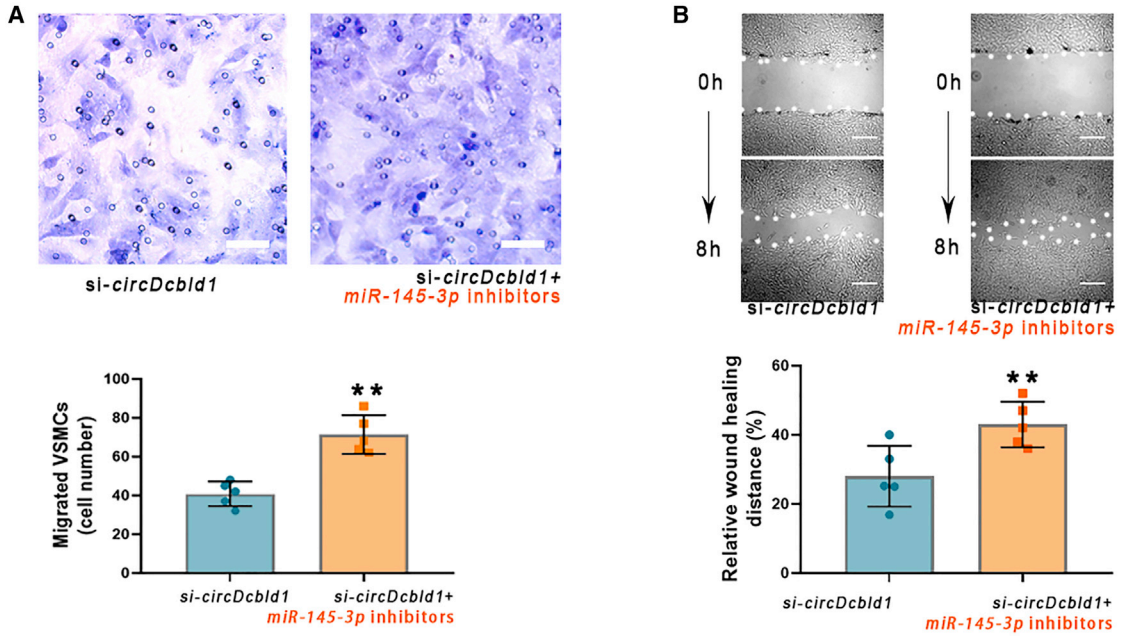

Figure 5. The Transfection of *miR-145-3p* Inhibitors Reverted the Decrease of VSMC Migration Induced by Low Expression of *circDcbld1*

As previously described, low expression of *circDcbld1* reduced VSMC migration. (A and B) In this study, the transfection of *miR-145-3p* inhibitors restored VSMC migration in (A) a Transwell assay (scale bars, 100 μm) and (B) a wound healing assay (scale bars, 500 μm; n = 9). **$p < 0.01$.

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 intraperitoneal 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.[39] 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,[11] 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 (8 × 15K; 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)[40] 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).

