Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Suv39h1 Regulates Phenotypic Modulation of Smooth Muscle Cells and Contributes to Vascular Injury by Repressing HIC1 Transcription

Yuyu Yang,* Qiumei Zhang,* Shuai Liu,* Haihang Yuan, Xiaoping Wu, Yi Zou, Yuanyuan Zhang, Junli Guo

BACKGROUND: Vascular smooth muscle cells (VSMCs), in response to a myriad of injurious stimuli, switch from a contractile state to a proliferative/migratory state in a process known as phenotypic modulation. Phenotypic modulation of VSMCs contributes to neointima formation and underscores a host of vascular pathologies, including atherosclerosis. In the present study, we investigated the involvement of Suv39h1 (suppressor of variegation 3-9 homolog 1), a lysine methyltransferase, in this process.

METHODS: *Suv39h1*$^{f/f}$ mice were crossbred to the *Myh11*-Cre^{ERT2} mice to generate VSMC-restricted Suv39h1 knockout mice (conditional knockout). Vascular injury was created by carotid artery ligation. Cellular transcriptome was evaluated by RNA sequencing and cleavage under targets and tagmentation with deep sequencing.

RESULTS: Suv39h1 upregulation was observed in animal and cell models of phenotypic modulation. Consistently, Suv39h1 silencing restored expression of contractile genes and attenuated proliferation/migration in VSMCs exposed to PDGF (platelet-derived growth factor)-BB. Importantly, Suv39h1 deletion significantly ameliorated neointima formation in mice in both the carotid artery injury model and the femoral artery injury model. Importantly, a small-molecule Suv39h1 inhibitor F5446 suppressed phenotypic modulation in vitro and mitigated vascular injury in mice. RNA sequencing identified HIC1 (hypermethylated in cancer 1) as a novel target for Suv39h1. HIC1 expression was repressed by Suv39h1 during VSMC phenotypic modulation, whereas HIC1 overexpression antagonized neointima formation in mice. Integrated transcriptomic analysis indicated that HIC1 might regulate VSMC phenotypic modulation by activating Jag1 (Jagged 1) transcription.

CONCLUSIONS: Our data suggest that Suv39h1 is a novel regulator of vascular injury and can be targeted for intervention of restenosis.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ cell proliferation ■ epigenomics ■ neoplasms ■ vascular smooth muscle cell

Cardiovascular disease is the leading cause of death worldwide. Despite advances in therapeutics and disease management in the era of precision medicine, cardiovascular disease–associated mortality, for which coronary artery disease (CAD) is blamed the most for, is projected to increase owing largely to an aging population and high prevalence of metabolic disorders.[1] Percutaneous coronary intervention, a pioneering technique that delivers symptom relief to patients with CAD, is considered a safe and effective alternative to coronary artery bypass grafting surgery. However, a quarter of patients with CAD undergoing percutaneous coronary intervention remain symptomatic.[2] Further, intimal hyperplasia (restenosis), a recurring complication following the

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Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.124.322048.
For Sources of Funding and Disclosures, see page 977.

Arterioscler Thromb Vasc Biol is available at www.ahajournals.org/journal/atvb

Nonstandard Abbreviations and Acronyms

α-SMA	α-smooth muscle actin
Ang II	angiotensin II
CAD	coronary artery disease
H3K9	H3 lysine residue 9
HIC1	hypermethylated in cancer 1
Jag1	Jagged 1
MRTF-A	myocardin-related transcription factor A
NF-κB	nuclear factor-κB
PDGF	platelet-derived growth factor
RNA-seq	RNA sequencing
SM22α	smooth muscle protein 22α
Suv39h1	suppressor of variegation 3-9 homolog 1
TGF-β	transforming growth factor-β
VSMC	vascular smooth muscle cell

Highlights

- Suv39h1 (suppressor of variegation 3-9 homolog 1) expression is elevated during vascular smooth muscle cell phenotypic switch.
- Conditional deletion of Suv39h1 in vascular smooth muscle cells attenuates neointima formation.
- Pharmaceutical inhibition of Suv39h1 attenuates neointima formation.
- Suv39h1 represses HIC1 (hypermethylated in cancer 1) transcription to promote vascular smooth muscle cell phenotypic switch.
- HIC1 regulates Jag1 (Jagged 1) transcription to suppress vascular smooth muscle cell phenotypic switch.

procedure, severely limits the effectiveness of percutaneous coronary intervention therapy and often necessitates repeated intervention.[3] The introduction of bare metal stent and later drug-eluting stent has significantly reduced but not completely abolished the incidence of restenosis. Common risk factors identified for restenosis include sex, senility, smoking, diabetes, and hypertension.[4] The cellular and molecular mechanism underlying restenosis, however, is not fully delineated.

Decades of vigorous investigations have led to the finding that restenosis is regulated by a complex interplay between different cell types and humoral factors. Under physiological conditions, quiescent vascular smooth muscle cells (VSMCs) assume a contractile phenotype characterized by rhythmic constriction and dilation. In response to elevated levels of inflammatory cytokines and PDGF (platelet-derived growth factor) following endothelial denudation, VSMCs can switch to a migraproliferative phenotype to encroach the intimal layer and become what is known as the neointima.[5] According to the theory of phenotypic modulation, accepted by most in the field as the pathophysiological basis for restenosis, VSMCs undergo profound transcriptomic overhaul paralleling behavioral alterations.[6] For instance, it is observed that contractile genes associated with the contractile phenotype, such as *Acta2* (encoding α-SMA [α-smooth muscle actin]) and *Cnn1* (encoding calponin), are downregulated, whereas proliferative markers, such as *Pcna* (encoding PCNA), are upregulated during VSMC phenotypic modulation.[7] These transcriptomic changes are programmed by cue-responsive transcription factors and the epigenetic machinery, which includes DNA/histone modifying enzymes, chromatin remodeling proteins, and noncoding RNAs.[8]

A consensus in epigenetic regulation of gene expression is that methylation and acetylation at histone H3 lysine residue 9 (H3K9) are exclusive to each other, whereas H3K9 acetylation is compatible with an open chromatin structure, and hence active transcription H3K9 methylation is associated with condensed chromatin and considered a marker for transcription repression.[9] H3K9 methylation status is dynamically regulated by a pair of dueling enzymes, a methyltransferase and a demethylase.[10] A previous study by the Mack laboratory has shown that the H3K9 demethylase Jmjd1a (also known as Kdm3a) directly binds to the promoters of contractile genes (eg, *Acta2*) and removes the repressive H3K9 to enable transcription by MRTF-A (myocardin-related transcription factor A), thus playing an essential role in VSMC differentiation from mesenchymal stem cells and, presumably, sustaining the contractile phenotype during restenosis.[11] In the present study, we investigated the role of Suv39h1 (suppressor of variegation 3-9 homolog 1), an H3K9 methyltransferase, in VSMC phenotypic modulation. Previous studies have provided evidence that implicates Suv39h1 as an important regulator of VSMC phenotype in different disease settings. For instance, Villeneuve et al[12,13] have reported that Suv39h1 attenuates the transition of VSMCs to a proinflammatory phenotype in response to hyperglycemia. Consistently, global Suv39h1 knockout mice have been shown to be more resistant to diabetes-associated vascular oxidative stress.[14] On the contrary, adenovirus-mediated Suv39h1 overexpression or depletion appears to influence neointima formation in both diabetic rats[15] and in C57/B6j mice exposed to chronic infusion with Ang II (angiotensin II).[16] Because a causal relationship between VSMC-autonomous Suv39h1, VSMC phenotypic switch, and neointima formation remains to be established, we sought to clarify this issue with a new mouse strain harboring VSMC conditional Suv39h1 deletion. Our data suggest that genetic deletion or pharmaceutical inhibition of Suv39h1 attenuates VSMC phenotypic switch and mitigates restenosis.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. The study materials will only be made available if sufficient material can be provided to other researchers for purposes of reproducing the results or replicating the procedure.

