A Novel Regulatory Mechanism of Smooth Muscle α-Actin Expression by NRG-1/circACTA2/miR-548f-5p Axis

Yan Sun,* Zhan Yang,* Bin Zheng, Xin-hua Zhang, Man-li Zhang, Xue-shan Zhao, Hong-ye Zhao, Toru Suzuki, Jin-kun Wen

**Rationale:** Neuregulin-1 (NRG-1) includes an extracellular epidermal growth factor–like domain and an intracellular domain (NRG-1-ICD). In response to transforming growth factor-β1, its cleavage by proteolytic enzymes releases a bioactive fragment, which suppresses the vascular smooth muscle cell (VSMC) proliferation by activating ErbB (erythroblastic leukemia viral oncogene homolog) receptor. However, NRG-1-ICD function in VSMCs remains unknown.

**Objective:** Here, we characterize the function of NRG-1-ICD and underlying mechanisms in VSMCs.

**Methods and Results:** Immunofluorescence staining, Western blotting, and quantitative real-time polymerase chain reaction showed that NRG-1 was expressed in rat, mouse, and human VSMCs and was upregulated and cleaved in response to transforming growth factor-β1. In the cytoplasm of HASMCs (human aortic smooth muscle cells), the NRG-1-ICD participated in filamentous actin formation by interacting with α-SMA (smooth muscle α-actin). In the nucleus, the Nrg-1-ICD induced circular ACTA2 (alpha-actin-2; circACTA2) formation by recruitment of the zinc-finger transcription factor IKZF1 (IKAROS family zinc finger 1) to the first intron of α-SMA gene. We further confirmed that circACTA2, acting as a sponge binding microRNA (miR)-548f-5p, interacted with miR-548f-5p targeting 3’ untranslated region of α-SMA mRNA, which in turn relieves miR-548f-5p repression of the α-SMA expression and thus upregulates α-SMA expression, thereby facilitating stress fiber formation and cell contraction in HASMCs. Accordingly, in vivo studies demonstrated that the localization of the interaction of circACTA2 with miR-548f-5p is significantly decreased in human intimal hyperplastic arteries compared with normal arteries, implicating that dysregulation of circACTA2 and miR-548f-5p expression is involved in intimal hyperplasia.

**Conclusions:** These results suggest that circACTA2 mediates NRG-1-ICD regulation of α-SMA expression in HASMCs via the NRG-1-ICD/circACTA2/miR-548f-5p axis. Our data provide a molecular basis for fine-tuning α-SMA expression and VSMC contraction by transcription factor, circular RNA, and microRNA. ((Circ Res. 2017;121:628-635. DOI: 10.1161/CIRCRESAHA.117.311441.)

**Key Words:** ACTA2 ■ circular RNA ■ miR-548 ■ neuregulin-1 ■ smooth muscle cells ■ TGF-beta1

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**V**ascular smooth muscle cells (VSMC) in the medial layer of the vascular wall regulate vessel tone and diameter, thus determining blood pressure and tissue perfusion.1 The actin cytoskeleton is an important component of vascular contractility and is also essential for VSMC proliferation and migration, which are involved in all the pathological changes taking place in the vascular wall, including restenosis, hypertension, and atherosclerosis.2 α-SMA (smooth muscle α-actin) participates in the formation of filaments that are major components of the cytoskeleton. Previous studies have demonstrated that the expression of α-SMA in VSMCs is activated by the interplay between serum response factor and accessory cofactors;3 the transforming growth factor (TGF)-β1 signaling regulates the expression of α-SMA gene.4 However, the cellular and molecular mechanisms that regulate α-SMA expression and VSMC contraction are not fully understood.

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Novel Regulation of α-SMA Expression

What Is Known?

- Neuregulin-1 (NRG-1) is expressed in the nervous system and its intracellular domain (NRG-1-ICD) modulates synaptic formation and transmission.
- Bioactive NRG-1 (extracellular epidermal growth factor–like domain) released by cardiac endothelial cells inhibits vascular smooth muscle cell proliferation.
- Circular RNAs and microRNAs regulate gene expression.

What New Information Does This Article Contribute?

- In vascular smooth muscle cells, NRG-1 is upregulated and cleaved in response to transforming growth factor-β1.
- In the cytoplasm, NRG-1-ICD participates in filamentous actin formation and cell contraction by interacting with α-SMA (smooth muscle α-actin), whereas NRG-1-ICD translocated into the nucleus induces circACTA2 formation via recruiting the transcription factor IKZF1 (IKAROS family zinc finger 1) to the first intron of α-SMA gene.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>circACTA2</td>
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<td>NRG-1-ICD</td>
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<td>α-SMA</td>
<td>smooth muscle α-actin</td>
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<td>TGF-β1</td>
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Circular RNAs (circRNAs), novel class of noncoding RNAs generated via back splicing, regulate eukaryotic gene expression by acting as cytoplasmic microRNA sponges, RNA-binding protein–sequestering agents, or nuclear transcriptional regulators. They appear to fine-tune multiple regulatory processes that are involved in vascular diseases associated with vascular smooth muscle cell dysfunction, including hypertension and restenosis. Here, we report a functional link between NRG-1-ICD and α-SMA via a novel circular RNA (circACTA2) generated from α-SMA gene. Our results show a novel mechanism that fine-tunes α-SMA expression by transcription factors, circular RNA and microRNA, suggesting that α-SMA expression is tightly regulated on multiple levels. This study also reveals a new pathway for circular RNA formation from the intron transcription start independent of linear transcripts. The NRG-1-ICD/circACTA2/miR-548f-5p regulatory axis may represent a new therapeutic target for dysfunction of vascular smooth muscle cells.

NRG-1 Expression Is Induced by TGF-β1 in VSMCs, and NRG-1-ICD Participates in Cytoskeleton Organization

NRG-1 was expressed in arteries of the C57BL/6 mice and was located in VSMCs (Figure 1A). NRG-1 was also abundantly expressed in mouse arterial endothelial cells (Figure 1A), as well as in human renal artery endothelial cells (Online Figure IA). NRG-1 expression and localization in α-SMA–positive VSMCs were markedly decreased in human renal arteries with intimal hyperplasia (Figure 1B). Notably, NRG-1 mRNA levels were significantly lower in the intimal hyperplastic arteries than in normal arteries (Online Figure IB). Abundant NRG-1 mRNA and protein were also detected in rat and human VSMCs, as well as in human cardiac microvascular endothelial cells (Online Figure IC and ID). TGF-β1 and PDGF-BB (platelet-derived growth factor-BB) increased and decreased NRG-1 expression, respectively, in a time-dependent manner (Figure 1C; Online Figure IIA through IID), suggesting that NRG-1 facilitates TGF-β1–induced HASMC (human aortic smooth muscle cells) differentiation, whereas NRG-1 downregulation might be responsible for PDGF-BB–induced proliferation and the intimal hyperplasia. Treating VSMCs with TGF-β1 increased...
soluble NRG-1β in the supernatants 1.5-fold over that treated with vehicle control, but PDGF-BB did not affect soluble NRG-1β level (Figure 1D; Online Figure IIE). These findings suggest that TGF-β1 not only induces NRG-1 expression but also promotes its cleavage by proteolytic enzymes.

In the NRG-1–overexpressed HASMCs, actin filaments were recruited into thick and long actin bundles, and their de-polymerization induced by cytochalasin B was markedly decreased (Figure 1E; Online Figure IIF), suggesting that NRG-1 participates in F-actin formation and stabilizes their structure. HRG-1β (heregulin-1β) did not affect the actin cytoskeleton (Online Figure IIIA), but NRG-1-ICD overexpression markedly enhanced stress fiber formation (Online Figure IIIB), suggesting that NRG-1 mediates cytoskeletal organization through its ICD domain. Moreover, NRG-1 overexpression promoted acetylcholine-induced HASMC contraction (Figure 1F).

Coimmunoprecipitation experiments showed that both NRG-1 and NRG-1-ICD were immunoprecipitated by an antibody to α-SMA, and TGF-β1 increased association of NRG-1 or NRG-1-ICD with α-SMA but not myosin (Online Figure III). Moreover, NRG-1 and NRG-1-ICD interacted with GST-α-SMA but not GST (Online Figure IVB). The colocalization of NRG-1 and α-SMA could be observed by immunofluorescence staining (Online Figure IVC). In situ proximity ligation analysis showed NRG-1 association with α-SMA in TGF-β1–treated cells (Figure 1G). These findings suggest that NRG-1-ICD promotes cytoskeletal organization via interacting with α-SMA.