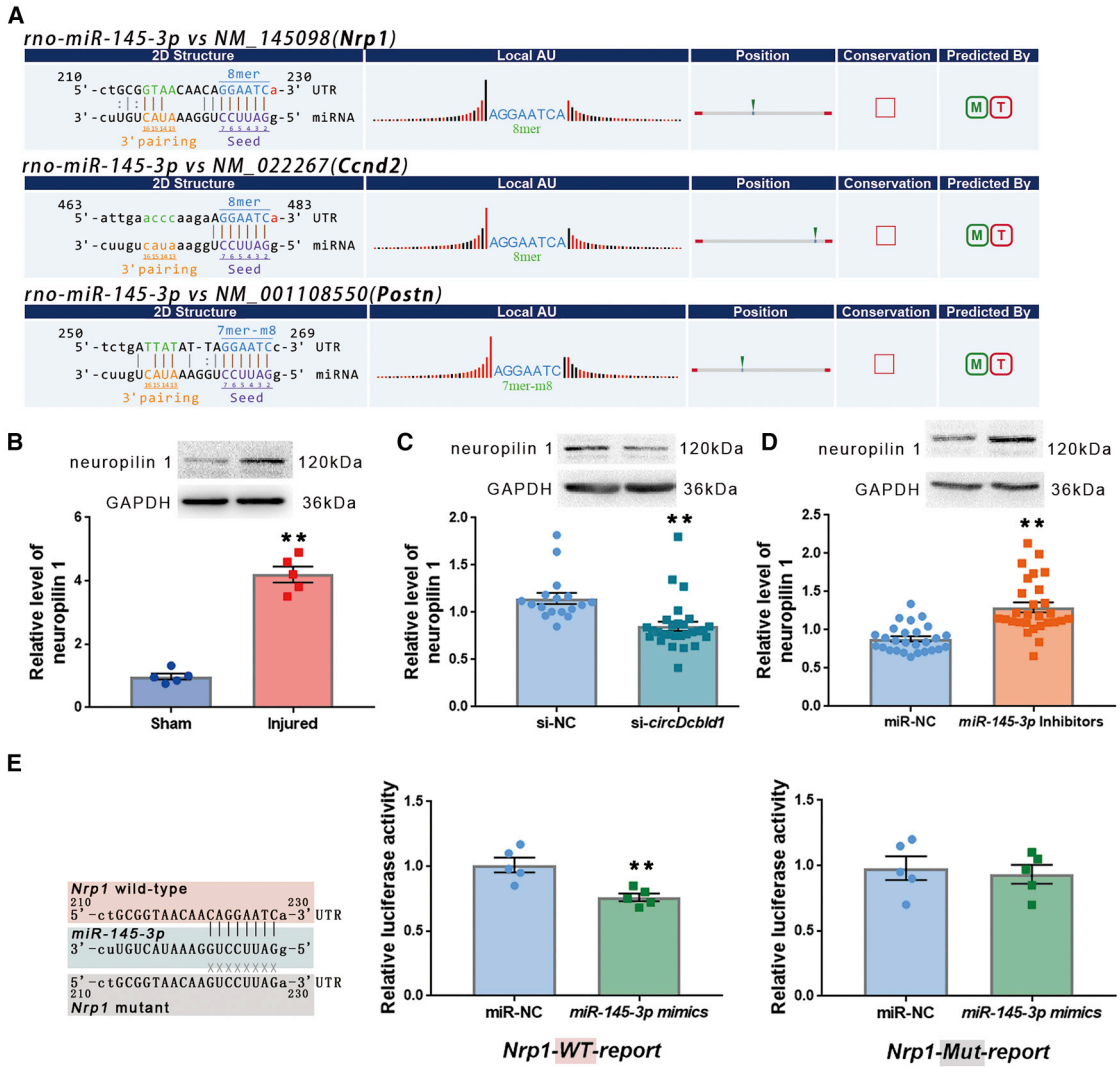

Figure 6. *Nrp1* is the Target of *miR-145-3p* in VSMCs

(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.

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′-GCCCTGTAGTGTTTCC TACTT-3′; reverse primer, 5′-GTGCAGGGTCCGAGGT-3′) were used for PCR amplification. Small nuclear RNA (snRNA) *RNU6B* (*U6*) was used as the internal reference (forward primer, 5′-CTC GCTTCGGCAGCACA-3′; reverse primer, 5′-AACGCTTCACGA ATTTGCGT-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.

