SUPPLEMENTAL MATERIAL

Title: Adenosine kinase is critical for neointima formation after vascular injury by inducing aberrant DNA hypermethylation

Short title: Adenosine kinase contributes to neointima formation

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Supplemental Methods

Generation of smooth muscle-specific adenosine kinase (*Adk*) knockout mice

Animals were used based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Institutional Animal Care and Use Committee at Augusta University. The *Adk* floxed mice were generated by Xenogen Biosciences Corporation (Cranbury, NJ, USA) (Suppl. Figure 2A). Myh11-Cre/ERT2 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Myh11 promoter/enhancer regions were inserted on the Y chromosome. To generate smooth muscle-specific *Adk* knockout mice, we first generated Myh11-Cre/ERT2/$Adk^{f/+}$ mice by crossing Myh11-Cre/ERT2 male mice with $Adk^{f/f}$ female mice. Myh11-Cre/ERT2/$Adk^{f/+}$ mice were then used to breed with $Adk^{f/f}$ female mice to achieve Myh11-Cre/ERT2/$Adk^{f/f}$ mice. To have these mice on an atherosclerotic background, Myh11-Cre/ERT2/$Adk^{f/f}$ mice were further bred with $Apoe^{-/-}$ mice, and $Apoe^{-/-}$/Myh11-Cre/ERT2/$Adk^{f/f}$ mice were generated.

Tamoxifen injection

Tamoxifen (Sigma, St. Louis, MO, USA) injection was started in 6-week-old mice for 5 consecutive days. Tamoxifen was first dissolved in 100% ethanol at 37°C to a concentration of 100 mg/ml and further diluted with sunflower oil to a final concentration of 10mg/ml. 10ul /1g body weight was given by intraperitoneal injection for 5 consecutive days. Two weeks after the last injection, the mice underwent common carotid artery ligation or transluminal injury with a guide wire.

Cell culture

Human coronary artery smooth muscle cells (HCSMCs) were purchased from ATCC, and cultured with vascular cell basal medium (ATCC, PCS-100-030) supplemented with Vascular Smooth Muscle Cell Growth Kit (ATCC, PCS-100-042) containing FBS (5%), asorbic acid (50ug/ml), L-glutamine (10mM), recombinant human (rh) Insulin (5ug/ml), rh EGF (5ng/ml), and rh FGF-β (5ng/ml). Cell passages of 3 to 5 were used in this study.

Cell growth and WST-1 proliferation assay

HCSMCs (passage 3) were used for WST1 proliferation assay (Cell Biolabs). Cells were infected with adenoviral *ADK* shRNA and adenoviral GFP control for 24 hours. For the cell number counting experiment, 3×10^4 cells were seeded into each well of a 12-well plate and cultured with full growth medium (the time point was marked as D0). Cell numbers were counted at 24 hours (D1), 48 hours (D2) and 72 hours (D3). For the WST1 proliferation assay, $3x10^3$ cells per well were seeded into each well of

a 96-well culture plate and incubated with full growth medium overnight. Cell growth arrest was achieved by incubating with vascular cell basal medium overnight. For some experiments, HCSMCs were treated with platelet-derived growth factor-BB (PDGF-BB, Sigma Aldrich; 10-20 ng/ml, 24 hours), 5-iodotubercidin (ITU, Tocris Bioscience; 0.1-10μM, 24 hours), 4-amino-5-(3-bromophenyl)-7-(6-morpholino-pyridin- 3-yl) pyrido[2,3-d]pyrimidine (ABT702, Tocris Bioscience; 0.1-10μM, 24 hours), or alloxazine (SC-252358; 0.1-10μM, 24 hours). 10ul of WST-1 cell proliferation assay reagent was added and incubated at 37°C. Absorbance was measured at 450nm.

