Long noncoding RNA *NEAT1* (nuclear paraspeckle assembly transcript 1) is critical for phenotypic switching of vascular smooth muscle cells

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In response to vascular injury, vascular smooth muscle cells (VSMCs) may switch from a contractile to a proliferative phenotype thereby contributing to neointima formation. Previous studies showed that the long noncoding RNA (lncRNA) *NEAT1* is critical for paraspeckle formation and tumorigenesis by promoting cell proliferation and migration. However, the role of *NEAT1* in VSMC phenotypic modulation is unknown. Herein we showed that *NEAT1* expression was induced in VSMCs during phenotypic switching in vivo and in vitro. Silencing *NEAT1* in VSMCs resulted in enhanced expression of SM-specific genes while attenuating VSMC proliferation and migration. Conversely, overexpression of *NEAT1* in VSMCs had opposite effects. These in vitro findings were further supported by in vivo studies in which *NEAT1* knockout mice exhibited significantly decreased neointima formation following vascular injury, due to attenuated VSMC proliferation. Mechanistic studies demonstrated that *NEAT1* sequesters the key chromatin modifier WDR5 (WD Repeat Domain 5) from SM-specific gene loci, thereby initiating an epigenetic "off" state, resulting in down-regulation of SM-specific gene expression. Taken together, we demonstrated an unexpected role of the lncRNA *NEAT1* in regulating phenotypic switching by repressing SM-contractile gene expression through an epigenetic regulatory mechanism. Our data suggest that *NEAT1* is a therapeutic target for treating occlusive vascular diseases.

long noncoding RNA | smooth muscle cells | phenotypic switching | gene expression | epigenetic regulation

Vascular smooth muscle cells (VSMCs) are a major component of the vascular wall. In response to vascular injury, VSMCs may switch from a contractile to a "synthetic" phenotype that is characterized by increased motility and proliferation as well as reduced expression of contractile proteins such as calponin (*CNN1*), SM22α (transgelin, *TAGLN*), SM MHC (myosin heavy chain, *MYH11*), SM α-actin (*ACTA2*), and Hic-5 (*TGFB1I1*) (1). Many occlusive vascular diseases in humans, including intimal hyperplasia after angioplasty, atherosclerosis, and restenosis following vascular interventions, are largely dependent upon VSMC phenotype modulation, contributing to progression of intimal lesions that compromise vessel patency (1, 2). Although many studies have shown that, following arterial injury and in atherosclerosis neointima, VSMCs mostly originate from the local vessel wall (3–10), there remains a lack of knowledge of the factors that can control the switch of SM phenotype in vascular diseases.

Previous studies have shown that serum response factor (SRF) is a critical transcription factor for regulating expression of many SM-specific contractile genes (11). SRF is a multifunctional protein that not only binds a highly conserved *cis*-regulatory element $CC(A/T)_6GG$, termed a CArG box that can be found in most SM-specific gene loci, but also interacts with a wide variety of accessory cofactors (11). WDR5 (WD Repeat Domain 5), a long noncoding RNA (lncRNA) binding protein (12) and a critical adaptor protein in histone 3 lysine 4 (H3K4) methyltransferase complexes (13), has been shown to play a crucial role in SM differentiation by promoting active chromatin, thereby allowing SRF to bind to CArG boxes of SM-specific genes (14). However, how the function of WDR5 is regulated to control SM-contractile protein gene expression is largely unknown.

In sharp contrast to the <3% of human genomic DNA that is transcribed and ultimately translated into protein, >80% of genomic DNA is capable of being transcribed but does not encode for protein (15, 16). Depending upon the length of the transcript, noncoding RNA can be divided into small (<200 nt) and long noncoding RNA (lncRNA, >200 nt) (17). Originally, the large number of noncoding transcripts were regarded as

Significance

Many occlusive vascular diseases in humans are largely dependent upon vascular smooth muscle cell (VSMC) phenotypic switching from a contractile to a proliferative phenotype, contributing to the formation of intimal lesions that eventually block the blood flow. Previous studies showed that the long noncoding RNA (lncRNA) *NEAT1* is critical for tumorigenesis. In this report, we showed that *NEAT1* expression was not only induced in VSMCs during phenotypic switching but functionally was critical for the smooth muscle phenotypic change. Our study demonstrates an unexpected role of the lncRNA *NEAT1* in VSMCs and suggests that *NEAT1* is a novel therapeutic target for treating occlusive vascular diseases in humans.

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Data deposition: The RNA-seq data generated in this study have been deposited in the Sequences Read Archive at the NCBI (accession no. SRP154294).

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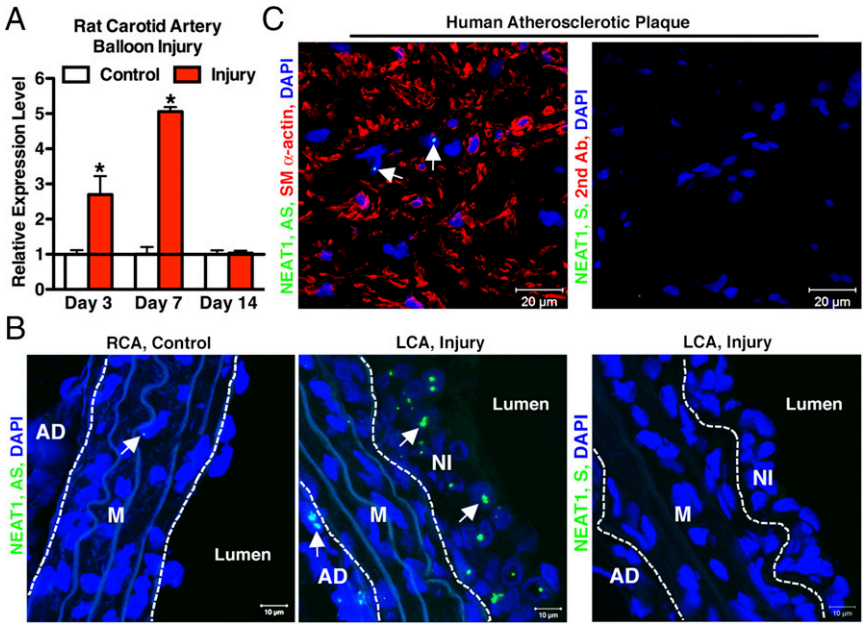