NRG-1-ICD Upregulates α-SMA Expression via Binding to the First Intron of the α-SMA Gene
To determine whether NRG-1-ICD can translocate to the nucleus to regulate α-SMA expression, we examined the effects of TGF-β1 on NRG-1-ICD nuclear translocation. Under basal conditions, NRG-1-ICD predominantly localized to HASMC cytoplasm, whereas marked nuclear translocation was observed in TGF-β1–treated cells (Figure 1H). Using chromatin immunoprecipitation sequencing, we found 65 genes differentially bound by NRG-1-ICD between control and TGF-β1–treated HASMCs. Of these, 14 were increased, including α-SMA gene (also named ACTA2) in TGF-β1–treated HASMCs (Online Figure VA). NRG-1-ICD was recruited to the region between +5642 and +5787 bp downstream of transcription start site of α-SMA gene. **P<0.01 vs 0 h.

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increased luciferase activity by 4-fold (Online Figure VC). TGF-β1 paralleled increased NRG-1-ICD and α-SMA expression in a time-dependent manner (Online Figure VD). Moreover, gain- and loss-of-function experiments for NRG-1-ICD showed that NRG-1-ICD overexpression increased, whereas NRG-1-ICD knockdown decreased, α-SMA expression (Figure 1J; Online Figure VE and VF). Adenovirus-mediated overexpression of mouse NRG-1-ICD could rescue α-SMA expression in si-NRG-1-ICD–transfected HASMCs (Online Figure VG). Unexpectedly, NRG-1-ICD overexpression did not affect α-SMA mRNA levels (Online Figure VH).

The finding that NRG-1 upregulated α-SMA expression but did not affect its mRNA level raises the possibility that NRG-1-ICD might regulate α-SMA expression at post-transcriptional level. As NRG-1-ICD could bind to the first intron of α-SMA gene, we hypothesized that NRG-1-ICD probably upregulated α-SMA expression via inducing circRNA formation. Convergent and divergent primers were used to amplify total RNAs and circular RNA transcripts derived from the α-SMA gene. One novel circRNA (termed circACTA2) was characterized by divergent RT-PCR (Online Figure VIA). RNase R digestion dramatically reduced α-SMA mRNA level but had lesser effect on circACTA2 (Online Figure VIB). Further, we designed additional divergent primers to amplify full-length circACTA2. circACTA2 presence was verified by RT-PCR (Online Figure VIC) and its length is 730 bp, whose sequences are complementary to exon-5 to exon-9 of α-SMA gene (Online Figure VID and VIE).

Using northern blotting, we analyzed circACTA2 and α-SMA mRNA and demonstrated that circACTA2 was derived from circularization of the exon-5 to exon-9 of the α-SMA gene (Figure 2A). Next, we used a single-chain guide RNA (sgRNA) recognizing NRG-1-ICD–binding sequences to conduct CRISPR interference (CRISPRi) and showed that CRISPRi for the NRG-1-ICD–binding sequences decreased the recruitment of NRG-1-ICD by 50% of the control (Figure 2B). CRISPRi for the NRG-1-ICD–binding sequences reduced the circACTA2 but not α-SMA mRNA levels (Figure 2C). Further, a pairs of sgRNAs (sgRNA1+sgRNA2) were used to mediate the deletion of NRG-1-ICD–binding sequences, and lack of these sequences reduced NRG-1-ICD recruitment and circACTA2 formation by 50%, respectively, but did not have a significant effect on α-SMA mRNA (Online Figure VIF through VI I). TGF-β and NRG-1-ICD overexpression, singly or in combination, increased the expression of circACTA2, whereas NRG-1-ICD knockdown abolished TGF-β–induced circACTA2 overexpression (Figure 2D; Online Figure VII). These findings suggest that NRG-1-ICD mediates TGF-β–induced circACTA2 formation by binding to the first intron of α-SMA gene.

NRG-1-ICD Induces CircACTA2 Formation via Recruiting Ikzf1 (IKAROS Family Zinc Finger 1) to the First Intron of α-SMA Gene

To test whether NRG-1-ICD induces circACTA2 expression by recruiting other transcription factors to the first intron of α-SMA gene, we used a biotinylated double-strand DNA fragment to pull down proteins binding to NRG-1-ICD–binding sequences from HASMCs. Transcription factors Ikaros, PHB2, and ASCC3 were pulled down, and coimmunoprecipitation assay revealed that Ikaros associated with NRG-1-ICD and TGF-β1 increased their interactions (Figure 2E). Immunofluorescence staining also showed colocalization of the interaction of Ikaros with NRG-1-ICD in TGF-β1–treated cells (Figure 2F). The Ikaros-binding sequence was detected at a 2-step chromatin immunoprecipitation assay, and TGF-β1 increased the concurrent recruitment of Ikaros and NRG-1-ICD to the Ikaros-binding site (GGGAAA; Figure 2G). In situ proximity ligation analysis demonstrated that NRG-1-ICD interaction with Ikaros was increased after TGF-β1 exposure (Online Figure VIIA). These results suggest that NRG-1-ICD forms a stable complex with Ikaros.

To investigate whether RNA-binding protein QKI (quaking) and RNA-editing enzyme ADAR1 are responsible for TGF-β–induced circACTA2 formation, we generated NRG-1-ICD–overexpressing and QKI or ADAR1 knockdown HASMCs and found that TGF-β1 or NRG-1-ICD overexpression increased QKI expression but decreased ADAR1 expression (Figure 2H; Online Figure VIIB and VIIC). Notably, QKI or ADAR1 knockdown (Online Figure VIID and VIE) decreased or increased circACTA2 formation, respectively, but did not affect α-SMA mRNA level (Figure 2I and 2J). These data demonstrate that both QKI and ADAR1 participate in NRG-1-ICD–induced circACTA2 formation.

circACTA2 Upregulates α-SMA Expression by Relieving miR-548f-5p Repression of α-SMA Expression

To determine the relationship between α-SMA expression and circACTA2 formation, we overexpressed or knocked down circACTA2 in mouse SMCs (Online Figure VIII A and VIIIIB) and showed that overexpression and knockdown of circACTA2 markedly increased or decreased α-SMA protein level, respectively (Figure 3A). Next, we designed 2 siRNAs: one siRNA targeting the backsplice sequence for circACTA2 (si-circACTA2) and another targeting sequence shared by both linear and circular transcripts (si-both; Online Figure IXA). si-circACTA2 knocked down only circACTA2 but did not affect α-SMA mRNA level, whereas si-both knocked down both circACTA2 and α-SMA mRNA expression (Online Figure IXA), circACTA2 overexpression increased α-SMA expression (Online Figure IXB), whereas knockdown abrogated TGF-β1–induced α-SMA upregulation (Online Figure IXC). These data suggest that circACTA2 plays an important role in mediating TGF-β1/NRG-1-ICD–induced α-SMA expression. RNAhybrid and miRanda-based heteroduplex analyses showed that circACTA2 contains sequences complementary to microRNA (miR-548f-5p seed sequence (Online Figure IXD). Luciferase assay revealed that miR-548f-5p mimic significantly decreased luciferase activity mediated by wild-type circACTA2 sequence but not its mutant (Online Figure IXE).

We used biotin-labeled circACTA2 probe to pull down microRNA(s) complementary to circACTA2 sequences and revealed a 20-fold enrichment of miR-548f-5p in the circACTA2 pulled down sediments (Figure 3B). Consistently, circACTA2 was also enriched in miR-548f-5p pulled down sediments (Figure 3C). The results of RNA in situ hybridization were consistent with interactions between circACTA2 and miR-548f-5p (Figure 3D).

miR-548f-5p level was significantly increased in the renal arteries with intimal hyperplasia (Online Figure IXF; Figure 3E),
and circACTA2 expression and its interaction with miR-548f-5p were lesser in the intimal hyperplastic arteries (Figure 3F and 3G). Human circACTA2 overexpression could not reduce wire injury–induced intimal hyperplasia of mouse femoral arteries (Online Figure XA and XB), likely because of the fact that circACTA2 nucleotide sequences are species-specific.

Human α-SMA 3′ untranslated region contains a miR-548f-5p–binding site at nucleotides 55 to 77 (Online Figure XIA). In a luciferase assay, miR-548f-5p mimic decreased α-SMA-untranslated region–directed luciferase activity by 60% (Figure 3H). miR-548f-5p mimic and anti-miR-548f-5p (Online Figure XIB) markedly reduced or increased α-SMA protein expression, respectively (Figure 3I; Online Figure XIC), in a dose-dependent manner (Online Figure XID and XIE). TGF-β1 treatment and NRG-1-ICD overexpression significantly reduced miR-548f-5p level (Figure 3J). These findings indicate that miR-548f-5p inhibits α-SMA expression by targeting its 3′ untranslated region.