Flow cytometric analysis of cell cycle with propidium iodide DNA staining

HCSMCs were infected with adenoviral *ADK* shRNA and adenoviral GFP control for 48 hours. Cells were digested and washed with phosphate-buffered saline and then fixed with 70% ice-cold ethanol overnight. The cells were stained with propidium iodide (PI)/RNase staining buffer (550825, BD Biosciences) and analyzed using Becton Dickinson FACSARIA II SORP cell sorter flow cytometer. GFP-positive cells in adenoviral *ADK* shRNA and adenoviral GFP control infection were gated for cell cycle analysis. The G0/G1, S and G2/M phases were quantified.

Isolation of mouse aortic vascular smooth muscle cells

Briefly, after euthanization by CO2 asphyxiation, the aorta and heart of animals were carefully dissected in Hank's buffered saline (HBBS) and then perfused with PBS. After carefully removing the heart and the adventitia, the aorta was cut into 1-2 mm pieces and then incubated with 1 mg/ml trypsin (Invitrogen) for 10 min to remove any remaining adventitia and endothelium. After incubation overnight at 37 °C in DMEM containing 10% fetal bovine serum (FBS), tissues were digested with 425 U/ml collagenase type II (Worthington Biochemical Corporation, Lakewood, USA) for 5 hrs. After washing, VSMCs were incubated in DMEM containing 10% fetal bovine serum (FBS) in T25 tissue culture flasks (Corning) coated with 0.25 μg/cm^2 laminin (Sigma) to promote maintenance of the contractile differentiation state.

Quantitative RT-PCR (qRT-PCR) analysis

Total RNA of human vascular smooth muscle cells with different treatments or mouse thoracic artery (adventitia layer was removed) were isolated using RNeasy Mini kit (QIAGEN), and qRT-PCR was done as described previously[1]. Briefly, 400 ng of RNA was utilized as template for reverse transcription reactions with random hexamer primers using iScript cDNA synthesis kit (Bio-Rad). Quantitative real time PCR was carried out on an ABI 7500 Real Time PCR system (Applied Biosystems). The information of primer sequences has been provided in Suppl. Table 2. All samples were amplified in

duplicate, and the experiments were repeated at least twice. Relative gene expression levels were analyzed using the 2-ΔΔCt method against internal control hypoxanthine phosphoribosyl transferase 1 (HPRT) for mouse and beta actin for human.

Protein preparation and Western blotting

Aortas were dissected and the periadventitia layers were removed under a stereoscope. Tissues were cut into small pieces and ground with homogenizer in RIPA buffer containing 1% proteinase inhibitor cocktail (Pierce) and 1% phenylmethanesulfonylfluoride (PMSF). Protein was extracted from human vascular smooth muscle cells with different treatments using RIPA buffer (Adox). Concentration of protein was quantified using BCA assay (Thermo Scientific). 10-30μg total protein was analyzed using 7-10% SDS-PAGE electrophoresis. After blocking with 5% non-fat milk, the membranes were incubated with antibodies overnight at 4°C. Antibodies used in this study were: ADK (ab38010, Abcam, 1:1000), β-actin (Cell Signaling Technology, 1:3000), PCNA (Cell Signaling Technology, 1:1000), CyclinD1 (Cell Signaling Technology, 1:1000), SM α-actin (Adox, Sigma, 1:5000), GAPDH (SC-25778, Santa Cruz Biotechnology, 1:3000), SM22α (AB-14106, Abcam, 1:5000), Vinculin (Adox, Sigma, 1:3000), KLF4 (GTX52307, GeneTex, 1:1000) and P18(SC-865, Santa Cruz Biotechnology, 1:1000). Images were taken using the ChemiDoc MP system (Bio-Rad), and band densities were quantified using Image Lab software (Bio-Rad).