Fig. 1. *NEAT1* is induced in balloon-injured rat carotid arteries. (*A*) The qRT-PCR was performed to assess *NEAT1* expression in rat carotid artery post-balloon injury at the time as indicated; $n = 5$ rats for each time point. $^*P < 0.05$. Error bars indicate standard deviation. (*B*) Seven days after rat carotid artery balloon injury, control right common carotid artery (RCA) (*Left*) or injured left common carotid (LCA) (*Middle*) were sectioned for RNA-FISH to visualize *NEAT1* (green), as indicated by arrows. Cell nuclei were stained with DAPI (blue). The section hybridized with *NEAT1* sense (*S*) probe was used as a negative control (*Right*). Dashed lines denote external or internal elastic lamina. AD, adventitia layer; M, media layer; NI, neointima layer. The representative images shown are chosen from three rats. (*C*) RNA-FISH/IF were performed to stain *NEAT1* (green, arrows) and SM α-actin (red) in human carotid endarterectomy specimen (*Left*). Sections with *NEAT1* S probe and second antibody (*Right*) served as negative control. The representative images shown are selected from three human specimens.

"junk" products, but emerging evidence suggests that a significant portion of lncRNAs are critical for control of gene expression during development and under pathological conditions (17). Despite some recent progress (18–21), most of the lncRNAs that are functionally important in regulating VSMC phenotypic switching remain to be identified.

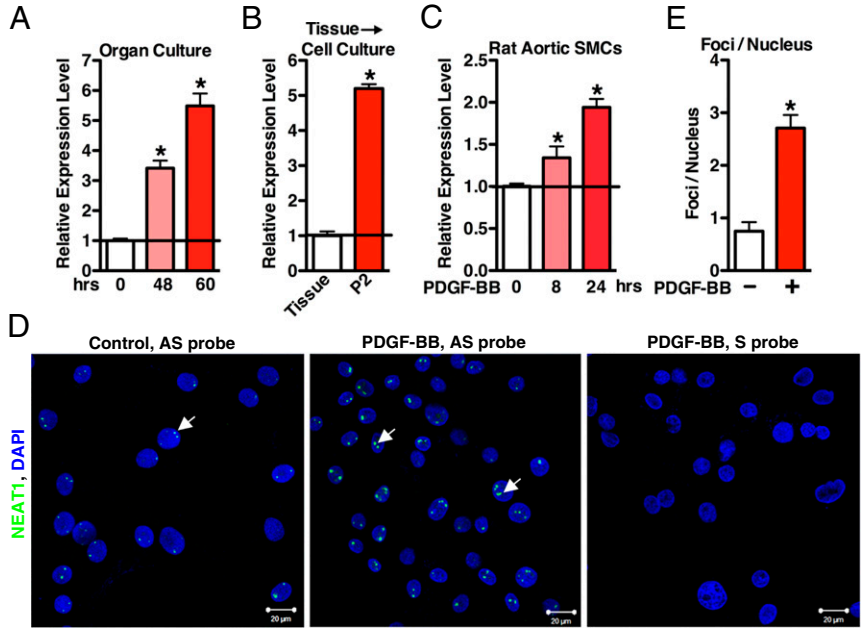

Fig. 2. *NEAT1* is induced in response to SM phenotypic switching in vitro. (*A*) The qRT-PCR was performed to evaluate *NEAT1* expression using the aortic tissues either from the freshly isolated rat thoracic aorta (0 h) or rat thoracic aorta cultured in 20% FBS for the times as indicated; $n = 4$. $^*P < 0.05$. (*B*) The qRT-PCR was performed to assess *NEAT1* expression in fresh rat aortic tissue or second passage enzymatically dispersed rat aortic VSMCs; $n = 4$. $^*P < 0.05$. (*C*) Rat primary aortic VSMCs were treated with PDGF-BB (25 ng/mL) for 8 h or 24 h as indicated, and *NEAT1* expression was measured by qRT-PCR; $n = 3$. $^*P < 0.05$. (*D*) Rat aortic SMCs were treated without (*Left*) or with (*Middle*) PDGF-BB (25 ng/mL) for 24 h, and then RNA-FISH assays were performed to visualize *NEAT1* expression. Arrows indicate the representative *NEAT1* signal. Cells treated with PDGF-BB and hybridized with *NEAT1* sense (*S*) probe served as the negative control (*Right*). (*E*) The numbers of RNA-FISH *NEAT1* foci per cell with or without PDGF-BB treatment in HCASMCs (in *D*) were manually counted and plotted; $n = 3$. $^*P < 0.05$.