NRG-1-ICD/circACTA2/miR-548f-5p Axis Regulates HASMC Contraction
circACTA2 knockdown markedly decreased α-SMA expression upregulated by NRG-1-ICD overexpression (Figure 4A; Online Figure XIIA). Conversely, circACTA2 overexpression

Figure 2. Neuregulin-1 (NRG-1) intracellular domain (NRG-1-ICD) induces circACTA2 expression through recruiting IKZF1 (IKAROS family zinc finger 1). A, Northern blotting detected α-SMA (smooth muscle α-actin) mRNA and circular ACTA2 (alpha-actin-2; circACTA2). B and C, Chromatin immunoprecipitation (ChIP) quantitative polymerase chain reaction (PCR) detected NRG-1-ICD binding to α-SMA gene first intron (B) or quantitative real-time PCR (qRT-PCR) detected circACTA2 and α-SMA mRNA (C) after CRISPRi-mediated genomic block. *P<0.05, ***P<0.001 vs control. D, qRT-PCR detected circACTA2 in HASMCs (human aortic smooth muscle cells) infected with Ad-NRG-1-ICD or Ad-control (Ad-Ctl) and then treated with or without transforming growth factor (TGF)-β1. ***P<0.001 vs Ad-Ctl, ###P<0.001 vs Ad-Ctl+TGF-β1. E, NRG-1-ICD interaction with Ikzf1 (IK-1) was examined by reciprocal coimmunoprecipitation (IP). F, Immunofluorescence staining examined NRG-1-ICD and IK-1 in HASMCs. Green, red, and blue staining indicates IK-1, NRG-1-ICD, and the nuclei, respectively. Bar=50 μm. G, Sequential ChIP and PCR detected NRG-1-ICD and IK-1 binding to the Ikzf1-binding site. H, Western blotting analyzed QKI (quaking) and ADAR1 (adenosine deaminase RNA-specific binding protein 1) expression in HASMCs treated as indicated in (H). I and J, qRT-PCR detected circACTA2 and α-SMA mRNA in HASMCs transfected with si-QKI (I) or si-ADAR1 (J). **P<0.01 vs si-control (si-Con).
further increased α-SMA upregulation by NRG-1-ICD overexpression (Figure 4A; Online Figure XIIB). Thus, we concluded that circACTA2 is a crucial mediator of NRG-1-ICD–induced α-SMA expression. Correspondingly, overexpression of NRG-1-ICD or circACTA2 alone or in combination significantly promoted HASMC contraction induced by acetylcholine or norepinephrine (Figure 4B; Online Figure XIIC).

miR-548f-5p silencing significantly increased α-SMA expression regardless of circACTA2 knockdown, whereas circACTA2 knockdown partially antagonized the effect of anti-miR-548f-5p (Figure 4C; Online Figure XIID). Further, circACTA2 overexpression and miR-548f-5p silencing co-operatively upregulated α-SMA level (Figure 4C; Online Figure XIIE). These findings suggest that circACTA2 may function as a sponge to bind miR-548f-5p and thereby relieving miR-548f-5p repression of α-SMA expression.

Discussion

In the present study, we found that VSMCs isolated from rat, mouse, and human aortas express abundant NRG-1, inconsistent with previous observations by other authors reporting that VSMCs did not seem to express NRG-1.21 Our results showed that TGF-β1 and PDGF-BB increased and decreased NRG-1 expression, respectively, suggesting that NRG-1 up-regulation facilitates TGF-β1–induced VSMC differentiation. Importantly, treating VSMCs with TGF-β1 increased NRG-1 soluble protein, indicating that NRG-1 is cleaved by proteolytic enzymes and releases its extracellular EGF-like domain.

We found that NRG-1-ICD participated in F-actin formation and stabilization of actin filaments by interacting with α-SMA and thereby enhanced contraction of HASMCs. Notably, we found that NRG-1-ICD translocated into the nucleus could mediate the TGF-β1–induced α-SMA expression in HASMCs. To explore whether NRG-1-ICD mediates TGF-β1–induced α-SMA expression through acting as a transcriptional regulator, we used chromatin immunoprecipitation sequencing to identify NRG-1-ICD–bound target genes and confirmed NRG-1-ICD binding to sequences of the first intron of α-SMA gene. TGF-β1 obviously increased NRG-1-ICD...
α-circACTA2 was derived from exons 5 to 9 of the α-SMA gene. Moreover, the Ikzf1-biding site (GGGAAA) exists in the NRG-1-ICD–binding sequences. Although NRG-1-ICD lacks a DNA-binding domain, it can bind to proteins with zinc-finger domains and shows strong transcriptional activity. In this study, we demonstrated that NRG-1-ICD formed a stable complex with Ikzf1 that is bound to the NRG-1-ICD–binding sequences of the α-SMA gene. The increase of nuclear NRG-1-ICD in response to TGF-β1 results in enhancement of circACTA2 formation by Ikzf1.

circACTA2, as a miR-548f-5p sponge, interacted with miR-548f-5p, which targets the 3′ untranslated region of the α-SMA mRNA, and increased α-SMA expression. Although both the IncRNAs (ceRNAs) and circRNAs regulate miRNA function, their efficiency may be different. The covalently closed structure of circRNAs leads to a higher transcript stability, allowing circRNAs to accumulate and maintain the regulatory function for a longer period of time. Despite the recent advances in our understanding of circRNA functions, the excise regulatory mechanisms between circRNA and miRNA remains to be further elaborated.

Altogether, this is the first study to demonstrate that NRG-1-ICD induces a novel circRNA, circACTA2, which subsequently interacts with miR-548f-5p, thus relieving the miR-548f-5p–mediated repression of α-SMA expression. In turn, α-SMA is expressed at higher levels and interacts with NRG-1-ICD in the VSMC cytoplasm. Thus, the NRG-1-ICD/ circACTA2/miR-548f-5p axis may represent a new therapeutic target for VSMC dysfunction.

Figure 4. Neuregulin-1 (NRG-1) intracellular domain (NRG-1-ICD)/circACTA2/miR-548f-5p axis regulates HASMC (human aortic smooth muscle cells) contraction. A, Western blotting detected α-SMA in HASMCs transfected with the indicated RNA or constructs. B, HASMCs were transfected as above, and cell length was measured using video motion edge detector. **P<0.01 vs before acetylcholine (Ach) stimulation, #P<0.05 vs Ad-NRG-1-ICD or Ad-circACTA2. C, α-SMA was analyzed by Western blotting in HASMCs transfected with the indicated constructs. D, HASMC length was measured as described above. *P<0.05, **P<0.01 vs before Ach stimulation, #P<0.05 vs anti-miR-548f-5p or Ad-circACTA2.

TGF-β1 markedly increased the concurrent recruitment of NRG-1-ICD and Ikzf1 to the NRG-1-ICD–binding sequences. The increase of nuclear NRG-1-ICD in response to TGF-β1 results in enhancement of circACTA2 formation by Ikzf1.

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Disclosures
None.

References


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Material and Methods

Human tissue harvest

Human vascular samples were obtained from thirteen patients, seven with hypertension and six without hypertension. Patients who had hypertension at least 10 years and managed blood pressure by using hypotensor. The renal arteries used in this study obtained from 2015 to 2017 at the second hospital of Hebei Medical University (Shijiazhuang, China). The protocols for human studies were approved by the ethical committees. Each of the surgical patients gave informed signed consent before donating tissue. One part of human renal arteries were fixed overnight in 10% neutral buffered formalin and processed for routine embedding in paraffin. Another part of renal arteries were snap-frozen in liquid nitrogen, stored at -80 °C, and subsequently used for nucleic acid extraction.

Cell culture and treatment

Human aortic smooth muscle cells (HASMCs) (ScienCell, no. 6110) were grown in Smooth Muscle Cell Medium containing apo-transferrin, insulin, fibroblast growth factor-2, insulin-like growth factor-1, hydrocortisone, and 2% foetal bovine serum (FBS) (ScienCell, no. 1101). Mouse aortic vascular smooth muscle cells (MASMCs) (ATCC, No.CRL-2797TM) were routinely cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, Rockville, MD) containing 100 units/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal bovine serum (GEMINI, USA), in a humidified incubator at 37 °C with 5% CO₂. Rat aortic smooth muscle cells (RASMCs) were isolated from thoracic aorta of male Sprague-Dawley rats. The cells were plated in low DMEM containing 10% FBS. The cells used in all studies were from passages 3 to 6. Cells were maintained in 5% CO₂ at 37 °C within a humidified atmosphere and determined to be SMCs by morphology and expression of α-SMA. Human cardiac microvascular endothelial cells (HCMECs) were obtained from Sciencell (no. 6000) and maintained in Endothelial Cell Medium (ScienCell, no. 1001). 2 ng/ml TGF-β1 was used to stimulation VSMCs. Before stimulation with TGF-β1 (R&D Systems, Minneapolis, MN, USA) and infection with adenoviruses (Ads), VSMCs were incubated in serum-free medium for 24 h. Human embryonic kidney 293A cells were obtained from ATCC (Manassas, VA) and maintained in high glucose DMEM supplemented with 10% FBS.