Hematoxylin/eosin staining, immunofluorescence (IF) and immunohistochemistry (IHC) analyses

Hematoxylin/eosin (H&E) staining, immunofluorescence (IF) and immunohistochemistry (IHC) were performed as previously described[2, 3]. Mouse arteries were fixed in 4% paraformaldehyde for 24 hours at 4°C and embedded in paraffin. Cultured human vascular smooth muscle cells on slides were also fixed with 4% paraformaldehyde for 20 minutes at 4°C. Slides with cultured cells or deparaffinized sections were treated with citric acid buffer (10mM, pH 6.0) and heated to 98°C for 10 minutes in a microwave for antigenic unmasking. For IHC staining, the slides were blocked with avidin blocking solution (Vector Laboratories) and incubated with primary antibodies overnight at 4°C, followed by incubation with biotinylated secondary antibody at room temperature for 1 hour (Vector Laboratories, 1:200) and ABC solution (Vector Laboratories) for 30 minutes at room temperature. Expression of the target molecules was visualized after DAB solution was added to the slides. Nuclei were stained with hematoxylin. The staining was monitored under microscopy. For IF staining, slides were blocked with 10% goat serum (50062Z, Thermo Scientific), then incubated with primary antibodies overnight at 4°C, followed by Alexa Fluor 488 or Alexa Fluor 594-conjugated secondary antibody for 1 hour at room

temperature. Nuclei were stained with 4', 6'-diamidino-2-phenylindole (DAPI). Images were collected using confocal microscopy (LS510, Zeiss). Primary antibodies used in this study were anti-α smooth muscle actin (Adox, Sigma, 1:1000), anti-Ki67 (Thermo Scientific, 1:50), and anti-PCNA (Cell Signaling Technology, 1:100). The H&E staining was performed according to a standard protocol. The images from IHC and H&E-stained slides were captured using an Olympus BX51 microscope. The neointima area and the media layer area were analyzed by Image J software.

Complete left common carotid artery ligation

Complete ligation of mouse left common carotid arteries was performed as previously described[4]. Briefly, mice were anesthetized with ketamine (80mg/kg body weight) and xylazine (5mg/kg body weight) by intraperitoneal injection. The left common carotid arteries were exposed and completely ligated near the bifurcation with 6-0 silk. The right common carotid artery was exposed but not ligated. Sections (5μm) between 0.2 to 2.0 mm proximal to the ligation site were obtained from blocks of paraffin-embedded arteries. Morphometric analysis was performed on H&E-stained slides in a blinded manner. The intima areas and medial layer area were measured using Image J software. Briefly, the area of the intima was calculated by subtracting the luminal area from the internal elastic lamina area, and the media layer area was obtained by subtracting the internal elastic lamina area from the external elastic lamina area. Arteries from at least eight animals were processed for data analysis.

Mouse artery vascular guide wire injury

Mouse vascular guide wire injury was performed as previously described[2, 5]. Mice were anesthetized using ketamine (80 mg/kg body weight) and xylazine (5 mg/kg body weight) by intraperitoneal injection. The left external branch of the common carotid artery was ligated and a 0.014-inch flexible angioplasty guide wire accessed into the common carotid artery by 5 passes through an external branch. 21 days after surgery, the injured arteries were harvested, embedded in paraffin and sectioned at 5μm thickness for H&E staining. Image J was used for analysis of the neointima area. Sections from at least six animals in each group were used for data analysis.

Adenosine and S-adenosylhomocysteine (SAH) measurement

Adenosine and SAH of human vascular smooth muscle cells were measured in the Proteomics Core Laboratory by liquid chromatography–mass spectrometry (LC-MS). HCSMCs were treated as indicated. The supernatants of cell medium were collected. After aspiration of the culture medium, the cells were washed with ice cold PBS and then scraped immediately, and the cell pellets were collected. 120 μl ice

cold 90% methanol was added to the cell pellets. The samples were placed on ice for 10 minutes and centrifuged at 13000 g for 5 minutes. 1μl supernatant was used for adenosine and SAH measurement using LC-MS. For assay of adenosine in carotid artery, each 0.4cm isolated artery was minced and homogenized in 200 ul ice cold 90% methanol. After placed on ice for 10 minutes, the tissue samples were centrifuged at 13,700 rpm for 27 minutes at 4°C. 1μl supernatant was used for adenosine measurement using LC-MS.