Animals

All animal protocols were reviewed and approved by the Intramural Committee on Ethical Treatment of Experimental Animals (approval No. IACUC-2109008-1). All animal procedures conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the National Institutes of Health guidelines. *Suv39h1*$^{f/f}$ mice[17] and *Myh11*-Cre^{ERT2} mice[18] have been previously described. The mice were kept under specific pathogen-free conditions and maintained at a standard temperature and humidity. To induce Suv39h1 deletion, the mice were intraperitoneally injected with tamoxifen (Sigma-Aldrich; T5648; 50 mg/kg) for 5 consecutive days. Vascular injury was induced in 12-week-old male mice by carotid artery ligation as previously described.[19] Only male mice were used because it is generally agreed in the field that female mice are less susceptible to lesion development following injuries. In certain experiments, the mice were injected intraperitoneally with F5446 (MedChemExpress; HY-150190, 5 mg/kg) thrice a week following the surgery. In certain experiments, murine HIC1 (hypermethylated in cancer 1) cDNA or short hairpin RNA targeting HIC1 (GCCUGGAUCUGUCCAAGAATT) was cloned into the ssAAV-SM22-CMV vector and injected via tail vein (1×10^{11} vg) before the surgery.

Cell Culture and Treatment

Primary VSMCs were isolated from 3- to 5-week C57/B6 mice (male and female) and maintained in DMEM supplemented with 15% fetal bovine serum as previously described.[20] Human primary aortic smooth muscle cells were purchased from Lonza and maintained in cultured media supplied by the vendor. Typically, cells with passages between 3 and 6 were used. Small interfering RNAs were purchased from GenePharma. Cells were harvested 24 to 48 hours after the transfection. Transient transfections were performed with Lipofectamine RNAiMax.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were performed essentially as described before.[21–23] In brief, chromatin in control and treated cells was cross-linked with 1% formaldehyde. Cells were incubated in lysis buffer (150 mmol/L NaCl, 25 mmol/L Tris pH 7.5, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) supplemented with protease inhibitor tablet and PMSF (phenylmethylsulfonyl fluoride). DNA was fragmented into ≈200 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-FLAG (Sigma; F1804) or preimmune IgG.

RNA Isolation and Real-Time Polymerase Chain Reaction

RNA was extracted with the RNeasy RNA isolation kit (Qiagen) as previously described.[24] Reverse transcriptase reactions were performed using a SuperScript First-Strand Synthesis System (Invitrogen) as previously described.[25] Real-time polymerase chain reactions were performed on an ABI Prism 7500 system with the following primers: human *SUV39H1*, 5′-CCTGCCCTCGGTATCTCTAAG-3′ and 5′-ATATCCACGCCATTTCACCAG-3′; mouse *Suv39h1*, 5′-CTGTGCCGACTAGCCAAGC-3′ and 5′-ATACCCACGCCACTTAACCAG-3′; human *SUV39H2*, 5′-TCTATGACAACAAGGGAATCACG-3′ and 5′-GAGACACATTGCCGTATCGAG-3′; mouse *Suv39h2*, 5′-CTGCCCAGGATAGCATTGTTC-3′ and 5′-CAAGTCTCGGCTCCACATTTAC-3′; human *TAGLN*, 5′-AGTGCAGTCCAAAATCGAGAAG-3′ and 5′-CTTGCTCAGAATCACGCCAT-3′; mouse *Tagln*, 5′-CCAACAAGGGTCCATCCTACG-3′ and 5′-ATCTGGGCGGCCTACATCA-3′; human *CNN1*, 5′-CTGTCAGCCGAGGTTAAGAAC-3′ and 5′-GAGGCCGTCCATGAAGTTGTT-3′; mouse *Cnn1*, 5′-TCTGCACATTTTAACCGAGGTC-3′ and 5′-GCCAGCTTGTTCTTTACTTCAGC-3′; human *MYH11*, 5′-CGCCAAGAGACTCGTCTGG-3′ and 5′-TCTTTCCCAACCGTGACCTTC-3′; mouse *Myh11*, 5′-ATGAGGTGGTCGTGGAGTTG-3′ and 5′-GCCTGAGAAGTATCGCTCCC-3′; mouse *Hic1*, 5′-AACCTGCTAAACCTGGACCAT-3′ and 5′-CCACGAGGTCAGGGATCTG-3′; mouse *Jag1* (Jagged 1), 5′-ATGCAGAACGTGAATGGAGAG-3′ and 5′-GCGGGACTGATACTCCTTGAG-3′. Ct values of target genes were normalized to the Ct values of housekeekping control gene (18s, 5′-CGCGGTTCTATTTTGTTGGT-3′ and 5′-TCGTCTTCGAAACTCCGACT-3′ for both human and mouse genes) using the ddCt method and expressed as relative mRNA expression levels compared with the control group, which is arbitrarily set as 1 as previously described.[26]

Statistical Analysis

For comparison between 2 groups, 2-tailed *t* test was performed. For comparison among ≥3 groups, 1-way ANOVA or 2-way ANOVA with post hoc Scheffé analyses was performed by the SPSS software (IBM SPSS v18.0, Chicago, IL). The assumptions of normality were checked using the Shapiro-Wilk test, and equal variance was checked using Levene test; both were satisfied.

RESULTS

Upregulation of Suv39h1 Accompanies Phenotypic Modulation in VSMCs

To demonstrate a correlation between Suv39h1 and VSMC phenotypic modulation, the following experiments were performed. C57B/6j mice were subjected to carotid artery ligation and euthanized 3 weeks after the surgery. An upregulation of Suv39h1 expression was observed in the ligated (injured) arteries compared with the contralateral (uninjured) arteries accompanying a downregulation of SM22α (smooth muscle protein 22α; *Tagln*), a contractile marker; no significant alteration in Suv39h2 expression was detected (Figure 1A and 1B). Aortic arteries isolated from the atherosclerotic mice (*Apoe*$^{-/-}$ mice fed a Western diet for 12 weeks) displayed higher Suv39h1 expression than those isolated from the control mice and lower SM22α expression (Figure 1C and 1D).