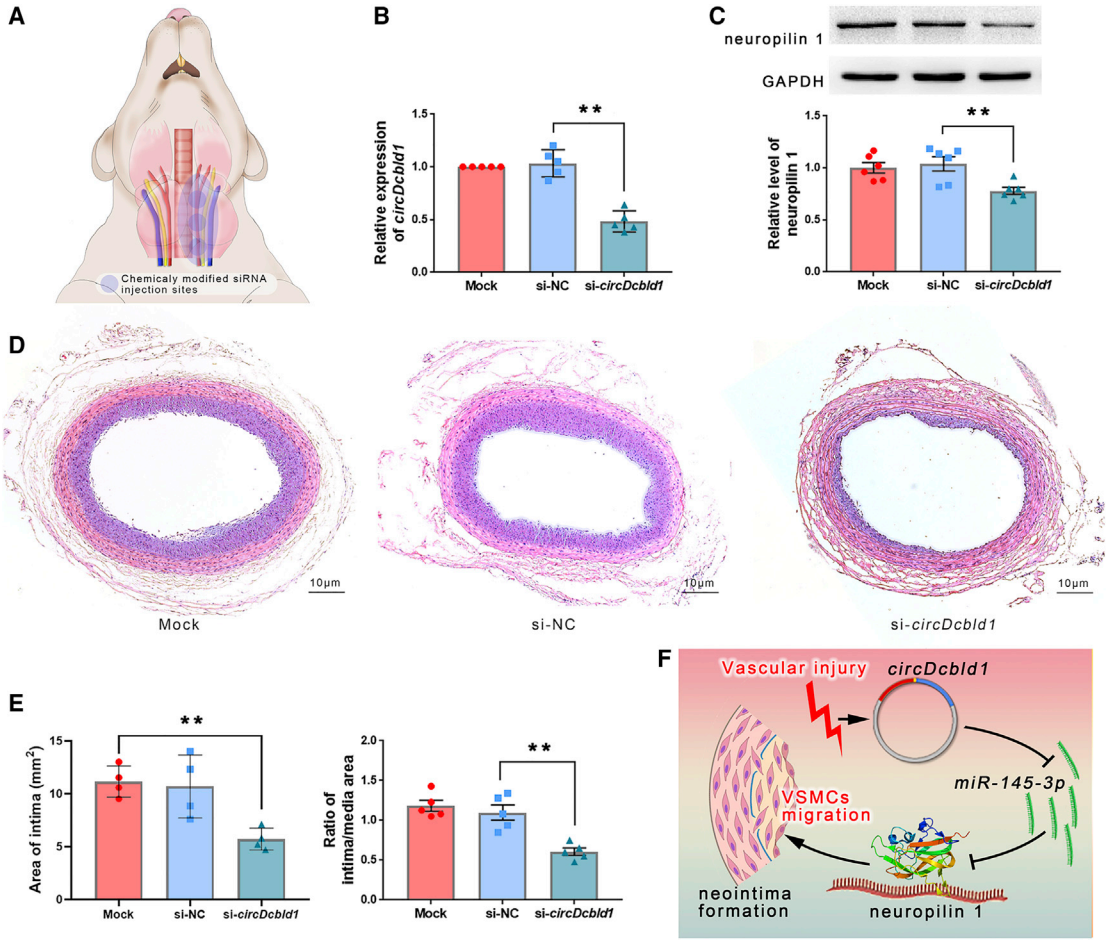

Figure 7. The Knockdown of *circDcbld1* Alleviated Intimal Hyperplasia in the Rat CCA

(A) Following balloon injury to the CCA, the chemically modified *circDcbld1* siRNA was injected into the surrounding tissue of the artery and repeated every 3 days until 14 days after the surgery. (B) PCR showed that chemically modified siRNA inhibited the expression of *circDcbld1* in the CCA. (C) With the knockdown of *circDcbld1*, the level of neuropilin-1 in the CCA was also reduced. (D) With the knockdown of *circDcbld1*, intimal hyperplasia of the CCA was alleviated. (E) The area of the intima and the intima/media ratio was declined. (F) Schematic diagram of the mechanism of *circDcbld1* involved in intimal hyperplasia. In a physiological environment, *Nrp1* is an inducer of VSMC migration. *miR-145-3p* inhibits VSMC migration by targeting *Nrp1*. Vascular injury causes an increase in *circDcbld1* expression and a decrease in *miR-145-3p*. Meanwhile, *circDcbld1* binds with cytoplasmic *miR-145-3p* through the molecular sponge mechanism, resulting in a further decrease in *miR-145-3p*'s biological activity. In this manner, *circDcbld1* promotes neointimal formation by upregulating VSMC migration. **$p < 0.01$.

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′-GGAUUCCUGGAAAUACUGUUC-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 (Ribio, 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, cells 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 H_2O 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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