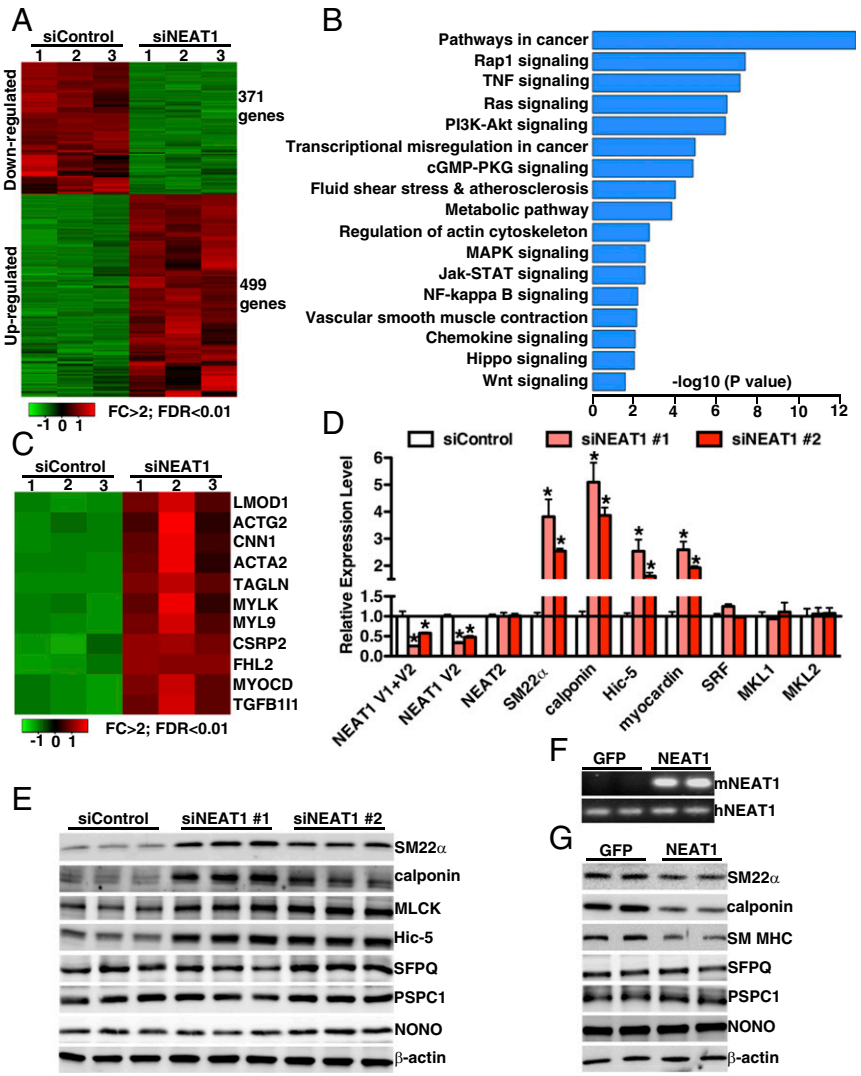

Fig. 3. *NEAT1* attenuates SM-specific gene expression. (*A*) Heat map of mRNAs differentially expressed between control and *NEAT1* knockdown HCASMCs, as revealed by RNA-seq with cutoff of FDR < 0.01 and FC (fold change) >2; $n = 3$ each group. (*B*) GO analysis of the differentially expressed genes between silencing control and silencing *NEAT1* in HCAMSCs. (*C*) Heat map of significantly up-regulated SRF-dependent contractile genes in silencing *NEAT1* compared with silencing control in HCASMCs. HCASMCs were transduced with scrambled control siRNA or two different *NEAT1* siRNA duplexes and harvested for (*D*) qRT-PCR or (*E*) Western blotting analysis as indicated. $*P < 0.05$. (*F*) RT-PCR was performed to validate the exogenous mouse *NEAT1* expression using the mouse or human *NEAT1* specific primers after control GFP or mouse *NEAT1* V1 adenovirus was transduced into HCASMCs for 48 h. (*G*) Western blot was performed to examine protein expression in GFP or *NEAT1* overexpressing HCASMCs.

Paraspeckles are nuclear bodies that are typically detected as foci in close proximity to nuclear speckles and are enriched with RNA-binding proteins such as splicing factor proline/glutamine-rich (SFPQ), paraspeckle component 1 (PSPC1), and non-POU domain containing octamer binding (NONO) that binds to the lncRNA *NEAT1* (nuclear paraspeckle assembly transcript 1) (22). In humans, *NEAT1* has two splice variants, *NEAT1*_1 (V1) (~3,700 nt) and *NEAT1*_2 (V2) (~23K nt) (22, 23). Although the homology of *NEAT1* exons among mammals is relatively low, the function of *NEAT1* in paraspeckle formation is conserved from mouse to human (22, 23). Both of the *NEAT1* variants can be found in paraspeckles, where they bind resident proteins to maintain architectural integrity of the paraspeckle (22, 24), although a recent study showed that *NEAT1* V1 can be also found in microspeckles (25). Previous studies have demonstrated that paraspeckles are not only subnuclear structures but also regulate the expression of a number of genes via the sequestration of specific proteins and RNAs (26–28). In particular, the paraspeckle lncRNA *NEAT1* is critical for IL-8 production in response to viral infection (28) and for promoting tumorigenesis in breast and prostate cancers (29, 30). However, little is known about the functional role of *NEAT1* in VSMCs during VSMC phenotypic modulation.

In this study, we discovered that the lncRNA *NEAT1* is induced in response to arterial injury and is a previously unrecognized coordinator of VSMC phenotype, which can down-regulate contractile gene expression and promote VSMC proliferation and migration through regulating the lncRNA binding protein WDR5.

Methods

Rat balloon angioplasty was carried out as previously described (31, 32). RNA-FISH (fluorescence in situ hybridization) was performed following the protocol as previously described (33). *NEAT1* KO (knockout) mice were generated and maintained in C57BL/6 strain background, and left carotid artery ligation injury was carried out as previously described (32, 34). The use of experimental rats and mice for arterial injury procedures, including BSL-2 viral work, was approved by the Institutional Animal Care and Use Committee and Biosafety committees at Augusta University. Full materials and methods are detailed in *SI Appendix*.