Adenovirus expression vector and plasmid constructs

Adenoviruses encoding NRG-1 (Ad-NRG-1), NRG-1-ICD (Ad-NRG-1-ICD), α-SMA (Ad-α-SMA), circACTA2 (Ad-circACTA2) and GFP control (Ad-Ctl) were entrusted to Hanbio, shanghai. The expression plasmid of NRG-1-ICD was created by the placement of human NRG-1 cDNA into the pcDNA3.1 vector. The ICD of mouse NRG-1 cDNA was cloned into the pEGFP-C2 vector. Sequence of α-SMA gene contains NRG-1-ICD binding site or its mutant sequences (Supplementary Table I) were amplified by PCR and inserted into pGL3-Basic vector (Promega). Sequences of circACTA2 or α-SMA genes 3’UTR contain miR-548f-5p target site or its mutant sequences (Supplementary Table I) were amplified by
PCR and inserted into pmir-GLO Dual-Luciferase miRNA Target Expression Vector (Promega).

**Animal experiments**

All animal studies were approved by the Institutional Animal Care and Use Committee of Hebei Medical University (approval ID: HebMU 20080026) and all efforts were made to minimize suffering. 8- to 12-week-old male wild-type C57BL/6 mice were anesthetized with 1.5% isoflurane, and the thoracic aortas were isolated for analysis of immunofluorescence staining. For the circACTA2-overexpression model, femoral artery wire injury was performed as previously described.\(^2\) Immediately after injury, the femoral artery was cannulated, and the biclamped segment was incubated with 20 μl of adenovirus (1 × 10\(^{10}\) pfu/ml) coding GFP or circACTA2 for 15 min. After 14 days, all mice were anesthetized and perfused with cold saline, and the arteries were harvested for analysis of RNA, morphology, and histology.

**Morphometry and histology**

Mice were euthanized, perfused and then fixed with 4% paraformaldehyde in 0.9% NaCl for 3 min through the left ventricle under physiological pressure. The femoral arteries were harvested, fixed with formalin and embedded in paraffin. Human renal arteries were fixed overnight in 10% neutral buffered formalin and processed for routine embedding in paraffin. Ten consecutive 4-μm-thick sections were prepared for hematoxylin and eosin staining. Images were acquired using a Leica microscope (Leica DM6000B, Switzerland) and digitized with LAS V.4.4 (Leica). Morphometric analysis of the neointimal area and measurement of the intima/media (I/M) ratio were performed in a blind manner.

**Immunostaining**

Immunofluorescence staining was performed with 4 μm paraffin cross-sections from the thoracic aorta of mouse and the renal artery of human. After deparaffinized with xylene and rehydrated, the slides were pre-incubated with 10% normal goat serum and then incubated with primary antibodies anti-NRG-1 (sc-348, santa cruz), anti-α-SMA (ab7817, abcam), anti-CD31 (ab28364, abcam). Secondary antibodies were fluorescein-labeled antibody to rabbit IgG (021516, KPL, USA) and rhodamine-labeled antibody to mouse IgG (031806, KPL, USA), or fluorescein-labeled antibody to mouse IgG (021815, KPL, USA) and rhodamine-labeled antibody to rabbit IgG (031506, KPL, USA). In each experiment, DAPI (157574, MB biomedical) was used for nuclear counterstaining. Images were captured by confocal microscopy (DM6000 CFS, Leica) and processed by LAS AF software.

HASMCs were prepared as described previously. The cells were fixed in 4% paraformaldehyde for 5 min at room temperature and then were washed with PBS, followed by incubation in 10% normal goat serum blocking solution for 30 min in a humidified chamber at room temperature. The cells were incubated in anti-NRG-1, anti-α-SMA, anti-NRG-1-ICD (sc-393009, santa cruz), anti-Ikaros (ab26083, abcam) for 2 h at room temperature, washed with PBS, and incubated in fluorescein-conjugated secondary antibodies for 60 min. The cells were then washed with PBS, mounted with DAPI, and visualized with a laser scanning confocal microscope.
Western blot analysis

Proteins from cultured cells were prepared with lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP-40). Equal amounts of protein were separated on SDS-PAGE, and electrotransferred to a PVDF membrane (Millipore). Membranes were blocked with 5% milk in TTBS for 2 h at room temperature and incubated primary antibodies overnight at 4 °C. Antibodies that were used are as follow: anti-NRG-1 (1:500, sc-348), anti-NRG-1-ICD (1:500,sc-393009), anti-α-SMA (1:500, sc-130617), anti-QKI (1:1000, ab126742), anti-ADAR1(1:1000, ab168809) or anti-β-actin (1:1000, sc-47778). Membranes were then incubated with the HRP-conjugated secondary antibody (1:5000, Rockland) for 1 h at room temperature. The blots were treated with the Immobilon™ Western (Millipore), and detected by ECL (enhanced chemiluminescence) Fuazon Fx (Vilber Lourmat). Images were captured and processed by FusionCapt Advance Fx5 software (Vilber Lourmat). All experiments were replicated three times.

Co-immunoprecipitation assay

Co-immunoprecipitation was performed as previously described. In brief, the cell lysates were immunoprecipitated with anti-sm-α-actin (sc-32251; Santa Cruz Biotechnology), anti-Myosin (ab24648; abcam), anti-Ikaros (12016-1-AP; Proteintech), anti-NRG-1-ICD (sc-393009; Santa Cruz Biotechnology) respectively for 1 h at 4 °C, then incubated with protein A-agarose overnight at 4 °C. Protein A-agarose-antigen-antibody complexes were collected by centrifugation at 12,000 g for 2 min at 4 °C, and washed 5 times with 1 ml immunoprecipitation-HAT buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 0.1 mM PMSF) for 20 min each time at 4 °C. The bound proteins were resolved using SDS-PAGE, followed by Western blotting with anti-NRG-1, anti-NRG-1-ICD, and anti-Ikaros antibodies.

GST pull-down assay

Glutathione S-transferase (GST), GST-α-SMA fusion protein were produced by BL21 Escherichia coli under induction by isopropylthio-b-galactoside at 28 °C. The proteins were purified by affinity absorption with glutathione-Sepharose 4B beads (Amersham Biosciences, Uppsala, Sweden). The recombinant GST and GST-α-SMA proteins on the glutathione beads were incubated with total cell lysates treated with TGF-β1 or not at 4 °C overnight, followed by extensive washing. The proteins on the beads were resolved by SDS-PAGE and probed by Western blotting with anti-NRG-1, anti-NRG-1-ICD, and anti-GST antibodies.

Isolation of RNA and PCR

Cultured cells were lysis by using the QIAzol Lysis Reagent (Catalog no.79306). Total RNA was extracted from above sample according to the manufacturer's instructions (miRNeasy Mini Kit; Catalog no.217004). The quality of the RNA was determined using a Nanodrop 2000 (Thermo). For RNase R treatment, 5μg of total RNA was incubated 20 min at 37 °C with or without 20 U/μl of RNase R (Epicientre Technologies, Madison, WI), and the resulting RNA was subsequently purified using an RNeasy MinElute cleaning Kit (Qiagen). For microRNA: reverse transcription and qRT-PCR was performed using the miRNA
Detection Kit by Genepharma (Shanghai, China) and internal control U6 according to the manufacturer’s protocol. For large mRNA: cDNA was synthesized using an M-MLV First Strand Kit (Life Technologies) with random hexamer primers. qRT-PCR of mRNAs or circRNAs was performed using Platinum SYBR Green qPCR Super Mix UDG Kit (Invitrogen), and real-time PCR experiments were carried on a ABI 7500 FAST system (Life Technologies). Relative amount of transcripts was normalized with GAPDH and calculated using the 2−ΔΔCt formula as previously described.5 RT-PCR of mRNAs or circRNAs, 5 μl 1:5 diluted cDNA or gDNA was amplified (22–37 cycles, depending on the template) in a 25 μl PCR reaction using the KOD Xtreme™ HotStart Polymerase Kit (71975-3, Novagen). To confirm the PCR results, the PCR products were purified through QIA quick PCR purification kit (Qiagen). Direct PCR product Sanger sequencing was performed by GENEWIZ services. Supplementary Table II summarizes the primer sequences.