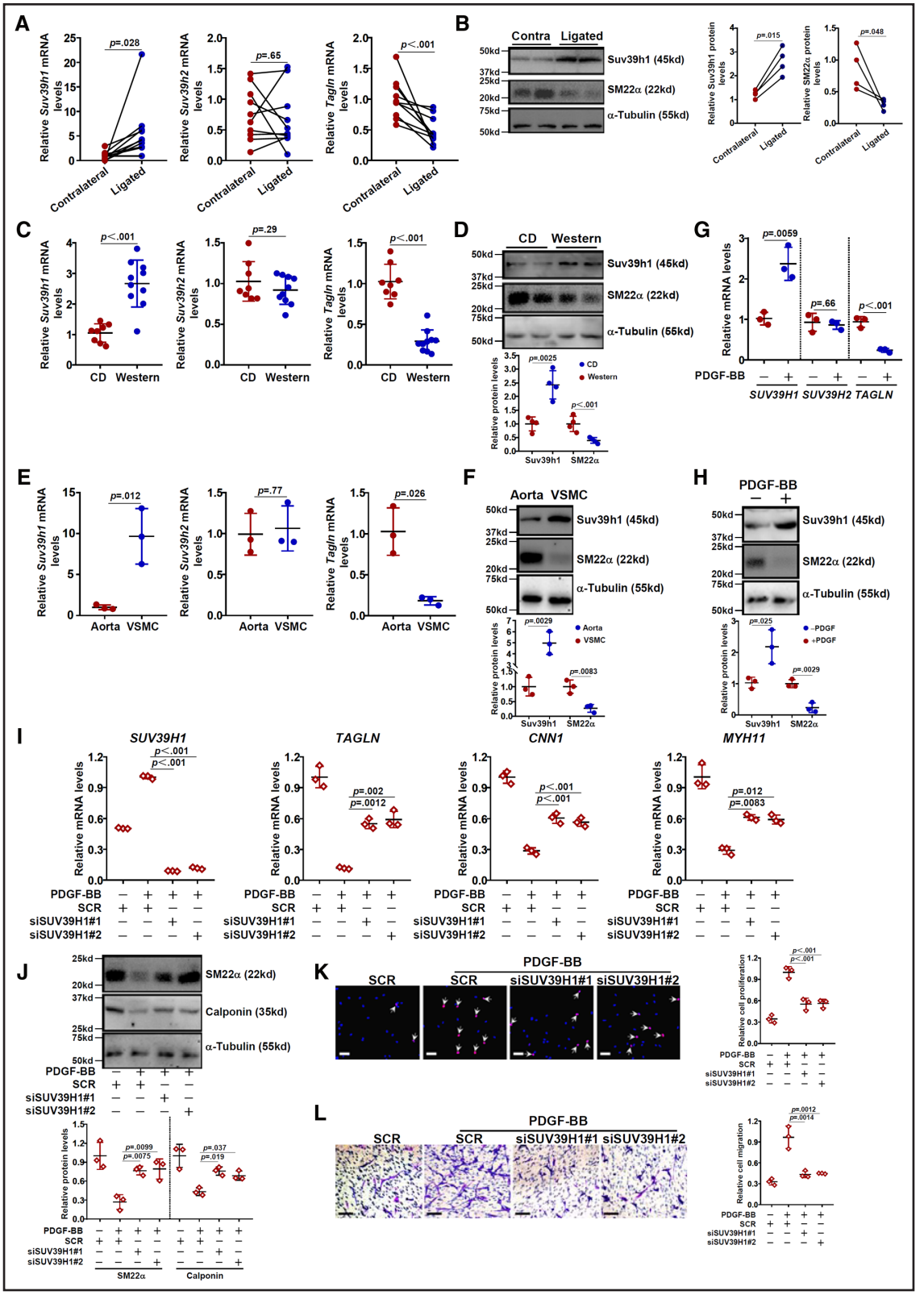

Figure 1. Upregulation of Suv39h1 (suppressor of variegation 3-9 homolog 1) accompanies phenotypic modulation in vascular smooth muscle cells (VSMCs).

A and **B**, C57/B6 mice were subjected to carotid artery ligation or the sham procedure as described in Materials and Methods. Suv39h1 expression in the injured and the contralateral arteries was examined by qPCR and Western blotting. n=4 to 10 mice for each group, paired 2-tailed Student test. **C** and **D**, *Apoe*$^{-/-}$ mice were fed a Western diet or a control diet (CD) for 12 weeks. Suv39h1 expression in the aortas was examined by qPCR and Western blotting. n=4 to 8 mice for the CD group and n=4 to 10 mice for the WD group, unpaired 2-tailed Student test. **E** and **F**, Suv39h1 expression in freshly dissected rat aortas and in cultured rat primary aortic SMCs was examined (*Continued*)

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Similarly, cultured VSMCs expressed higher levels of Suv39h1 and lower levels of SM22α than nascent vessels (Figure 1E and 1F). Finally, exposure of VSMCs to PDGF-BB led to significant upregulation of Suv39h1 and downregulation of SM22α (Figure 1G and 1H).

To demonstrate a causative relationship between Suv39h1 and VSMC phenotypic modulation, 2 independent pairs of siRNAs targeting Suv39h1 were transfected into human primary VSMCs. Suv39h1 knockdown partially reversed downregulation of contractile genes (Figure 1I). Suv39h1 silencing also suppressed cell proliferation (Figure 1J) and cell migration (Figure 1K). Likewise, in primary VSMCs isolated from the *Suv39h1*$^{f/f}$ mice, Cre-mediated Suv39h1 deletion simultaneously normalized contractile gene expression and cellular migraproliferative behaviors (Figure S1).

Suv39h1 Deletion Attenuates Neointima Formation in Mice

To further establish a role for VSMC-specific Suv39h1 in neointima formation, the *Suv39h1*$^{f/f}$ mice were crossbred with the *Myh11*-Cre^{ERT2} mice to generate conditional Suv39h1 knockout mice (*Suv39h1*$^{\Delta SMC}$, conditional knockout [CKO]). Both the wild-type mice and the CKO mice were subjected to carotid artery ligation and euthanized for analysis 4 weeks after the surgery (Figure 2A). H&E staining indicated that intimal expansion was significantly attenuated in the CKO mice than in the WT mice with a reduction in the neointima/media ratio (Figure 2C) and relative neointima area (Figure 2D). Ki67 immunohistochemical staining confirmed that reduction in intimal expansion in the CKO mice could be attributed to weakened VSMC proliferation (Figure 2E). On the contrary, contractile gene expression, as measured by α-SMA immunofluorescence staining, was better preserved in the CKO mice than in the WT mice (Figure 2F). A similar observation was made in an alternative model wherein the mice were subjected to wire injury to the femoral artery: Suv39h1 deletion in VSMCs markedly ameliorated neointima formation (Figure S2).

Pharmaceutical Inhibition of Suv39h1 Attenuates Phenotypic Switch of VSMCs In Vitro and Neointima Formation in Mice

F5446 was a recently identified small-molecule Suv39h1 inhibitor.[27] Treatment with F5446 led to a decrease in trimethylated H3K9 levels in primary murine VSMCs; based on the dose response, 50 and 100 nmol/L were chosen for the remainder of the study (Figure S3A). Cotreatment with F5446 alleviated repression of contractile genes by PDGF-BB in a dose-response manner (Figure 3A). F5446 treatment also dose-dependently weakened cell proliferative (Figure 3B) and migratory (Figure 3C) capabilities. To test whether Suv39h1 inhibition might influence neointima formation in vivo, C57B/6j mice were subjected to carotid artery ligation followed by F5446 administration (Figure 3D). As expected, F5446 decreased trimethylated H3K9 levels in the vessels (Figure S4). F5446 administration significantly alleviated intimal hyperplasia as evidenced by H&E staining (Figure 3E), calculated neointma/media ratio (Figure 3F), and relative neointima area (Figure 3G). F5446 intervention also suppressed VSMC proliferation, as assessed by Ki67 staining (Figure 3H), and preserved contractile gene expression, as assessed by α-SMA staining (Figure 3I) and Western blotting (Figure 3J). It was also observed that F5446 administration downregulated the expression of several proinflammatory mediators in the vessels indicating that Suv39h1 might regulate vascular inflammation (Figure S5). Taken together, these data illustrate that similar to Suv39h1 deletion, Suv39h1 inhibition might afford vasculoprotective effects in mice.

HIC1 Is a Novel Target Downstream of Suv39h1

To determine the mechanism whereby Suv39h1 might contribute to phenotypic modulation of VSMCs, RNA sequencing (RNA-seq) was performed to compare the cellular transcriptome in VSMCs with or without Suv39h1. As shown in Figure 4A, Suv39h1 deletion led to significant alterations in VSMC transcriptome. Overall, more genes were upregulated than downregulated by Suv39h1 consistent with the notion that Suv39h1 is generally a repressor of transcription (Figure 4B). GO (gene ontology) analysis indicated that genes influenced by Suv39h1 deletion were mostly involved in migraproliferative and synthetic behaviors of VSMCs (Figure 4C). To narrow down the potential Suv39h1 targets that might contribute to the phenotypic switch of VSMCs, 2 previously published RNA-seq data sets, both performed with arteries isolated from mice subjected to vascular injury, were included in the screening; 6 genes, whose expression levels were downregulated during vascular injury and upregulated by Suv39h1 deletion, were identified with the transcription factor HIC1 ranked top of the list (Figure 4D).