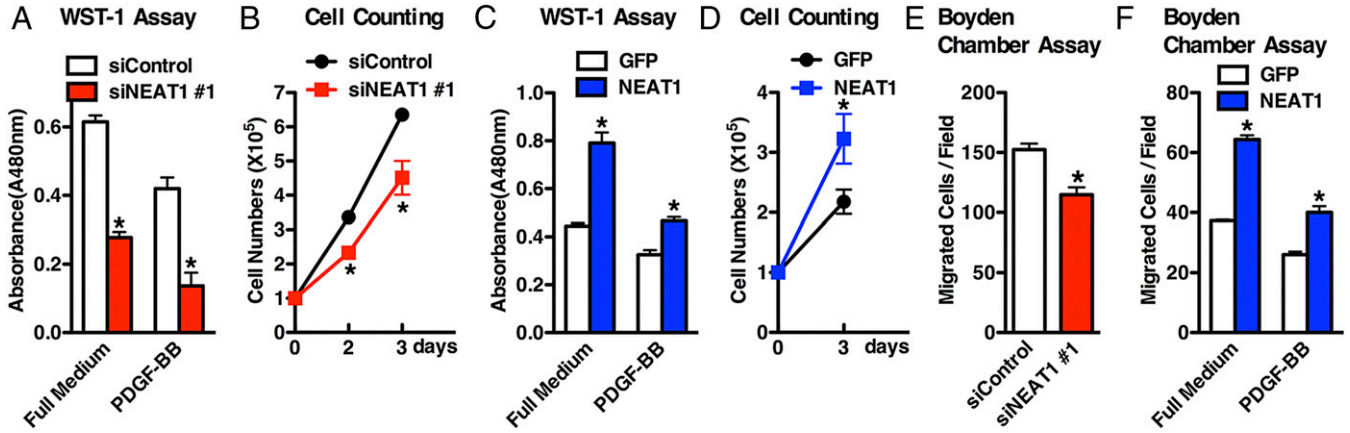

Fig. 4. *NEAT1* is required and sufficient to promote VSMC proliferation and migration. (*A*) Control or *NEAT1* silencing duplex #1 was transfected into HCASMCs in full medium or PDGF-BB–containing medium (25 ng/mL), and cell proliferation was measured using the cell proliferation WST-1 kit (Roche); $n = 3$. $^*P < 0.05$. (*B*) HCASMCs after transfection with control or *NEAT1* #1 silencing duplex were counted at each time point as indicated; $n = 3$. $^*P < 0.05$. Adenovirus encoding *NEAT1* or control GFP were transduced into HCASMCs, and then the proliferation was measured as described in *A* by (*C*) WST-1 assay or (*D*) cell counting; $n = 3$. $^*P < 0.05$. (*E*) Quantification of Boyden chamber assay to assess cell migration after 8-h plating of silencing duplex transfected or (*F*) viral transduced HCASMCs; $n = 3$. $^*P < 0.05$.

Results

***NEAT1* Expression Is Increased in VSMCs Following Arterial Injury.** A mounting body of evidence suggests the lncRNA *NEAT1* plays a critical role in tumorigenesis by promoting cell proliferation and migration, but little is known about whether *NEAT1* has any function in VSMCs. As an initial step to examine the function of *NEAT1* in VSMCs, we performed qRT-PCR assay to assess *NEAT1* expression by using the RNA samples harvested from control or carotid arteries injured by a balloon denudation, a model resembling human angioplasty procedures in which VSMCs convert from a contractile to synthetic phenotype and contribute to neointima formation (35). The qRT-PCR results revealed that *NEAT1* was significantly elevated twofold and fivefold 3 and 7 d postinjury, respectively, during the onset of maximal VSMC proliferation and migration (35, 36) (Fig. 1*A*). RNA-FISH assays further revealed that *NEAT1* was rarely detectable in the medial VSMCs of control intact arteries (Fig. 1*B*, *Left* and *SI Appendix*, Fig. S1), but were readily detectable in the nuclei of neointimal cells (Fig. 1*B*, *Middle*, arrows), which are mostly dedifferentiated VSMCs (35). Similarly, we also found that *NEAT1* is readily detectable in human advanced carotid atherosclerotic plaque (Fig. 1*C*, *Left*), suggesting a conservation of the potential *NEAT1* function between rodent and humans. Together, these data demonstrate that *NEAT1* expression is induced in neointimal VSMCs in response to arterial injury.

***NEAT1* Expression Is Induced During SM Phenotypic Modulation in Vitro.** To extend the in vivo findings (Fig. 1) to in vitro, we next sought to determine the expression level of *NEAT1* during SM phenotypic switching in in vitro settings. In culture, rat aortic tissue undergoes a dramatic switch toward a synthetic proliferative phenotype (37, 38) that is associated with significantly increased expression of *NEAT1* in a time-dependent manner (Fig. 2*A*). During dedifferentiation of VSMCs induced by primary culture (37), *NEAT1* expression was significantly induced (Fig. 2*B*). In response to PDGF-BB, a growth factor known to further inhibit SM contractile protein expression while promoting VSMC proliferation and migration (39), *NEAT1* expression was significantly up-regulated in a time-dependent manner (Fig. 2*C*). RNA-FISH assays further revealed the induced expression of *NEAT1* in PDGF-BB–treated rat aortic primary VSMCs (Fig. 2 *D* and *E*). Taken together, these data demonstrate that *NEAT1* expression is induced in phenotypically modulated synthetic VSMCs in vitro.

***NEAT1* Suppresses SM-Specific Gene Expression.** Since *NEAT1* expression was elevated in tandem with the reduction of SM-specific markers during SM phenotypic switching in vivo and in vitro, we next sought to investigate the function of *NEAT1* in SM-specific gene expression by loss- or gain-of-function assays. First, we examined the effects of *NEAT1* depletion on VSMC transcriptome by performing RNA-sequencing (RNA-seq) analysis. RNA-seq data revealed that silencing *NEAT1* results in significant down-regulation of both V1 and V2 isoforms but not its adjacent lncRNA NEAT2 (also known as MALAT1; *SI Appendix*, Fig. S2). Using a twofold cutoff and false discovery rate (FDR) of <0.01 threshold for inclusion, RNA-seq data further revealed that silencing *NEAT1* in HCASMCs (human coronary artery smooth muscle cells) significantly down-regulated 371 genes while up-regulating 499 genes (Fig. 3*A* and Dataset S1). Gene ontology (GO) enrichment analysis revealed that these genes are associated with SM contraction in addition to many signaling pathways related to tumorigenesis such as Hippo signaling (32). Interestingly, among those genes up-regulated after silencing *NEAT1*, there are a large set of SRF-dependent genes encoding SM contractile proteins such as calponin (*CNN1*), SM22α (*TAGLN*), SM MHC (*MYH11*), SM α-actin (*ACTA2*), Hic-5 (*TGFB1I1*), and myocardin (*MYOCD*), a potent SRF cofactor critical for promoting VSMC myogenesis (40) (Fig. 3*C*). To further validate the findings, depletion of *NEAT1* using an additional siRNA duplex also significantly increased expression of SM22α, calponin, Hic-5, and myocardin at both mRNA and protein levels in HCASMCs, without affecting the expression of paraspeckle *NEAT1*-binding proteins such as SFPQ, PSPC1, and NONO (Fig. 3 *D* and *E*). Moreover, silencing *NEAT1* did not significantly alter other key factors critical for regulation of SM-specific gene expression, including SRF, MKL1, and MKL2 (Fig. 3*D*). The induction of endogenous SM-specific gene expression resulting from silencing *NEAT1* in VSMCs is unlikely an off-target effect, since two different *NEAT1* silencing duplexes have similar results. Furthermore, overexpression of mouse *NEAT1* V1 was sufficient to reduce SM-specific gene expression in HCASMCs while having no effect on paraspeckle protein expression (Fig. 3 *F* and *G*). As a recent study showed that the paraspeckle protein SFPQ mediates *NEAT1* function to regulate IL-8 expression in response to viral infection (28), we next sought to determine whether SFPQ plays a role in regulating SM-specific gene expression by transfection of silencing duplexes against SFPQ into HCASMCs. Data from this experiment revealed that, in contrast to silencing *NEAT1*, depletion of SFPQ did not have any effects on the endogenous SM contractile