Chromatin immunoprecipitation assay and sequential ChIP

The chromatin immunoprecipitation (ChIP) assay was performed as described previously.6 In brief, HASMCS were treated with 1% formaldehyde for 10 min to cross link proteins with DNA. The cross-linked chromatin was then prepared and sonicated to an average size of 400–600 bp. The samples were diluted 10-fold and then precleared with protein A-agarose/salmon sperm DNA for 30 min at 4 °C. The DNA fragments were immunoprecipitated overnight at 4 °C with the anti-NRG-1-ICD, or anti-IgG (as negative control) antibodies. After cross-linking reversal, NRG-1-ICD occupancy on the α-SMA gene intron was examined. All results were determined by quantitative qRT-PCR. ChIP primer sequences (Supplementary Table II) will be provided upon request. Sequential 2-step ChIP assays then were performed as described previously.3 In brief, chromatin fragments were immune-precipitated by overnight incubation with antibodies for NRG-1-ICD, Ikaros, or control IgG at 4 °C. After several washes, the precipitates were incubated with 50 μl of buffer containing 0.5% SDS and 0.1 M NaHCO3 for 10 min at 65 °C. The supernatant was collected after spinning; diluted with 1 mM EDTA, 150 mM NaCl, 50 mM HEPES (pH 7.5), 0.1% SDS, 1% Triton X-100, and 0.1% sodium deoxycholate; and incubated overnight with antibodies for Ikaros, NRG-1-ICD, or control IgG. After they were washed, the protein-DNA complexes were eluted from the beads and treated with proteinase K overnight. DNA was purified on a minicolumn, and the NRG-1-ICD-binding site +5642/+5787 bp of the α-SMA intron was amplified by RT-PCR with the primer described above. Each experiment was replicated at least 3 times.

ChIP-sequencing (ChIP-seq)

DNA from NRG-1 ChIP experiment was sequenced using the Illumina Hiseq 2000 platform at BGI in Shenzhen, China. Library preparation, cluster generation and sequencing by synthesis were performed according to manufacturer’s protocol. All raw reads were aligned using Burrows-Wheeler Aligner to the human reference genome (hg19). Aligned reads were processed by Model-based Analysis of ChIP-seq for peak calling. Significant peaks were defined using the criteria of a threshold of minimally 9 reads and p-value less than 10−8. ChIP experiments were independently repeated and ChIP-seq called peaks verified by qRT-PCR.
Small interfering RNA transfection

Small interfering RNAs (siRNAs) targeting human α-SMA (si-α-SMA) and circACTA2 (si-circACTA2) were designed by BioCaring Biotechnology (Shijiazhuang, China) and synthesized by Gene Pharma (Shanghai, China). The siRNA sequences were on supplementary table III, Non-specific siRNA (si-Control), NRG-1-ICD siRNA (si-NRG-1-ICD), ADAR1 siRNA (si-ADAR1) and QKI siRNA (si-QKI) were purchased from Santa Cruz Biotechnology. Transfection was performed using Lipofectamine 2000 following the manufacturer’s instructions. Twenty-hours following transfection, HASMCs were treated with TGF-β1, GFP-mNRG-1-ICD, Ad-NRG-1-ICD, or anti-miR-548f-5p. Cells were then harvested and lysed for western blotting or PCR.

Luciferase assay

Human embryonic kidney 293A cells were maintained as previously described. For luciferase assays, 293A cells were cotransfected with a miR-548f-5p mimic (Gene pharma; Shanghai) or NC mimic (200 pmol) combined with 100 ng of luciferase reporter or an empty vector; and 293A cells were also transfected with a luciferase-harboring α-SMA intron (NRG-1-ICD binding site), pcDNA3.1-NRG-ICD using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Luciferase activity was measured using a Dual-Glo Luciferase Assay System (Promega, Madison, WI) with a Flash and Glow (LB955, Berthold Technologies) reader 24 h after transfection. The specific target activity was expressed as the relative activity ratio of firefly luciferase to Renilla luciferase. All constructs were evaluated in a minimum of three separate wells per experiment.

Biotin pull-down of RNA

To detected circACTA2 and miR-548f-5p interaction, biotin pull-down was carried out as previously described. In brief, HASMCs were cross-linked with 1% formaldehyde in PBS for 10 min at room temperature, then quenched with 0.125 M glycine for 5 min. The cells were resuspended in lysis buffer (50 mM Tris, pH 7.0, 10 mM EDTA, and 1% SDS; with freshly added 1 mM DTT, complete protease inhibitor, and 0.1 U/μl RNase inhibitor) on ice for 10 min and were sonicated. The cell lysate was diluted in two times volume with hybridization buffer (750 mM NaCl, 1% SDS, 50 mM Tris, pH 7.0, 1 mM EDTA, 15% formamide, 1 mM DTT, protease inhibitor, and 0.1 U/μl RNase inhibitor). 100 pmol Biotin probes (sequences in Supplementary Table IV) were added. Streptavidin Dynabeads (Life Technologies) were blocked for 2 h at 4 °C in lysis buffer containing 1 mg/ml yeast tRNA and 1 mg/ml BSA and wash twice with 1 ml lysis buffer. 100 μl washed/blocke Dynabeads was added per 100 pmol of biotin probes, and the whole mix was then rotated for 30 min at 37 °C. Beads were captured by magnets (Life Technologies) and washed five times with wash buffer (2× SSC, 0.5% SDS, and 0.1 mM DTT and PMSF). Beads were then subjected to RNA elution with buffer (Tris 7.0, 1% SDS).

Phalloidin staining for actin stress fibers

HASMCs were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 at room temperature for 10 min, followed by tetramethyl rhodamine isothiocyanate
(TRITC)-phalloidin (Sigma) staining for 30 min in the dark. Staining with 4',6-diamidino-2-phenylindole (DAPI) was performed to visualize nuclear localization. Confocal microscopy was performed with a Confocal Laser Scanning Microscope System (Leica).

**ELISA**

The concentration of NRG-1β was detected in the culture medium from HASMCs treated with PDGF-BB or TGF-β1 for 12 h. Culture medium using a NRG-1β Elisa kit (BlueGene Biotech, Putuo District, Shanghai, China). The absorbance at 450 nm was measured with a microplate reader (SPECTRAFluor Plus, Tecan).

**Northern blotting**

PCR primers including T7 promoter sequences were designed to target the linear transcript of α-SMA, or the circular ACTA2 transcript exclusively (sm-α-actin: 522 bp; circACTA2: 530 bp). Supplementary Table V summarizes the primer sequences. In vitro transcription using 10 μg unpurified PCR-template was performed using DIG Northern Starter Kit (Roche; 12039672910) including subsequent DNase I digestion of the template according to the manufacturer’s instruction. HASMC or MASMC RNA was mixed with RNA loading dye and loaded onto 2% agarose gel in MOPS buffer. Subsequently, the gel was equilibrated in 20 × SSC for 2 × 15 min and gels are blotted by capillary transfer with 20 × SSC overnight. Subsequently, membranes were UV cross-linked and prehybridized with DIG Easy Hyb for 30 min with gentle agitation in an appropriate container. For hybridization, the membranes were then incubated with 100 ng/ml DIG-labelled RNA antisense probe in hybridization buffer at 68 °C overnight. After washing (two times for 5 min with 2 × SSC, 0.1% SDS followed by two times for 15 min with 0.1 × SSC 0.1% SDS at 68 °C), the membranes were detected according to the manual (Roche, DIG Northern Starter Kit).

**Fluorescence in situ hybridization**

Cultured cells were prepared as described previously. The cells were fixed in 4% paraformaldehyde for 5 min at room temperature and then were washed with PBS. Paraffin cross-sections (4 μm thick) from renal arteries were deparaffinized and rehydrated for hybridization. In situ hybridization was performed using specific probes of circACTA2 and miR-548f-5p according to user manual of miRCURY LNATM microRNA ISH Optimization Kit (Exiqon). Hybridization was performed using fluorescence-labeled probes (Supplementary Table VI) in hybridization buffer (Exiqon) by incubation at 55 °C for 1 h in a thermoblock (Labnet). After stringent washing with SSC buffer, then DAPI (157574, MB biomedical) was used for nuclear counter staining. Images were captured by confocal microscopy (DM6000 CFS, Leica) and processed by LAS AF software. Images were acquired using a Leica microscope (Leica DM6000B, Switzerland) and digitized with a software of LAS V.4.4 (Leica).

**Proximity ligation assay**

HASMCs were grown in 6 well chamber slides to 70%-80% confluence. After starvation in serum free medium for 24 hours, cells were treated with or without TGF-β1 for 12 h. For
fixation, 4% paraformaldehyde was added and incubated for 10 min. 0.25% Triton X-100 was used for permeabilization. Proximity ligation assay was performed using the Rabbit PLUS and Mouse MINUS Duolink in situ proximity ligation assay (PLA) kits with primary antibodies (anti-α-SMA and anti-NRG-1, or anti-IK-1 and anti-NRG-1-ICD) according to the manufacturer’s protocol. Subsequently, slides were dehydrated, air-dried, and embedded in DAPI-containing mounting medium. Fluorescence was detected using a laser scanning confocal microscope.