Figure 1 Continued. by qPCR and Western blotting. n=3 biological replicates, unpaired 2-tailed Student test. **G** and **H**, Human primary aortic SMCs were treated with or without PDGF (platelet-derived growth factor)-BB (20 ng/mL) for 48 hours. Suv39h1 expression was examined by qPCR and Western blotting. n=3 biological replicates, unpaired 2-tailed Student test. Data are expressed as mean±SD. *P<0.05, 2-tailed Student *t* test. **I** through **K**, Human primary aortic SMCs were transfected with siRNA targeting Suv39h1 or scrambled siRNA (SCR) followed by treatment with PDGF-BB (20 ng/mL) for 48 hours. Contractile gene expression was examined by qPCR (**I**). Cell proliferation was examined by EdU incorporation (**J**). Cell migration was examined by transwell assay (**K**). n=3 biological replicates. Data are expressed as mean±SD, 2-way ANOVA with post hoc Scheffé. EdU indicates ethynyl-2' deoxyuridine; and qPCR, quantitative polymerase chain reaction.

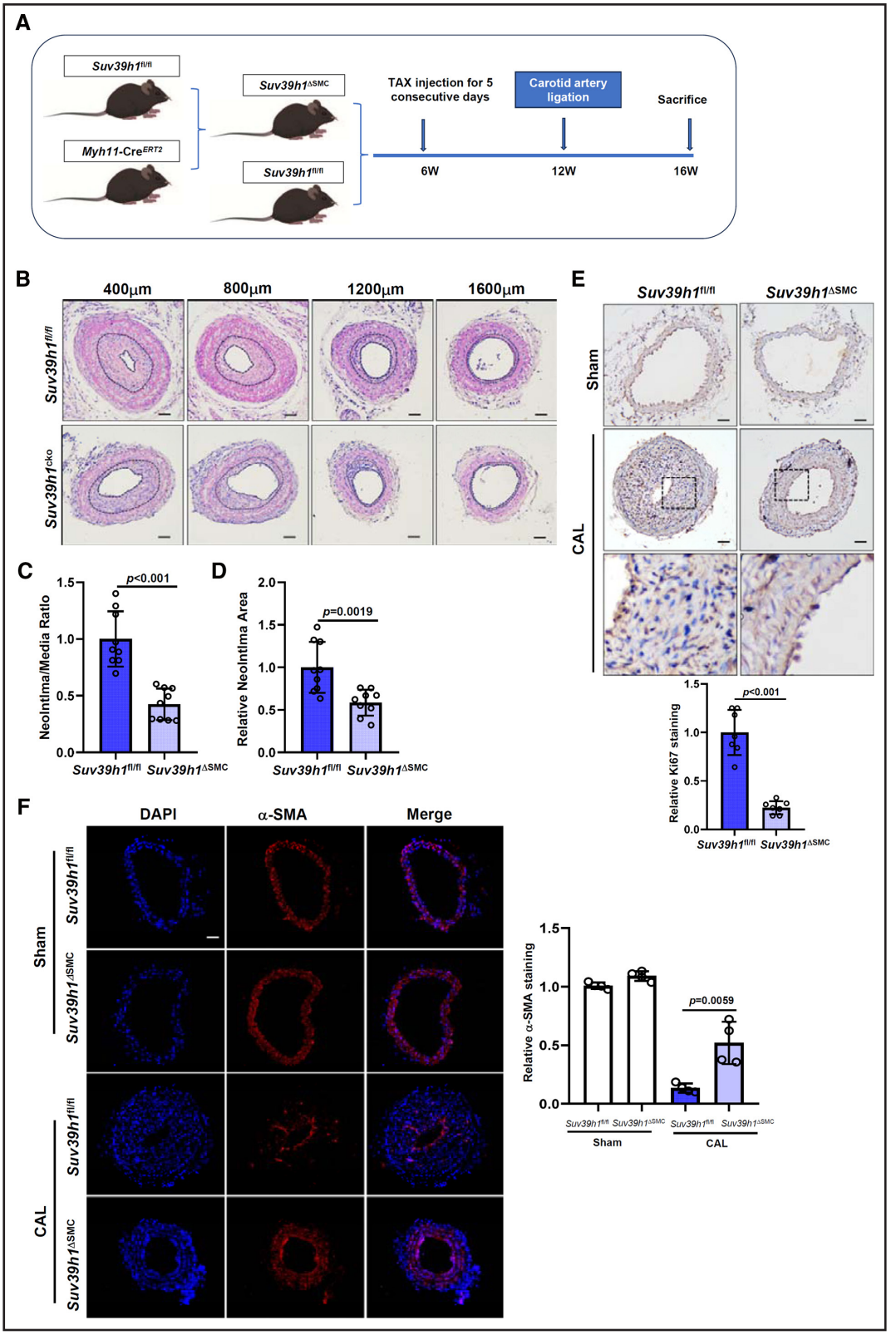

Figure 2. Suv39h1 (suppressor of variegation 3-9 homolog 1) deletion attenuates neointima formation in mice.
A through **F**, SMC conditional Suv39h1 knockout (CKO) mice and wild-type (WT) control mice were subjected to carotid artery ligation as described in Methods. **A**, Scheme of protocol. **B**, Representative H&E staining images of vessels. **C**, Neointima/media ratios. **D**, Relative medial areas. **E**, Ki67 staining. **F**, α-SMA (α-smooth muscle actin) staining. n=7 to 9 mice for each group. Data are expressed as mean±SD, unpaired 2-tailed Student test. CKO indicates conditional knockout; and H&E, hematoxylin and eosin.