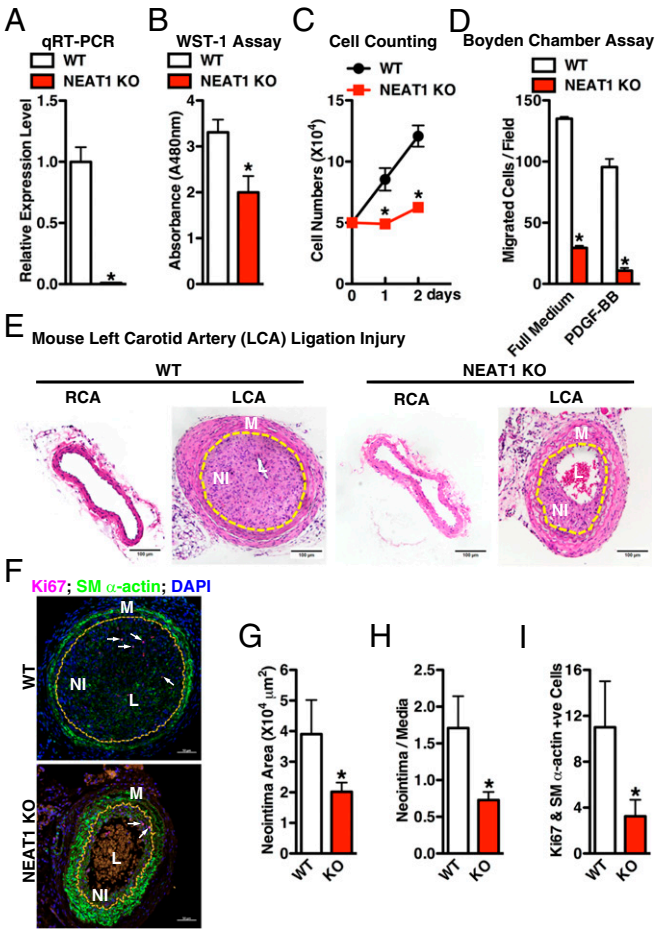

Fig. 5. KO of *NEAT1* impairs VSMC proliferation and migration and attenuates neointima formation induced by ligation injury in mice. (*A*) The qRT-PCR was performed to measure *NEAT1* expression in thoracic aorta from WT or *NEAT1* KO mice; $n = 4$. $^*P < 0.05$. Primary aortic VSMCs prepared from WT or *NEAT1* KO mice were subjected to (*B*) WST-1 assay and (*C*) cell number counting to measure SMC proliferation or (*D*) Boyden chamber transwell assay to measure SMC migration, as indicated; $n = 3$. $^*P < 0.05$. (*E*) Adult WT or *NEAT1* KO mice were subjected to carotid artery ligation injury (LCA) for 28 d. Representative hematoxylin- and eosin-stained sections show less neointima formation in the KO mice compared with WT. Neointima lesions are outlined with yellow lines. L, lumen; M, media layer; NI, neointima layer. (*F*) Costaining of proliferative marker Ki67 (red) and SM-specific marker SM α-actin (green) was performed in the ligation-injured LCA (28 d postinjury) from WT or *NEAT1* KO mouse. Nuclei were counterstained with DAPI (blue). Arrows point to the representative Ki67 positive cells in neointima. Neointima lesions were outlined with yellow lines. Statistical analysis of (*G*) neointima area and (*H*) neointima/media layer ratio of sections shown in *F* using ImageJ software; $n = 7$. $^*P < 0.05$. (*I*) Quantification of the Ki67 positive SMCs within the neointima area of the WT or *NEAT1* KO LCA as shown in *F*; $n = 7$. $^*P < 0.05$.

gene expression (*SI Appendix*, Fig. S3). Taken together, these data demonstrate that *NEAT1* plays a critical role in repressing SM-specific gene expression independent of its paraspeckle binding protein SFPQ.

***NEAT1* Promotes VSMC Proliferation and Migration.** KO of *NEAT1* in female mice impairs alveolar cell proliferation, resulting in the defects of mammary gland development (41). Recent studies also demonstrated that *NEAT1* promotes prostate and breast cancer cell proliferation and survival (29, 30). Consistent with this, *NEAT1* is induced in arterial injury (Fig. 1) where neointimal VSMCs are known to be proliferative. We therefore directly tested whether *NEAT1* is able to affect VSMC proliferation by silencing endogenous *NEAT1* or overexpression of *NEAT1* in HCASMCs. Data from these experiments revealed that the rates of HCASMC proliferation were inhibited by silencing endogenous *NEAT1* expression and increased by *NEAT1* overexpression, supporting a positive role for the *NEAT1* in regulating VSMC proliferation (Fig. 4 *A–D*). Similarly, by using Boyden chamber assay, we found that silencing endogenous *NEAT1* in HCASMCs significantly impaired VSMC migration (Fig. 4*E*), while overexpression of *NEAT1* significantly promoted VSMC migration in both full medium and PDGF-BB–treated conditions (Fig. 4*F*). Taken together, these data demonstrate that *NEAT1* plays an integral role in SM phenotypic modulation by suppressing SM contractile gene expression while promoting VSMC proliferation and migration.