**Cell contraction assay**

The contractility of HASMCs was evaluated as described previously.\(^8\) The cells were stimulated with 100 nM Acetylcholine (Ach) for 30 s, and the length of individual cells (n = 100) was analyzed using the Image-Pro Express a software package. The extent of cell contraction was calculated as the ratio of the change in relative length to the initial relative length. Dynamic of cell contraction was recorded by using Confocal Laser Scanning Microscope System (Leica).

**Contractile response of HASMCs**

HASMCs were added in the insert of the transwell, and the infiltration rate of isothiocyanate fluorescein-labeled bovine serum albumin (BSA) was measured to reflect the contraction of HASMCs. Briefly, 5 μl of both FITC-labeled BSA (4 mg/ml) and NE at a final concentration of 10\(^{-5}\) mol/L was added into the upper compartment of transwells with cultured HASMCs. 100 μl of medium was collected from the lower compartment, fluorescence was measured at 5, 10, 15, 25, 35, 45, 60, and 70 min after NE was added, and 100 μl of fresh medium was supplemented into the lower compartment after medium withdrawal each time.\(^9\)

**CRISPRi assay**

The designation and construction of CRISPRi were performed as described previously.\(^10\) In brief, the sequence encoding *Staphylococcus aureus* Cas9 and C-terminal SV40 nuclear localization sequences (NLS) were amplified by PCR from a dSaCas9 (#61594, Addgene). Using standard ligation-independent cloning, we cloned these sequences into MSCV-Puro (Clontech) and used sanger sequencing for sequence confirmation. For CRISPRi sgRNA construction, four sgRNAs were designed to target near the NRG-1-ICD binding site of the first intron of α-SMA. The sgRNA oligos (Supplementary Table VII) were phosphorylated, annealed and inserted into the BPK2660 (Plasmid #70709, Addgene) vector using BsmBI ligation strategy.\(^11\) The dSaCas9 and four sgRNA vectors were co-transfected into the VSMC cells in six-well plate using Lipofectamine 2000 (Invitrogen) as the manufacturer’s protocols. Next day the cells were re-seeded in 10 cm dishes. After 6 days of transfection, puromycin was added 0.5 mg/ml to cell culture and further grown for 14 days. Individual puromycin resistant colonies were picked up manually and then expanded in 12-well plates. Total RNA was extracted using QIAzol Lysis Reagent (79306), and the expression of circACTA2 was detected by qRT-PCR.

**Target DNA deletion by CRISPR/Cas9 technology.**

For the CRISPR/Cas9 assay, sgRNA design and clone were carried out as described
In brief, sgRNAs were constructed by first annealing each pair of oligos (Supplementary Table VIII) and ligating them to BsaI-linearized SaCas9 (Plasmid#61591, Addgene). The vectors were confirmed by sanger sequence. VSMC cells were seeded in 6-well plates at a density of $2 \times 10^5$ cells per well. After 24 h, the cells were transiently transfected with 2 μg CRISPR/Cas9 plasmid using Lipofectamine 2000 (Life Technologies). Genomic DNA and total RNA was extracted after 72 h transfection using DNA Extraction Kit (Omiga) or QIAzol Lysis Reagent (79306). PCR was conducted to amplify the targeting region by using DNA template. The expression of circACTA2 was detected by qRT-PCR.

**DNA pull-down assay**

DNA pull-down was performed essentially as described in previous. DNA probes for pull down assay were produced by PCR using the following primers (Supplementary Table IX). The resulting PCR products were purified with PCR Purification Kit (QIAGEN). HASMCs treated with or without TGF-β1 (2 ng/mL) for 12 h were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.1% NP-40) containing protease inhibitors. Cell extracts (200 μg) were precleared with ImmunoPure streptavidin-agarose beads (20 μl/sample, Promega) for 1 h at 4 °C. After centrifugation for 2 min at 5000 g, the supernatant was incubated with 100 ng of purified biotinylated oligonucleotides and 10 μg of poly (dl-dC)•poly (dl-dC) overnight at 4 °C while gently being rocked. 30 μl of streptavidin-agarose beads was added, followed by an additional 1 h of incubation at 4 °C. The protein-DNA-streptavidin-agarose complex was washed four times with lysis buffer, separated on a 10% SDS-PAGE. Differentially expressed proteins were identified using Mass Spectrometry. Analysis was carried out on the Ultrafil Xtreme LC-MS/MS mass spectrometer at BGI in Shenzhen, China.

**Statistical analysis of experimental data**

All of the data are presented as the means±SEM. Differences between two groups were assessed using analysis of variance followed by a Student’s t-test. For multiple comparisons or repeated measurements ANOVA or repeated ANOVA followed by Tukey’s posthoc test was used. A value of P<0.05 was considered statistically significant, and denoted with 1, 2, or 3 asterisks when lower than 0.05, 0.01, or 0.001, respectively. Statistical analysis was performed using Graphpad Prism 5 software (GraphPad Software, San Diego, CA, USA).
References


13. Guo P, Dong XY, Zhang X, Zhao KW, Sun X, Li Q, Dong JT. Pro-proliferative factor klf5 becomes

**Supplementary Table I.** Primers for plasmid constructs

<table>
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<tr>
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<th>Sequences 5' to 3'</th>
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**Supplementary Table II.** Primers for Real-time PCR and RT-PCR

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Supplementary Table III. The siRNA sequences

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Supplementary Table IV. Oligo probe sequences

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Supplementary Table V. Primer sequences for Northern blot probe

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<td>linear α-SMA-F</td>
<td>CGATAGAAGATGGCATCATCAGACAGCTG</td>
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Supplementary Table VI. FISH probes sequences
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<tr>
<td>miR-548f-5p-FAM-probe</td>
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<td>circATCA2-cy3-probe</td>
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**Supplementary Table VII.** CRISPRi assay gRNA oligos

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**Supplementary Table VIII.** CRISPR/Cas9 technology oligos

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<td>Intron-α-SMA-sg2-R</td>
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**Supplementary Table IX.** DNA pull-down primers

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<td>the control site, biotin-F</td>
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<td>the control site, biotin-R</td>
<td>CATTGGCTTAAAGCTAAGGCCCAC</td>
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Online Figure I. NRG-1 is abundantly expressed in rat and human VSMCs, and its expression is significantly down-regulated in human renal arteries with intimal hyperplasia. 

A, Immunofluorescent staining of NRG-1 (red), CD31 (green) and DAPI (blue) on human renal arteries with intimal hyperplasia and normal renal arteries. Scale bar=100 μm.

B, NRG-1 mRNA was determined by qRT-PCR in human renal arteries with intimal hyperplasia (n=7) and normal renal arteries (n=6). ***P<0.001 vs control.

C, The expression of NRG-1 in rat aortic smooth muscle cell (RASMC), human aortic smooth muscle cell (HASMC), and human cardiac microvascular endothelial cell (HCMEC) was analyzed by Western blotting.

D, RT-PCR detection of NRG-1 mRNA expression in HCMECs, HASMCs, and RASMCs.
Online Figure II