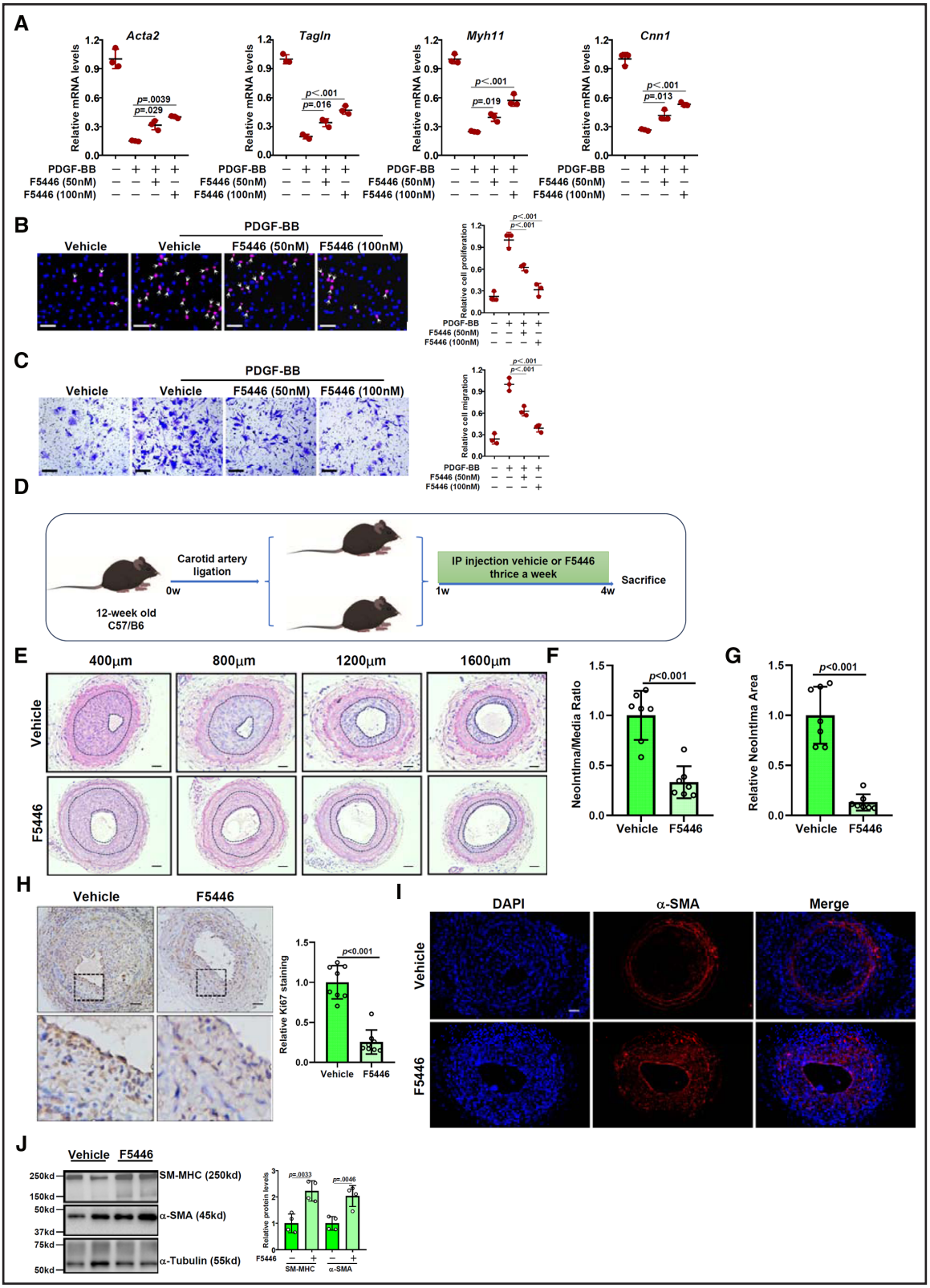

Figure 3. Pharmaceutical inhibition of Suv39h1 (suppressor of variegation 3-9 homolog 1) attenuates phenotypic switch of vascular smooth muscle cells (VSMCs) in vitro and neointima formation in mice.

A through **C**, Primary murine VSMCs were treated with PDGF (platelet-derived growth factor)-BB (20 ng/mL) in the presence or absence of F5446 for 48 hours. **A**, Contractile gene expression was examined by qPCR. **B**, Cell proliferation was examined by EdU incorporation. **C**, Cell migration was examined by transwell assay. n=3 biological replicates. Data are expressed as mean±SD, 2-way ANOVA with post hoc Scheffé. **D** through **J**, C57B6/j mice were subjected to carotid artery ligation followed by administration of F5446 (5 mg/kg). **D**, Scheme of (*Continued*)

qPCR data confirmed that HIC1 expression was downregulated in the injured arteries and in VSMCs exposed to PDGF-BB treatment (Figure S6). Suv39h1 deletion, however, rescued HIC1 from PDGF-BB–induced repression (Figure 4E and 4F). In addition, Suv39h1 inhibition by F5446 also normalized HIC1 expression (Figure S7). To further demonstrate a functional interplay between Suv39h1 and HIC1, HIC1 was depleted simultaneously with Suv39h1; codeletion of Suv39h1 and HIC1 offset the effect of Suv39h1 deficiency and allowed PDGF-BB to repress contractile gene expression (Figure 4G) and to provoke cell migraproliferative behaviors (Figure 4H and 4I). Next, short hairpin RNA targeting HIC1 was under the control of SM22α promoter and packaged into AAV9 for injection into the Suv39h1 CKO mice. Indeed, HIC1 deletion in the vessels of the Suv39h1 CKO mice normalized neointima formation (Figure S8). Similarly, HIC1 silencing overcame Suv39h1 inhibition by F5446 and enabled PDGF-BB to promote phenotypic switch of VSMCs (Figure S9).

HIC1 Regulates Phenotypic Switch of VSMCs In Vitro and Neointima Formation in Mice

The following experiments were designed to further demonstrate the relevance of HIC1 in phenotypic switch of VSMCs and neointima formation. HIC1 overexpression by adenoviral transduction modestly but significantly attenuated repression of contractile genes by PDGF-BB treatment (Figure 5A). HIC1 overexpression also dampened cell proliferation (Figure 5B) and cell migration (Figure 5C).

Next, a VSMC-specific HIC1 vector was designed by placing HIC1 cDNA under the control of SM22α promoter and packaged into AAV9 for tail vein injection (Figure 5D). qPCR data showed that HIC1 expression was significantly higher in the vessels from mice injected with AAV-HIC1 than AAV-empty vector (Figure S10). Consistent with the in vitro observations, HIC1 overexpression significantly mortified neointima formation as evidenced by H&E staining (Figure 5E), neointma/media ratio (Figure 5F), relative neointima area (Figure 5G), Ki67 staining of proliferating VSMCs (Figure 5H), and α-SMA staining (Figure 5I). These data combined illustrate an inhibitory role of HIC1 in VSMC phenotypic switch.

HIC1 Regulates Phenotypic Switch of VSMCs by Activating Jag1 Transcription

Next, RNA-seq and cleavage under targets and tagmentation with deep sequencing (CUT&Tag-seq) were performed to investigate the potential mechanism whereby HIC1 might regulate phenotypic switch of VSMCs. HIC1 overexpression led to marked transcriptomic overhaul in VSMCs with over 5000 genes being differentially expressed (Figure 6A and 6B). GO analysis indicated that the differentially expressed genes were predominantly represented by pathways that regulate cellular migraproliferative behaviors (Figure 6C). Due to the lack of a specific IP (immunoprecipitation) antibody for HIC1, VSMCs were transduced with adenoviral HIC1 with a FLAG tag followed by CUT&Tag-seq with anti-FLAG antibody. As shown in Figure 6D, ≈20% of the FLAG-HIC1 peaks were detected within the promoter regions (±3 kb relative to the transcription start site).

Using the following criteria, that is, (1) 2× fold change plus FPKM >5 per RNA-seq, (2) promoters occupied by HIC1 per CUT&Tag-seq, and (3) belonging to the top-rated GO pathway "cell migration," we were able to narrow potential HIC1 target genes down to 8, of which Jag1 ranked first (Figure 6E and 6F). qPCR (Figure 6G) and Western blotting (Figure 6H) verified that Jag1 expression was downregulated in VSMCs by PDGF-BB treatment but was reversed by HIC1 overexpression. Chromatin immunoprecipitation assay confirmed that HIC1 was associated with the Jag1 promoter in VSMCs (Figure 6I). Of note, higher Jag1 expression was detected in Suv39h1-deleted VSMCs and in the vessels from Suv39h1 CKO mice (Figure S11).

The relevance of the HIC1-Jag1 interplay in the phenotypic switch of VSMC was further examined by the following experiments. In VSMCs, HIC1 overexpression partially reversed the repression of contractile genes by PDGF-BB, but Jag1 knockdown completely abrogated the effect of HIC1 (Figure 6J). In addition, Jag1 silencing offset the prohibitive effects of HIC1 overexpression on cell proliferation (Figure 6K) and cell migration (Figure 6L). Likewise, Jag1 silencing was found to overcome Suv39h1 deficiency to promote VSMC phenotypic switch in vitro (Figure S12). Together, these data suggest that HIC1 might regulate phenotypic modulation of VSMC by, at least in part, regulating Jag1 transcription.

DISCUSSION

Phenotypic modulation of vascular modulation, to which the epigenetic machinery contributes, is the pathophysiological basis for restenosis. Here, we detail a role for Suv39h1, a lysine methyltransferase, in this process by providing evidence to show that targeting Suv39h1 genetically or pharmaceutically may yield novel therapeutic solutions for CAD intervention. Prior to our study,

Figure 3 Continued. protocol. **E**, Representative H&E staining images of vessels. **F**, Neointima/media ratios. **G**, Relative medial areas. **H**, Ki67 staining. **I**, α-SMA (α-smooth muscle actin) staining. **J**, Contractile gene expression was examined by Western blotting. n=8 mice for each group. Data are expressed as mean±SD, unpaired 2-tailed Student *t* test. EdU indicates ethynyl-2' deoxyuridine; H&E, hematoxylin and eosin; and qPCR, quantitative polymerase chain reaction.