KO of *NEAT1* Significantly Impairs VSMC Proliferation and Migration and Attenuates Neointima Formation After Carotid Artery Ligation Injury in Mice. In vitro gain- and loss-of-function assays suggest a unique role of the paraspeckle lncRNA *NEAT1* in suppressing SM-specific gene expression while promoting VSMC migration and proliferation (Figs. 3 and 4). Therefore, we next examined the functional role of *NEAT1* in neointima formation in vivo under pathological conditions using *NEAT1* KO mice (34). *NEAT1* KO mice do not exhibit apparent gross abnormalities at baseline except for dysfunction of the corpus luteum (42) and in mammary gland development in the female KO mice (41). We confirmed that *NEAT1* expression is completely depleted in the KO aorta (Fig. 5*A*). We further demonstrated that the VSMCs cultured from *NEAT1* KO mice show decreased cell proliferation capability (Fig. 5 *B* and *C*) and migration (Fig. 5*D*) compared with WT VSMCs. Moreover, the neointima formed in response to carotid artery ligation for 28 d was significantly reduced in KO mice compared with the WT counterparts (Fig. 5 *E–H*). Immunofluorescence (IF) staining of proliferation marker Ki67 and SM-contractile gene SM α-actin further revealed that the *NEAT1* deficiency attenuated VSMC proliferation after ligation injury (Fig. 5*I*). Taken together, these data demonstrate that the lncRNA *NEAT1* plays a critical role for SM phenotypic switching in vitro and in vivo.

***NEAT1* Induces an Inactive Chromatin State Within SM-Specific Gene Promoters by Binding to the Epigenetic Activator WDR5.** Data described above demonstrated a function of *NEAT1* in SM-contractile gene expression in VSMCs; therefore, we next sought to determine the mechanism by which *NEAT1* represses SM-specific gene expression. We first knocked down *NEAT1* in HCASMCs, and ChIP assays were performed to examine the chromatin status by using a variety of antibodies against modified histones, including transcriptionally active marks H3K4me3, H3K9ac and the repressive mark H3K27me3. Since SRF binding to the CArG boxes within SM-specific gene promoters is the prerequisite to induce expression of these genes, we also evaluated SRF binding to CArG box regions within SM-specific gene promoters by using anti-SRF antibody. Data from these ChIP assays revealed that depletion of *NEAT1* significantly promoted the enrichment of active histone modifications (H3K4me3 and H3K9ac) while decreasing the enrichment of inactive modification (H3K4me3) within CArG regions of SM-specific genes, calponin, and SM22α, resulting in increased binding of SRF to the CArG box regions of these genes (Fig. 6 *A* and *B*). To further dissect the mechanism by which *NEAT1* could affect these histone modifications, we next sought to determine which factors bind to *NEAT1* in VSMCs. Biotin RNA pull-down assays using nuclear protein extracted from PDGF-BB–treated or nontreated HCASMCs revealed that *NEAT1* lncRNA bound WDR5 (12, 13, 43), a critical adaptor protein within MLL (Mixed Lineage Leukemia) H3K4 methylase complexes that previously has been shown to activate SM-specific gene expression (14, 44), and that this binding increased in PDGF-BB–treated cells (Fig. 6*C*). In contrast, SRF was absent from the *NEAT1* RNA/protein complex (Fig. 6*C*) and was not colocalized with *NEAT1*, as shown by