Separation of the two molecules was performed on a Shimadzu Nexera UHPLC system equipped with a Phenomenex Kinetex C18 column (100x2.1mm, 1.7um) at a flow rate of 0.2ml/min and column oven temperature of 4°C, using an isocratic elution with 1% acetonitrile in 10mM ammonium acetate for 5 minutes. The effluent was ionized using positive ion electrospray on an ABSCIEX 4000 QTRAP mass spectrometer with the following instrument settings: ion spray voltage 5500V, curtain gas 20, temperature 450, gas 1 30, gas 2 20, and low/unit resolution for Q1/Q3. The optimal collision energy, declustering potential, entrance potential and exit potential were determined using purchased standards. The MS ran in MRM mode, monitoring the transitions of 268.0 / 136.0 and 385.1 / 136.2 for adenosine, SAM and SAH, separately. After the samples were analyzed, the integrated peak areas for these transitions were calculated using Multiquant software (version 2.0, ABSCIEX). Standard curves were generated in a range of 0 to1000 fM for the three molecules.

DNMT activity assay

HCSMCs were infected with adenoviral *ADK* shRNA and adenoviral GFP control and incubated for 48 hours. The nuclear proteins were prepared using a Nuclear Extraction Kit (AB113474, ABCAM). The DNA methyltransferase (DNMT) activity was quantified using a fluorimetric EpiQuick DNMT Activity Assay Ultra kit.

Global DNA methylation measurement

HCSMCs were infected with adenoviral *ADK* shRNA and adenoviral GFP control and incubated for 48 hours. The genomic DNA was isolated using QIAamp DNA Blood Mini Kit (QIAGEN). Global DNA methylation status in VSMCs was assessed using the MethylFlash Methylated DNA quantification kit.

Infinium methylation assay

HCSMCs were infected with adenoviral *ADK* shRNA and adenoviral GFP control and incubated for 48 hours. Genomic DNA methylation was evaluated using Infinium methylation assay. Briefly, the bisulfite conversion of genomic DNA of VSMCs was performed using EZ DNA Methylation Gold kit (Zymo

Research). Illumina Infinium 450K Methylation array was used to analyze 200ng converted DNA according to the manufacturer's protocols (Illumina). The quantitative value of DNA methylation was calculated from the ratio of fluorescent signals from the methylated alleles to the sum of the signals from all alleles, assigned via Genome Studio Methylation Software Module (Illumina). Minfi package in Bioconductor (http://bioconductor.org/packages/release/bioc/html/minfi.html) was used for quality control and normalization before statistical analysis. The average of the DNA methylation level across all the CpG sites within a sample was used as an index of the global DNA methylation level of the sample. Student *t*-test analysis from the Limma package was used to identify differentially methylated CpG sites between the control group and the *ADK* KD group in the methylation array data.

KLF4 DNA bisulfite sequencing

HCSMCs were infected with adenoviral *ADK* shRNA and adenoviral GFP control for 48 hours. Genomic DNA was isolated using QIAamp DNA Blood Mini Kit (QIAGEN). The genomic DNA was bisulfite converted using an EpiTect Bisulfite Kit (QIAGEN) as instructed. The human *KLF4* gene sequence was obtained from genome assembly GRCH38. Methylation of the fragment (2000bp upstream of the transcriptional start site (TSS) and 200 bp downstream of the TSS) was predicted by Methyl Primer Express software (Life Technologies). The fragment between -487 bp to +122 bp of the *KLF4* (NM_004235) promoter region was amplified. The information on primer sequences is provided in Suppl. Table 2. The resulting PCR products were first examined by agarose gel electrophoresis and then subcloned into a TA vector using a TOPO® Cloning Kit (Invitrogen). Ten positive clones from each PCR product were selected for DNA sequencing analysis with M13R primer in an ABI 310 automated sequencing system (ACGT Inc). DNA sequencing results were analyzed by BiQ analyzer 2.0 (http://biq-analyzer.bioinf.mpi-sb.mpg.de). The levels of methylated CpG sites between the -487 bp to -350 bp region of the *KLF4* promoter were evaluated between groups of *ADK* KD and control VSMCs.

Statistical analysis

The data were analyzed with GraphPad Prism Software by one-way ANOVA with Tukey's *post-hoc* test, two-way ANOVA with Bonferroni's post-hoc test, or Student's *t*-test to evaluate two-tailed levels of significance. The number of experiments performed is provided in figure legends. Values are expressed as the mean ± SEM and the null hypothesis was rejected at $P \leq 0.05$.