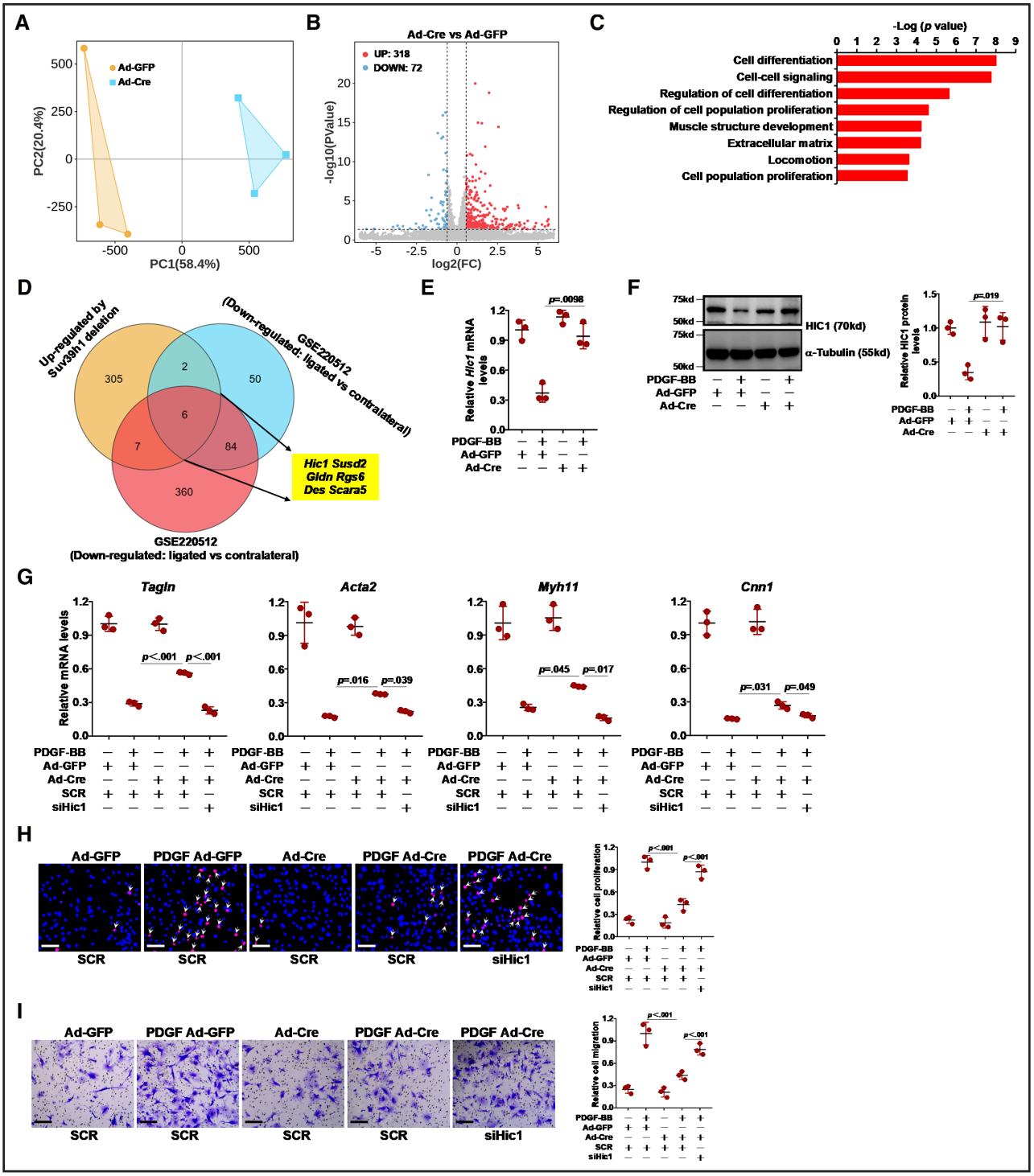

Figure 4. HIC1 (hypermethylated in cancer 1) is a novel target downstream of Suv39h1 (suppressor of variegation 3-9 homolog 1). **A** through **E**, Primary vascular smooth muscle cells (VSMCs) isolated from *Suv39h1*$^{f/f}$ mice were transduced with indicated adenovirus followed by treatment with PDGF (platelet-derived growth factor)-BB (20 ng/mL) for 48 hours. RNA sequencing was performed as described in Materials and Methods. **A**, PCA plot. **B**, Volcano plot. **C**, GO analysis. **D**, Venn diagram. **E** and **F**, Primary VSMCs isolated from *Suv39h1*$^{f/f}$ mice were transduced with indicated adenovirus followed by treatment with PDGF-BB (20 ng/mL) for 48 hours. HIC1 expression was examined by qPCR and Western blotting. **G** through **I**, Primary VSMCs isolated from *Suv39h1*$^{f/f}$ mice were transduced with indicated adenovirus and transfected with siRNA targeting HIC1 or scrambled siRNA (SCR) followed by treatment with PDGF-BB (20 ng/mL) for 48 hours. **G**, Contractile gene expression was examined by qPCR. **H**, Cell proliferation was examined by EdU incorporation. **I**, Cell migration was examined by transwell assay. n=3 biological replicates. Data are expressed as mean±SD, 2-way ANOVA with post hoc Scheffé. EdU indicates ethynyl-2'deoxyuridine; GO, gene ontology; qPCR, quantitative polymerase chain reaction; and PCA, principal component analysis.