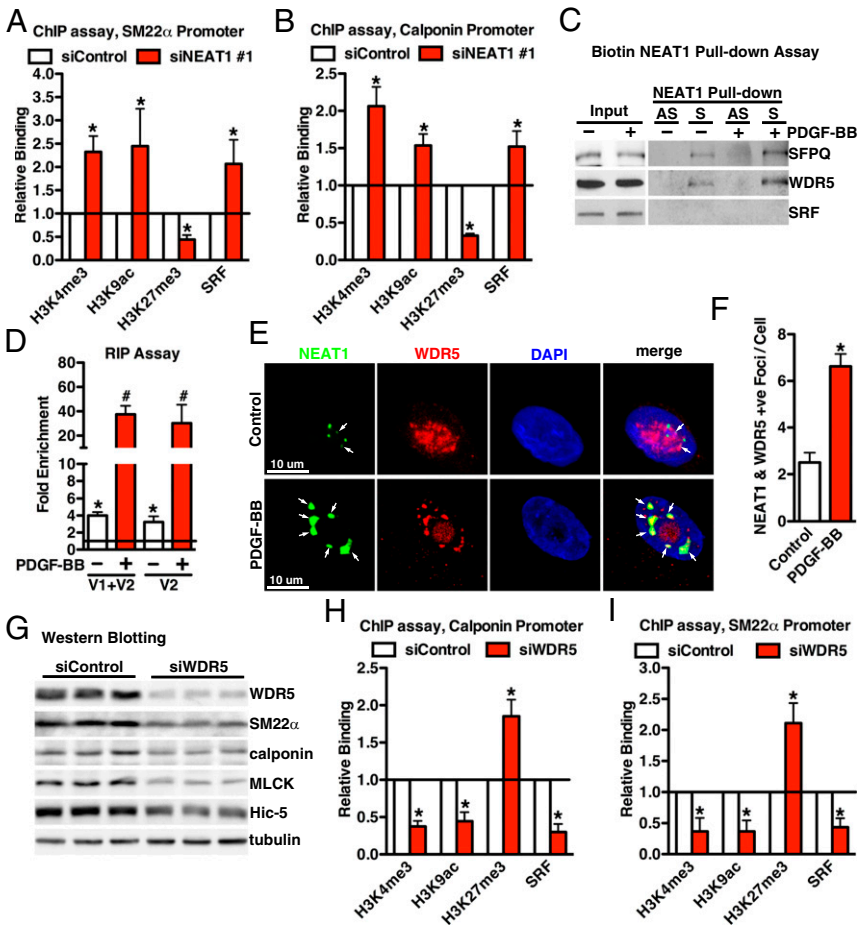

Fig. 6. *NEAT1* functions through binding to WDR5 in VSMCs. Control or *NEAT1* silencing duplex #1 was transfected into HCASMCs, and chromatin was harvested for ChIP using the antibodies as indicated to determine the histone modifications or SRF binding to the CArG regions within (*A*) SM22α or (*B*) calponin gene regulatory regions; $n = 3$. $^*P < 0.05$. (*C*) Biotin labeled sense (S) or antisense (AS) human *NEAT1* was incubated with the nuclear protein from HCASMCs treated with or without PDGF-BB (25 ng/mL, 24 h), and Western blotting was performed to examine the proteins binding to *NEAT1* by the indicated antibodies. (*D*) RIP assays were performed to determine the association between *NEAT1* and WDR5 with or without PDGF-BB treatment (25 ng/mL, 24 h). Primers designed for *NEAT1* V1 or V2 were utilized to measure the relative *NEAT1* binding to WDR5. Samples immunoprecipitated with IgG served as control (set to 1). (*E*) RNA-FISH/IF was performed to determine the colocalization of *NEAT1* (green) and WDR5 (red) with or without PDGF-BB treatment (25 ng/mL, 24 h). Arrows point to the colocalized *NEAT1* and WDR5. (*F*) Quantification of the colocalized *NEAT1* and WDR5 foci each cell as shown in *D*; $n = 40$ cells in each group. $^*P < 0.05$. (*G*) Western blot was performed to assess SM-specific gene expression after knockdown of WDR5 in HCASMCs for 48 h. (*H*) ChIP assays were performed as described in *A* and *B*, except HCASMCs were transfected with silencing duplexes against WDR5 to determine epigenetic marks or SRF binding within calponin or SM22α gene (*I*); $n = 3$. $^*P < 0.05$.

RNA-FISH/IF assay (*SI Appendix*, Fig. S4). Data from RNA immunoprecipitation (RIP) assays further demonstrated that *NEAT1* was associated with WDR5 at basal level, but also the binding was significantly enhanced by treatment with PDGF-BB (Fig. 6*D*). Consistently, RNA-FISH/IF revealed that PDGF-BB treatment not only induced *NEAT1* expression but promoted colocalization of *NEAT1* and WDR5 (Fig. 6 *E* and *F*). Since PDGF-BB can promote *NEAT1* binding with WDR5, we next directly assessed WDR5 function in VSMCs by knocking down endogenous WDR5 in HCASMCs. Protein lysate was harvested for Western blot 48 h after transfection of silencing RNA duplex against WDR5. Data from these experiments demonstrated that knockdown of WDR5 significantly decreased endogenous SM-specific protein expression (Fig. 6*G*). This effect is most likely due to the condensed chromatin configuration induced by depletion of WDR5 as shown by the decreased enrichment of active histone modifications (H3K4me3 and H3K9ac) and SRF binding along with enhanced inactive modification (H3K4me3) within CArG regions of SM-specific genes, calponin, and SM22α (Fig. 6 *H* and *I*). Taken together, these data suggest that *NEAT1* functions through binding with WDR5 to repress SM-specific gene expression in VSMCs.

In summary, our data suggest that, in contractile VSMCs, *NEAT1* is expressed at a low level. Under these conditions, the WDR5 component of the MLL histone methytransferase complex is free to facilitate an "open" configuration chromatin at the promoters of SM-specific genes, thereby promoting SRF binding and transcriptional activation (Fig. 7*A*). In contrast, in response to the stimuli that promote VSMC dedifferentiation and proliferation such as PDGF-BB, induced expression of lncRNA *NEAT1* sequestrates WDR5 from SM-specific gene loci, thereby initiating an epigenetic "off" state and consequently impairing SRF accessibility to the CArG boxes, resulting in down-regulation of SM-specific gene expression (Fig. 7*B*).

Discussion

This study provides evidence demonstrating a critical role for the paraspeckle-specific lncRNA *NEAT1* in promoting VSMC proliferation, migration, and dedifferentiation during phenotypic switching. Paraspeckles are mammal-specific nuclear bodies that can be found in most cells cultured in vitro (45). *NEAT1* is essential for the architectural integrity of paraspeckle by binding to paraspeckle proteins such as SFPQ (22, 24). Our current study

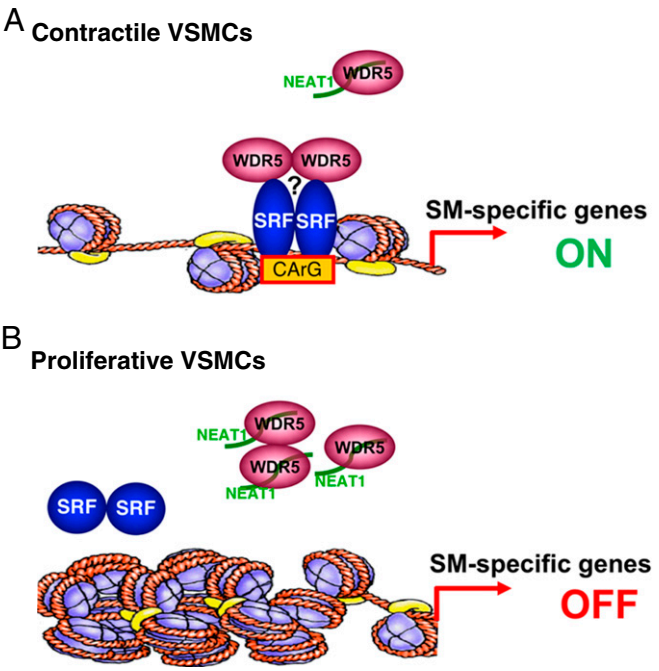

Fig. 7. Schematic summary of this study. (*A*) In contractile VSMCs, *NEAT1* is expressed at a low level, freeing the histone modifier WDR5 to trimethylate histone H3K4 at the promoters of genes encoding SM contractile proteins, thereby opening the chromatin at these loci, facilitating SRF binding and transcriptional activation. WDR5 may possibly function through other cofactors to modulate SM contractile gene expression, as indicated by a question mark. (*B*) In contrast, in response to vascular injury or mitogens such as PDGF-BB, the increased expression of lncRNA *NEAT1* sequestrates WDR5 from MLL complexes at the promoters of genes encoding SM-specific contractile proteins, initiating an epigenetic off state and consequently impairing SRF accessibility to the CArG boxes, resulting in down-regulation of SM-specific gene expression.