A. NRG-1 mRNA relative expression over TGF-β 1 treatment.

B. NRG-1 protein relative level over TGF-β 1 treatment.

C. NRG-1 mRNA relative expression over PDGF-BB treatment.

D. Western blot analysis of NRG-1 and β-actin expression levels.

E. Concentration of NRG-1 over PDGF-BB treatment.

F. Actin area % over Ad-Ctl, Ad-NRG-1, Ad-Ctl+CB, and Ad-NRG-1+CB treatments.
Online Figure II. TGF-β1 induces and PDGF-BB inhibits NRG-1 expression in VSMCs. A, Human aortic smooth muscle cells (HASMCs) were treated with TGF-β1 for the indicated times, and NRG-1 mRNA was detected by qRT-PCR. Data represent NRG-1 mRNA relative expression normalized to GAPDH mRNA. ***P<0.001 vs TGF-β1 for 0 h. B, Quantitative analysis of Figure 1C. Data are expressed as mean±SEM from three independent experiments. *P<0.05 vs TGF-β1 for 0 h. C, HASMCs were serum-starved and treated with PDGF-BB (10 ng/ml) for the indicated times, and NRG-1 mRNA was detected by qRT-PCR. **P<0.01, ***P<0.001 vs PDGF-BB for 0 h. D, Western blot detection of NRG-1 expression in HASMCs treated with PDGF-BB for the indicated times (left). The right panel shows densitometric analyses from three independent experiments. *P<0.05 vs PDGF-BB for 0 h. E, Levels of NRG-1β in the culture medium of HASMCs treated with PDGF-BB were determined by the ELISA. F, Quantitative analysis of Figure 1E, showing actin area from three independent experiments. *P<0.05, ***P<0.001 vs Ad-Ctl or Ad-Ctl+CB. All data are means±SEM of 3 independent experiments.
Online Figure III. NRG-1β does not affect the actin cytoskeleton, but NRG-1-ICD overexpression enhances stress fiber formation. A, HASMCs treated or not with HRG-1β for 24 h were fluorescently stained for F-actin with TRITC-phalloidin, and then imaged with a laser scanning confocal microscope. The right panel shows actin area analyses from three independent experiments. B, HASMCs were infected with Ad-Ctl or Ad-NRG-1-ICD for 24 h, stained fluorescently for F-actin with TRITC-phalloidin, and imaged with a laser scanning confocal microscope. The right panel shows actin area analyses from three independent experiments. ***P<0.001 vs Ad-Ctl.
Online Figure IV. NRG-1-ICD participates in cytoskeleton organization. A, HASMCs were incubated in serum-free medium for 24 h, followed by treatment with TGF-β1 for 12 h. The interactions between α-SMA and NRG-1 or NRG-1-ICD were examined by coimmunoprecipitation (IP). Myosin was used as a negative control for immunoprecipitation. B, GST-α-SMA fusion protein was purified from E.coli and used to pull down NRG-1 and NRG-1-ICD from HASMCs treated or not with TGF-β1. The pulled down proteins were examined by Western blot analysis with the indicated antibodies. C, HASMCs were grown on cell culture inserts and treated or not with TGF-β1. Expression of α-SMA and NRG-1 was examined by immunofluorescence staining. Green, red and blue staining indicates α-SMA, NRG-1 and the nuclei, respectively. Yellow staining indicates co-localization of α-SMA with NRG-1. Scale bars=75 μm.
Online Figure V

A
Con vs TGF-β1

Increased genes bound by NRG-1-ICD

- α-SMA
  - AMOTL1
  - IRAK3
  - SLC25A21
  - CRYM
  - ANKR26P1
  - MYO9B
  - IL1RN
  - PHACTR1
  - BTBD9
  - USP49
  - LRRRC1
  - CSGALNACT1
  - RALYLY

Reduced genes bound by NRG-1-ICD

- ACADSB
- SLC22A15
- KCNMB3
- ZNF800
- LINC0100

B

α-SMA

Exon1

NTRG-1-ICD-binding region (GGGAAA)

Exon2

+5642

C

pcDNA3.1
pcDNA3.1-NRG-1-ICD

Relative luciferase activity

WT Mut

** **

D

TGF-β1

NRG-1-ICD

α-SMA

β-actin

Relative level

0 h 6 h 12 h 24 h
Online Figure V. Treating HASMCs with TGF-β1 affects NRG-1-ICD binding to target genes and increases NRG-1-ICD and α-SMA expression. A, ChIP-seq data show the different genes bound by NRG-1-ICD between control and TGF-β1-treated HASMCs. B, Schematic illustration of NRG-1-ICD-binding region in the first intron of α-SMA gene, and it is located approximately 5642 bp–5787 bp downstream of the transcription start site. C, 293A cells were co-transfected with wild-type pGL3-NRG-1-ICD-binding site-luc or its mutant and NRG-1-ICD expression plasmid. Luciferase activity was measured. ***P<0.001 vs empty vector. D, HASMCs were incubated in serum-free medium for 24 h, followed by treatment with TGF-β1 for the indicated times. Western blot analysis detects NRG-1-ICD and α-SMA expression. The right panel shows densitometric analyses from three independent experiments. *P<0.05 vs TGF-β1 0 h or 6 h. E and F, Quantitative analysis of Figure 1J. Expression of α-SMA was quantitated by densitometry, and values were normalized to total β-actin. n=3. *P<0.05 vs Ad-Ctl+TGF-β1-untreated group (first bar), #P<0.05 vs Ad-Ctl+TGF-β1 (second
bar). **G**, HASMCs were transfected with si-NRG-1-ICD for 24 h, and then treated with TGF-β1 or transfected with GFP-mNRG-1-ICD plasmid (encoding mouse NRG-1-ICD). Western blot analysis detects NRG-1-ICD and α-SMA expression. α-SMA was quantitated as described above. *P<0.05 vs si-NRG-1-ICD+TGF-β1 (second bar).** **H**, HASMCs were infected with Ad-Ctl or Ad-NRG-1-ICD for 24 h, and α-SMA mRNA was detected by qRT-PCR. All data are the means±SEM of three independent experiments.
Online Figure VI

D

circACTA2 sequences

GAGACAGAGAGGAGCAGGAAAGTGTTTTAGAAGCATTTGCGGTGGACAATGGAA
GGCCCGGCTTCATCGTATTCCTGTTTGCTGATCCACATCTGCTGGAGGTGGACAGAGAGGCCAGGATGGAGCCACCGATCCAGACAGAGTATTTGCGCTCCGGAGGGGCAATGATCTTGATCTTCATGGTGCTGGGTGCTAGGGCCGTGATCTCCTTCTGCATTCGGTCGGCAATGCCAGGGTACATAGTGGTGCCCCCTG
ATAGGACATTGTTAGCA
TAGAGGTCCTTCCTGATGTCAATATCACACTTCATGATGCTGTTGTAGGTGGTTTCATGGATGCCAGCAGACTCCATCCCAAGGATGAGGATGCGGCAGTGGCCATCTCATTTTCAAAGTCAGCTACATAACACAGTTTCTCCTTGATGTCCCGGACAATCTCACGCTCAGCAGT
AGTAACGAAGGAATAGCCACGCTCAGTCAGGATCTTCATGAGGTAGTCAGTGAGATCTCGGCCAGCCAGATCCAGACGCATGATGGCATGGGGCAAGGCATAGCCCTCATAGATGGGGACATTGTGGGTGACACCATCTCCAGAGTCCAGCACGATGCCAGTTGTGCGTCTTGGAGGCATAGA
GAGCTACATAACACAGTTTCTTCCTTCTATGTGCCCGGACATCCAGGATGACACTGCGGTG
AGTAACGGAAACGTTTCATTTCCGGATGGGTGATCTTTGCCCCATCAGGCAACTCGTAACTCTTCTCAAGGGAGGATGAGGATGCGGCAGTGGCCATCTCATTTTCAAAGTGCGCTTCAGCAGACTCCATCCCGATGAGGATGCGGTG

B

Relative expression

circACTA2
α-SMA mRNA
Mock
RNase R

A

Divergent primers
Convergent primers

250 bp
2000 bp
500 bp
100 bp
750 bp
250 bp
100 bp

1
2

730 bp
E

circACTA2 junction sequences (5’→3’)

5’ → 3’

circACTA2 junction sequences (3’→5’)

5’ → 3’

F

PAM Intron-ACTA2-sgRNA1

3’ GAGGGGACCGGTAAGAGGAGGTCTTA 5’