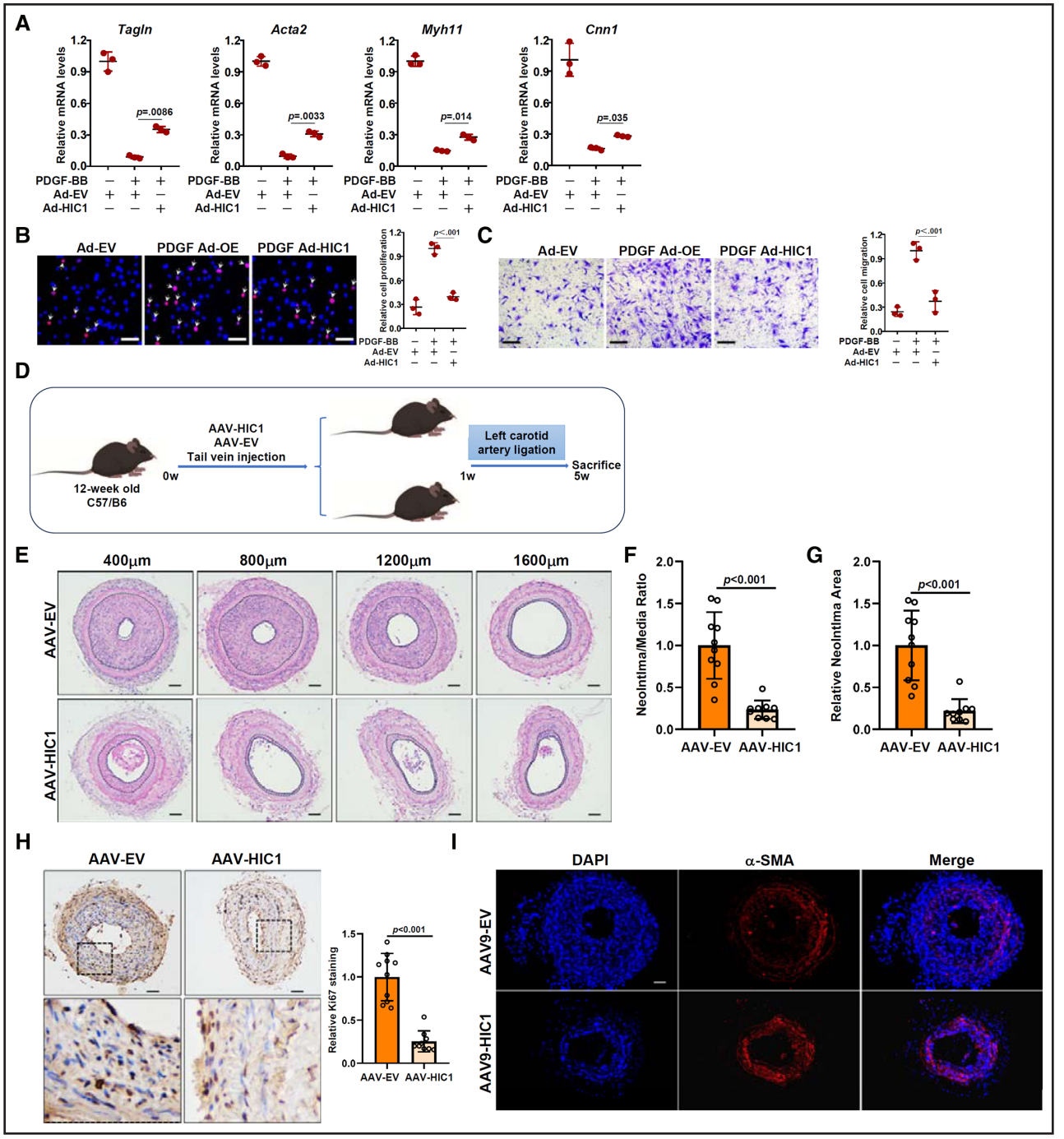

Figure 5. HIC1 (hypermethylated in cancer 1) regulates phenotypic switch of vascular smooth muscle cells (VSMCs) in vitro and neointima formation in mice.

A through **C**, Primary VSMCs were transduced with indicated adenovirus followed by treatment with PDGF (platelet-derived growth factor)-BB (20 ng/mL) for 48 hours. **A**, Contractile gene expression was examined by qPCR. **B**, Cell proliferation was examined by EdU incorporation. **C**, Cell migration was examined by transwell assay. n=3 biological replicates. Data are expressed as mean±SD, 2-way ANOVA with post hoc Scheffé. **D** through **I**, C57/B6 mice were injected via tail vein with AAV carrying HIC1 or an empty vector (EV) followed by carotid artery ligation. **D**, Scheme of protocol. **E**, Representative H&E staining images of vessels. **F**, Neointima/media ratios. **G**, Relative medial areas. **H**, Ki67 staining. **I**, α-SMA (α-smooth muscle actin) staining. n=10 mice for each group. Data are expressed as mean±SD, unpaired 2-tailed Student test. AAV indicates adeno-associated virus; EdU, ethynyl-2'deoxyuridine; H&E, hematoxylin and eosin; and qPCR, quantitative polymerase chain reaction.

there have been conflicting reports on how Suv39h1 might regulate the maintenance and disruption of VSMC homeostasis. For instance, Villeneuve et al[12] have shown that Suv39h1 expression is downregulated in vessels exposed to hyperglycemia from the diabetic (*db/db*) mice, which may account for elevated levels of cytokines

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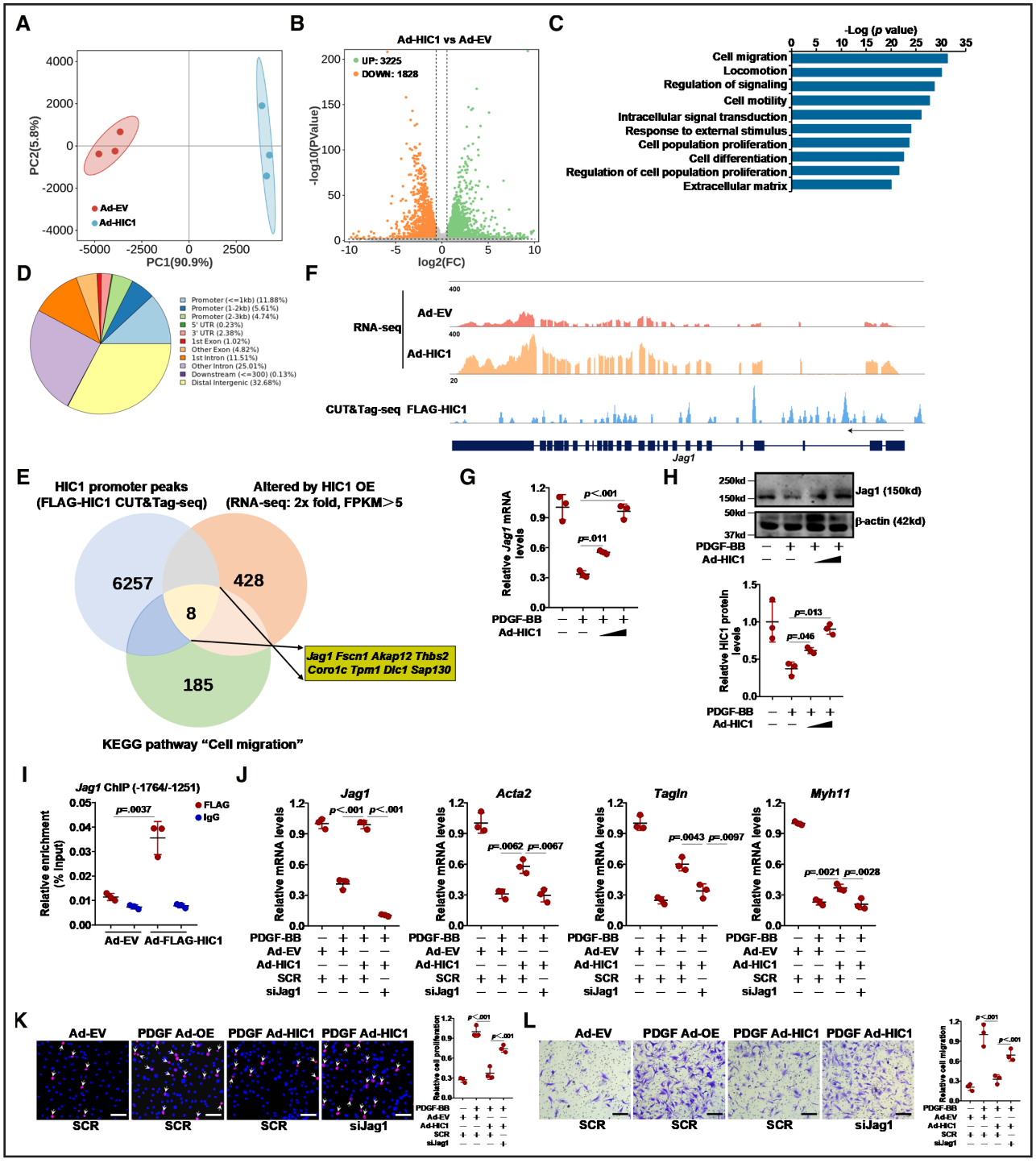

Figure 6. HIC1 (hypermethylated in cancer 1) regulates phenotypic switch of vascular smooth muscle cells (VSMCs) by activating Jag1 (Jagged 1) transcription.