revealed an unexpected role of *NEAT1* in SM phenotypic switching independent of its paraspeckle binding protein SFPQ (*SI Appendix*, Fig. S3). We found that expression of *NEAT1* is induced during phenotypic modulation in VSMCs in vitro and in vivo (Figs. 1 and 2). Loss- and gain-of-function *NEAT1* assays revealed that *NEAT1* not only promotes proliferation and migration of VSMCs but also decreases the expression of SM-specific contractile proteins (Figs. 3–5). In contrast to the recent study which showed that the paraspeckle protein SFPQ mediates *NEAT1* ability to regulate IL-8 expression in response to viral infection (28), our data show that deletion of *NEAT1* binding protein SFPQ has no effects on SM-contractile protein gene expression (*SI Appendix*, Fig. S3). It is unclear whether the effects of *NEAT1* on SM-specific gene expression are dependent upon its function for the paraspeckle formation. Interestingly, a previous study showed that NEAT2, a neighboring lncRNA to *NEAT1*, is highly expressed in endothelial cells and is critical for angiogenesis (46). Furthermore, knockdown of NEAT2 in VSMCs has been shown to attenuate VSMC proliferation and migration (47), similar to our findings for *NEAT1*. Together with the recent report demonstrating that knockdown of *NEAT1* in endothelial cells impaired the integrity and increased the permeability of the blood–tumor barrier (48), it appears that lncRNA expression from the clustered *NEAT1* and NEAT2 loci is important for both endothelial cells and VSMC phenotype.

Our mechanistic studies demonstrated that, in response to PDGF-BB, a potent mitogen promoting SM phenotypic modulation (39), *NEAT1* bound the key chromatin modifier WDR5 and led to decreased histone H3K4me3 and H3K9ac at the promoters of SM-specific genes, thereby initiating an epigenetic off state and resulting in down-regulation of SM-specific gene expression (Figs. 6 and 7). Our data also suggested that silencing of *NEAT1*-mediated SM gene expression occurs through promoting SRF binding to CArG boxes, not through its expression level (Figs. 3*D* and 6 *A* and *B*). A recent study showed that the SWI/SNF chromatin-remodeling complexes were identified as paraspeckle components that interact with *NEAT1* (49). Our previous studies demonstrated that Brahma (Brm) or Brahma-like gene 1 (Brg1), the ATPase subunits of the SWI/SNF ATP-dependent chromatin remodeling complex, is required for the expression of SM-specific genes by promoting SRF/myocardin complex binding to the promoters of these genes through facilitating an open chromatin state within these gene loci (50). Interestingly, Capture Hybridization Analysis of RNA Targets - Mass Spectrometry data from the previous report suggested an association between *NEAT1*, BRG1, and WDR5 in prostate cancer cells (29). Therefore, it is possible that sequestration of Brg1 by *NEAT1* in paraspeckles is an alternative mechanism accounting for the regulation of SM-specific gene mediated by *NEAT1* in VSMCs. Furthermore, our RNA-seq data demonstrated that silencing *NEAT1* in HCASMCs significantly augmented expression of myocardin mRNA (Fig. 3 *C* and *D*). Myocardin is a powerful SRF cofactor that is sufficient to promote SM-specific gene expression in multiple cells (40, 51). Interestingly, overexpression of myocardin facilitated an open status of chromatin in SM-specific gene loci to allow SRF binding and an active transcription of these genes (40), similar to the results after silencing *NEAT1* in VSMCs (Fig. 6 *A* and *B*). Although we did not have the direct evidence to show that myocardin bind to *NEAT1* in vitro, it is possible that *NEAT1* can recruit myocardin through other bridging factors in vivo, thereby displacing myocardin from the SM gene. Therefore, it is likely that the augmentation of SM-specific gene expression induced by silencing *NEAT1* results from a combination of myocardin induction and/or liberation of WDR5, Brg1, and myocardin from *NEAT1* complexes in VSMCs.

It has been documented that, in many solid tumors, including those found in prostate cancer, lung cancer, esophageal cancer, colorectal cancer, and hepatocellular carcinoma, the paraspeckle lncRNA *NEAT1* is overexpressed and *NEAT1* promotes tumor cell proliferation (52). In the current study, we found that *NEAT1* was also induced following vascular injury. Consistent with the function of *NEAT1* in promoting cancer cell proliferation, we found overexpression of *NEAT1* enhanced VSMC proliferation, while knockdown or KO of *NEAT1* attenuated VSMC proliferation and migration, thereby diminishing neointima formation after vascular injury (Figs. 4 and 5). GO analysis of mRNAs whose expressions were altered following knockdown of *NEAT1* revealed that *NEAT1* affects expression of many genes related to cell cycle and the Hippo-YAP pathway in VSMCs (Fig. 3*B*) (32), suggesting that *NEAT1*-mediated regulation of VSMC proliferation likely occurs through multiple mechanisms, including modulating of Hippo pathway activity and expression of cell cycle genes. Interestingly, we found that *NEAT1* expression was induced in adventitial cells after vascular injury (Fig. 1*B* and *SI Appendix*, Fig. S1). Since there is emerging evidence suggesting adventitial cells may be attributed to neointima formation in addition to media layer VSMCs (53), future studies are needed to investigate the potential role of *NEAT1* in adventitial cells.

Activation of hypoxia-inducible factor (HIF) is common in many types of solid tumors. A recent study demonstrated that *NEAT1* was a direct transcriptional target of HIF in many breast cancer cell lines and in solid tumors, thereby conferring HIF-dependent gene regulation and eventually leading to accelerated cellular proliferation (30). In addition, *NEAT1* was identified as a direct transcriptional target of ERα (estrogen receptor alpha) and as an important mediator for maintenance of prostate cancer progression (29). Furthermore, the transcription factor C/EBPβ was critical for *NEAT1* expression during acute promyelocytic leukemia cell differentiation induced by all-trans retinoic acid (54). In this study, we found that *NEAT1* was significantly induced

during SM phenotypic modulation in vivo and in vitro (Figs. 1 and 2). It will be important to investigate the underlying mechanisms by which *NEAT1* is induced in response to vascular injury and in response to mitogens such as PDGF-BB.

In summary, this study has revealed an unexpected role of *NEAT1* in promoting VSMC phenotypic modulation that is mediated, at least in part, through *NEAT1*'s regulation of MLL histone methyltransferase complexes. These findings suggest that *NEAT1* is a potential therapeutic target for treating occlusive vascular diseases.

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