GGCTCCCTCCGTGCCATCTCTCCACACATTCTTCTATCTCTGAGGACTCTTGTA

AGTCCTCCCTGCCATCATATTCTCCCTCAAGCTGTCCTCCTGCTCTCTACTCGAGGAGGTCTTG

CCTGGTAAGAGGACCTCCCCACCCCATCCACCTGCTGGACATGATGGGCTTGGAGAAGAGGTGAGCTG

CCCTCATGCTGTTCTGATGTCTCTTGCTTCCATGCATCCCTTTCCAGGGCCATGAGGAGGCT

3’ GAGGGGACCGGTAAGAGGAGGTCTTA

G

M C 1 2 1+2

500 bp

250 bp

100 bp

GAPDH

circACTA2

α-SMA mRNA

H

Binding of NRG-1-ICD

(I/P ratio of eluted immunoprecipitated

Con sgsRNA1 sgsRNA2

sgRNA1+sgRNA2

I

Relative expression

circACTA2

α-SMA mRNA

J

circACTA2

relative expression

si-Con

si-Con+TGF-1

si-NRG-1-HCD

si-NRG-1-HCD+TGF-1

***

###

2.0

2.5

0.0
Online Figure VI. circACTA2 sequences are complementary to exons 5-9 of the α-SMA gene, deletion of the NRG-1-ICD binding region reduces circACTA2 formation but does not affect α-SMA mRNA level. A, Divergent and convergent primers were used to verify that circACTA2 is a circRNA. Convergent primers were used to detect total α-SMA mRNAs. Divergent primers amplified circACTA2 in cDNA but not genomic DNA (gDNA). GAPDH serves as linear control and size marker in base pairs. B, qRT–PCR for the abundance of circACTA2 and α-SMA mRNA in HASMCs treated with RNase R. The amount of circACTA2 and α-SMA mRNA was normalized to the value measured in the mock treatment. *P<0.05, ***P<0.001 vs mock treatment. C, RT-PCR products of full-length circACTA2 were analyzed and visualized by agarose gel electrophoresis. D, circACTA2 sequences. E, Sanger sequencing confirms head-to-tail junction of circACTA2. F, Schematics illustrating the positions of the two guide RNAs in the first intron of α-SMA gene locus. G, PCR detects two sgRNAs-mediated deletion of the NRG-1-ICD binding region. H, ChIP-qPCR detects NRG-1-ICD binding to NRG-1-ICD binding region after CRISPRi-mediated genomic deletion. *P<0.05. I, qRT-PCR detects circACTA2 and α-SMA mRNA expression in HASMCs after two sgRNAs (sgRNA1+sgRNA2)-mediated deletion of the NRG-1-ICD binding region by CRISPR interference. *P<0.05 vs con or sgRNA1 or sgRNA2. J, HASMCs were transfected with si-NRG-1-ICD or si-Con for 24 h and then treated with or without TGF-β1, qRT-PCR detects circACTA2 expression. ***P<0.001 vs si-Con, **P<0.001 vs si-Con+TGF-β1. Data represent the means±SEM of 3 independent experiments.
Online Figure VII. Ikzf1, QKI and ADAR1 participate in NRG-1-ICD-induced circACTA2 expression. A, HASMCs treated with or without TGF-β1 for 12 h were used to detect the interaction of NRG-1-ICD with Ikzf1 (IK-1) using Duolink with two primary antibodies. Scale bar=100 μm. B and C, Quantitative analysis of Figure 2H. Western blot data of QKI (B) and ADAR1 (C) were quantitated by densitometry and values were normalized to total β-actin. Data are means±SEM, n=3. *P<0.05 vs Ad-Ctl+TGF-β1-untreated group (first bar), #P<0.05 vs Ad-Ctl+TGF-β1 (second bar). D and E, HASMCs were transfected with siRNA targeting QKI (si-QKI) or ADAR1 (si-ADAR1) for 24 h. Western blotting was performed using anti-QKI (D), anti-ADAR1 (E), and anti-β-actin antibodies.
Online Figure VIII. Overexpression of circACTA2 in mouse aortic smooth muscle cells. 
A and B, Mouse aortic smooth muscle cells (MASMCs) were infected with Ad-circACTA2 or Ad-Ctl for 24 h, qRT-PCR detects circACTA2 expression. Data represent the means±SEM of 3 independent experiments. ***P<0.001 vs Ad-Ctl (A). Northern blot analysis detects circACTA2 expression in MASMCs infected with Ad-Ctl or Ad-circACTA2 (B).
Online Figure IX

A

si-circACTA2

si-both

B

Ad-Ctl
Ad-circACTA2

TGF-β1
-  +  -  + kDa
α-SMA
β-actin

C

si-Con
si-circACTA2

TGF-β1
-  +  -  + kDa
α-SMA
β-actin

D

circACTA2 | matching | position
---|---|---
mir-548f-5p | UAGGGCCGUGAUCCUUCUGCA | 66
Online Figure IX. circACTA2 mediates TGF-β1/NRG-1-ICD-induced α-SMA expression by acting as miR-548f-5p sponge. A. Schematic illustration showing two targeted siRNAs. si-circACTA2 targets the back-splice junction of circACTA2, and si-both targets both the linear and circular transcripts. qRT-PCR detects circACTA2 and α-SMA mRNA in HASMCs transfected with two siRNAs. Data are the means±SEM of three independent experiments. ***P<0.001 vs si-Con or si-circACTA2. B. HASMCs were infected with Ad-Ctl or Ad-circACTA2 for 24 h and then treated with or without TGF-β1 for an additional 12 h. Western blot analysis was performed with anti-α-SMA and anti-β-actin antibodies. α-SMA was quantitated by densitometry and values were normalized to total β–actin (right panel). Data are means±SEM, n=3. *P<0.05 vs Ad-Ctl+TGF-β1-untreated group (first bar), #P<0.05 vs Ad-Ctl+TGF-β1 (second bar). C. Western blot detection of expression of α-SMA in HASMCs transfected with si-Con or si-circACTA2 for 24 h and then treated with or without TGF-β1. α-SMA was quantitated as described above. *P<0.05 vs si-Con+TGF-β1-untreated group (first bar), #P<0.05 vs si-Con+TGF-β1 (second bar). D. Prediction of miR-548f-5p binding sites in the circACTA2 sequence. E. 293A cells were co-transfected with wild-type pmirGLO-circACTA2 or its mutant pmirGLO-circACTA2 mut and miR-548f-5p mimic or miR-Ctl. Luciferase activity was measured. Data are the means±SEM of three independent experiments. ***P<0.001 vs pmirGLO or pmirGLO-circACTA2 mut. F. Representative hematoxylin and eosin staining of cross sections from human renal arteries with intimal hyperplasia and normal renal arteries. Scale bars=100 μm.
Online Figure X. circACTA2 overexpression does not reduce neointima formation induced by femoral artery wire injury in mouse. A. Representative hematoxylin and eosin-stained arterial sections from Ad-Ctl- and Ad-circACTA2-infected mouse models. n=6. Scale bars=100 μm. B. The ratio of intima to media (I/M) (left) and extent of arterial stenosis were calculated (right).
Online Figure XI

A

miR-548f-5p  UUUUGACACUAUGAAAACGU
α-SMA UTR  UUAUGCCUUCAGUUCUUUCCCA
mutant  UCCGG

B

C

D

miR-Ctl (nmol/L) 100  75   50    0
miR-548f-5p mimic (nmol/L) 0    25   50   100

E

Anti-miR-Ctl (nmol/L) 100  75   50    0
Anti-miR-548f-5p (nmol/L) 0     25   50    100
Online Figure XI. miR-548f-5p suppresses α-SMA expression by targeting 3′-UTR of α-SMA gene. A, The miR-548f-5p-binding site in the 3′UTR of α-SMA mRNA (green); the mutated site is shown in red. B, qRT-PCR detection of miR-548f-5p expression in HASMCs transfected with miR-548f-5p mimic or anti-miR-548f-5p and their corresponding control. Data represent the means±SEM of 3 independent experiments. ***P<0.001 vs miR-Ctl or anti-miR-Ctl. C, Quantitative analysis of Figure 3I. Western blot data were quantitated by densitometry and values were normalized to total β–actin. Data are means±SEM, n=3. *P<0.05 vs miR-Ctl, #P<0.05 vs Anti-miR-Ctl. D and E, HASMCs were transfected with miR-Ctl and different doses of miR-548f-5p mimic (25, 50 and 100 nmol/l), and α-SMA expression was detected by Western blotting (D) or transfected with anti-miR-Ctl and different doses of anti-miR-548f-5p (25, 50 and 100 nmol/l) and α-SMA expression was analyzed by Western blotting (E). The right panel shows densitometric analysis from 3 independent experiments. *P< 0.05 vs miR-Ctl or anti-miR-Ctl.
Online Figure XII

A

\[ \alpha\text{-SMA protein relative level} \]

<table>
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B

\[ \alpha\text{-SMA protein relative level} \]

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C

Accumulative infiltration rate of FITC BSA (%)

D

\[ \alpha\text{-SMA protein relative level} \]

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E

\[ \alpha\text{-SMA protein relative level} \]

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<tbody>
<tr>
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<td>+</td>
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F

Accumulative infiltration rate of FITC BSA (%)

Time after NE (min)
Online Figure XII. NRG-1-ICD/circACTA2/miR-548f-5p axis regulates HASMC contraction. A and B. Quantitative analysis of Figure 4A. Western blot data were quantitated by densitometry and values were normalized to total β-actin. Data are means±SEM, n=3. *P<0.05 vs si-Con+Ad-NRG-1-ICD-untreated group, *P<0.05 vs si-Con+Ad-NRG-1-ICD (A); *P<0.05 vs Ad-Ctl+Ad-NRG-1-ICD-untreated group, *P<0.05 vs Ad-Ctl+Ad-NRG-1-ICD (B). C and F, HASMCs were infected or transfected with the indicated constructs for 24 h and then stimulated with norepinephrine (NE). Contractile response of HASMCs to NE was determined by the infiltration rate of FITC-labeled BSA through the transwell. **P<0.01 vs Ad-Ctl. D and E, Quantitative analysis of Figure 4C. Western blot data were quantitated by densitometry and values were normalized to total β-actin. Data are means±SEM, n=3. *P<0.05 vs si-Con+anti-miR-548f-5p-untreated group, *P<0.05 vs si-Con+anti-miR-548f-5p (D); *P<0.05 vs Ad-Ctl+anti-miR-548f-5p-untreated group, *P<0.05 vs Ad-Ctl+anti-miR-548f-5p (E).