A through **C**, Primary VSMCs were transduced with indicated adenovirus followed by treatment with PDGF (platelet-derived growth factor)-BB (20 ng/mL) for 48 hours. **A**, PCA plot. **B**, Volcano plot. **C**, GO analysis. **D**, Primary VSMCs were transduced with adenoviral FLAG-HIC1 followed by treatment with PDGF-BB (20 ng/mL) for 48 hours. Cleavage under targets and tagmentation with deep sequencing (CUT&Tag-seq) was performed with anti-FLAG. **E**, Venn diagram. **F**, CUT&Tag-seq tracks of FLAG-HIC1 signals and RNA-sequencing tracks of the read coverage surrounding the *Jag1* gene loci. **G** through **I**, Primary VSMCs were transduced with indicated adenovirus followed by treatment with PDGF-BB (20 ng/mL) for 48 hours. Jag1 expression was examined by qPCR and Western blotting. Chromatin immunoprecipitation (ChIP) assay was performed with anti-FLAG or IgG. **J** through **L**, Primary VSMCs were transduced with Ad-HIC1 or Ad-EV and transfected with indicated siRNA targeting HIC1 or scrambled siRNA (SCR) followed by treatment with PDGF-BB (20 ng/mL) for 48 hours. **J**, Contractile gene expression was examined by qPCR. **K**, Cell proliferation was examined by EdU incorporation. **L**, Cell migration was examined by transwell assay. n=3 biological replicates. Data are expressed as mean±SD, 2-way ANOVA with post hoc Scheffé. AAV indicates adeno-associated virus; EdU, ethynyl-2'deoxyuridine; GO, gene ontology; H&E, hematoxylin and eosin; PCA, principal component analysis; and qPCR, quantitative polymerase chain reaction.

in VSMCs when they transition to an inflammatory phenotype. Consistently, Sudhahar et al[28] have reported that inflammatory response during the development of aortic aneurysm can be attributed to miR-125b–mediated repression of Suv39h1 in VSMCs. On the contrary, Zhang et al[15] have demonstrated that Suv39h1, upregulated in primary murine VSMCs exposed to high glucose, might be responsible for increased proliferation and migration of VSMCs in diabetic rats. The key difference between our study and these previous studies is that unlike ours, the studies by Sudhahar et al, Zhang et al, and Villeneuve et al either did not choose to genetically target Suv39h1 (thus only providing correlative evidence) or used a lentiviral delivery system to manipulate Suv39h1 expression (thus completely lacking specificity). Therefore, our data provide bona fide evidence to support a causal relationship between Suv39h1, VSMC phenotypic switch, and neointima formation.

Through RNA-seq, we identify HIC1 as a potential target downstream of Suv39h1. HIC1 has been most extensively studied in oncogenesis where it is considered as a tumor repressor frequently detected to be silenced by DNA methylation.[29] Because Suv39h1-dependent H3K9 methylation and DNA methylation are 2 intimately interlocked epigenetic mechanisms, it would be of interest to determine whether Suv39h1 might form a cross talk with DNA methyltransferases to regulate HIC1 transcription. Our data also illustrate that HIC1 overexpression dampens phenotypic switch of VSMCs in vitro and ameliorates neointima formation in vivo. Our observations are consistent with several previously published studies. It has been suggested that HIC1 expression can be upregulated by TGF-β (transforming growth factor-β), an inducer of contractile genes, and can be used to delineate a population of mesenchymal progenitor cells.[30,31] Of interest, Wang et al have shown that DNA methylation leads to HIC1 repression in pulmonary VSMCs isolated from patients with chronic thromboembolic pulmonary hypertension featuring a similar process of VSMC phenotypic switch associated with CAD.[32] Further, SMC-specific HIC1 deletion immediately after birth (P4/P5) severely impairs, accordingly to Uchida et al,[33] testicular SMC development along with downregulation of a panel of contractile genes, including α-SMA and calponin, in the testis in mice; it is not immediately clear whether or not VSMC development is influenced. When combined, these data point to a pivotal role for HIC1 in regulating SMC pathophysiology and provide strong rationale for continuing this line of investigation to target HIC1 for devising novel therapy for CAD intervention.

Through integrated transcriptomic analysis we further identify Jag1 as 1 of the HIC1 targets that might mediate the vasculoprotective effects of HIC1. Indeed, Jag1 knockdown partially offset the antimigraproliferative effects of HIC1 overexpression in cultured VSMCs. However, it should be pointed out that HIC1 targets other than Jag1 might contribute to VSMC phenotypic modulation by HIC1. For instance, the Miano group has previously reported that the immediate early gene *Akap12* (A-kinase anchoring protein 12) can be induced by retinoic aicd in VSMCs, that *Akap12* appears to be necessary to keep VSMCs in a quiescent state by limiting cell proliferation, and that *Akap12* downregulation is detected in vivo following carotid artery ligation mirroring Ki67 upregulation.[34] Alternatively, Kang et al[35] have recently discovered that Fscn1 (Fascin actin-bundling protein 1) is targeted by the miR-143/145 cluster and implicated in phenotypic switch of pulmonary SMCs in the context of pulmonary hypertension. It is possible that HIC1 may regulate VSMC phenotype by orchestrating the transcription of multiple, as opposed to singular, genes. These lingering issues should be thoroughly investigated in future studies.

One often overlooked but immensely intriguing point is that Suv39h1 might contribute to pathobiological processes by methylating and consequently modulating the activities of nonhistone factors. A recent proteomic survey has identified several potential Suv39h1 substrates including RAG2 (recombination activating gene 2), DOT1L (DOT1-like hisone lysine methyltransferase), and SET8 (SET domain containing protein 8). Of interest, each of these proteins has been implicated in VSMC phenotypic switch or neointima formation. For instance, it has been observed that RAG2-null (*Rag2*$^{-/-}$) mice develop more severe restenosis following vascular injury than the wild-type littermates.[36] In addition, SET8 is known to maintain the contractile phenotype of VSMCs by preserving cell viability in a model of vascular calcification.[37] On the contrary, DOT1L has been shown to pivot VSMCs to an inflammatory phenotype by regulating NF-κB (nuclear factor-κB) transcription.[38] It would be of significant interest if future work using proteomic tools can identify potential nonhistone substrates for Suv39h1 that might contribute to VSMC phenotypic switch and neointima formation.

There are a few limitations associated with the present study that might dampen its translational potential. First, the entire foundation of our proposal relies on data derived from cell culture and experimental animals. Therefore, the relevance of our finding to human pathology remains questionable. Second, Suv39h1 has recently been implicated in modulating the function of immune cells including macrophages and lymphocytes.[39–41] Because restenosis is invariably preceded by an acute-phase inflammatory response, the possibility that Suv39h1 might contribute to neointima formation in a VSMC nonautonomous manner cannot be definitively ruled out and warrants further investigation. Third, the specificity of the small-molecule compound F5446 previously described to inhibit Suv39h1 activity is not validated. Other Suv39h1 inhibitors have been reported and

tested in different disease models.[42–44] Our data certainly provide further incentive to design such compounds for safe and effective pharmacotherapy against restenosis.

ARTICLE INFORMATION

Received October 23, 2024; accepted April 10, 2025.

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Acknowledgments

Y. Yang, Y. Zhang, and J. Guo conceived the project; Y. Yang, Q. Zhang, S. Liu, H. Yuan, X. Wu, and Y. Zou designed experiments, performed experiments, collected data, and analyzed data; all wrote and edited the manuscript; Y. Yang, Y. Zhang and J. Guo secured funding and provided supervision.

Sources of Funding

This work was supported by grants from the National Natural Science Foundation of China (U22A20270, 32171180, 82260082, 82121001, 82170440, and 81870326), the National Key R&D Program of China (2024YFA1802300), Hainan Key Research and Development Fund (ZDYF2021SHFZ089 and ZDYF2023SHFZ109), the Innovational Fund for Scientific and Technological Personnel of Hainan Province (KJRC2023B07), and the Natural Science Foundation of Hainan Province (823MS144).

Disclosures

None.

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