Reference

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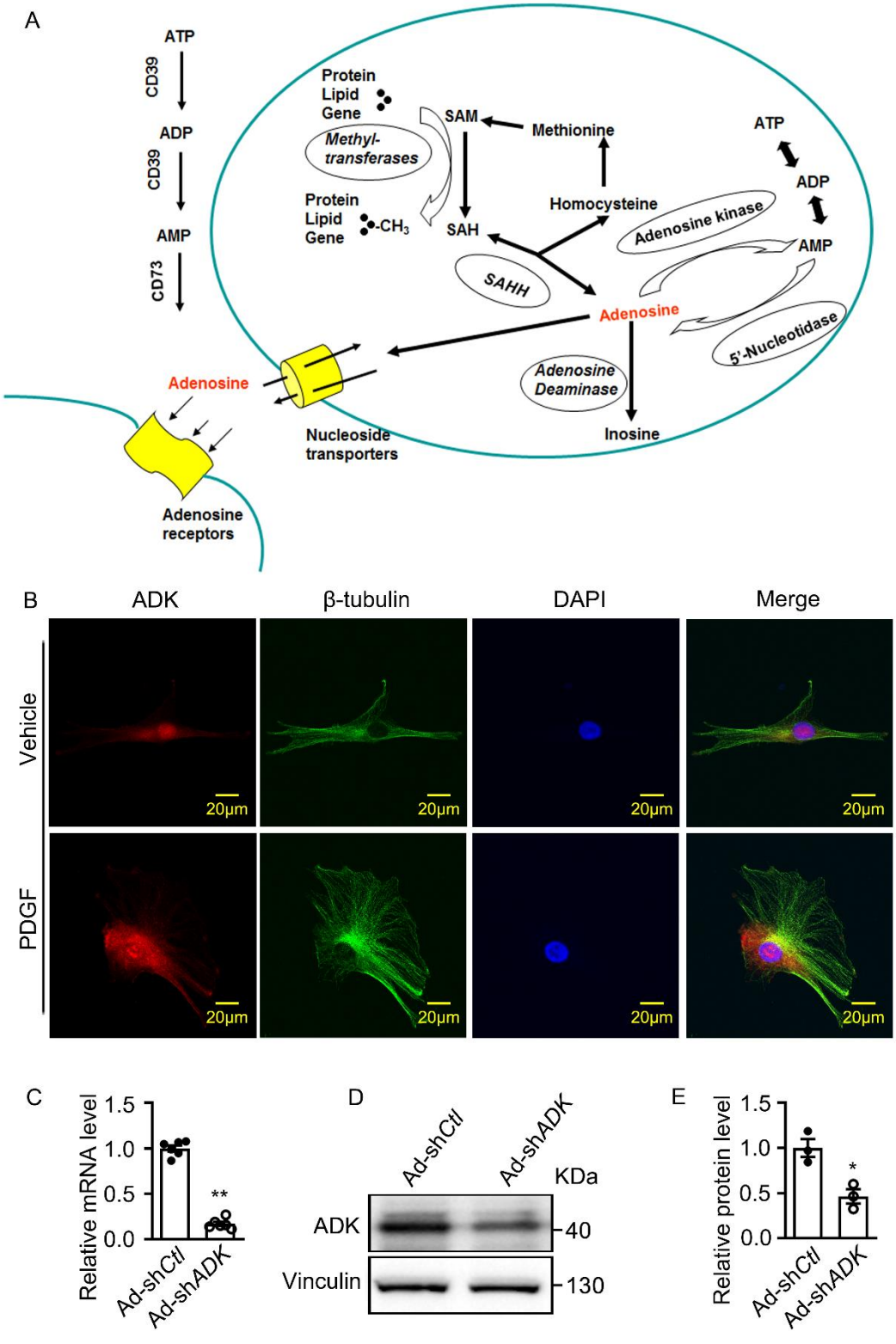
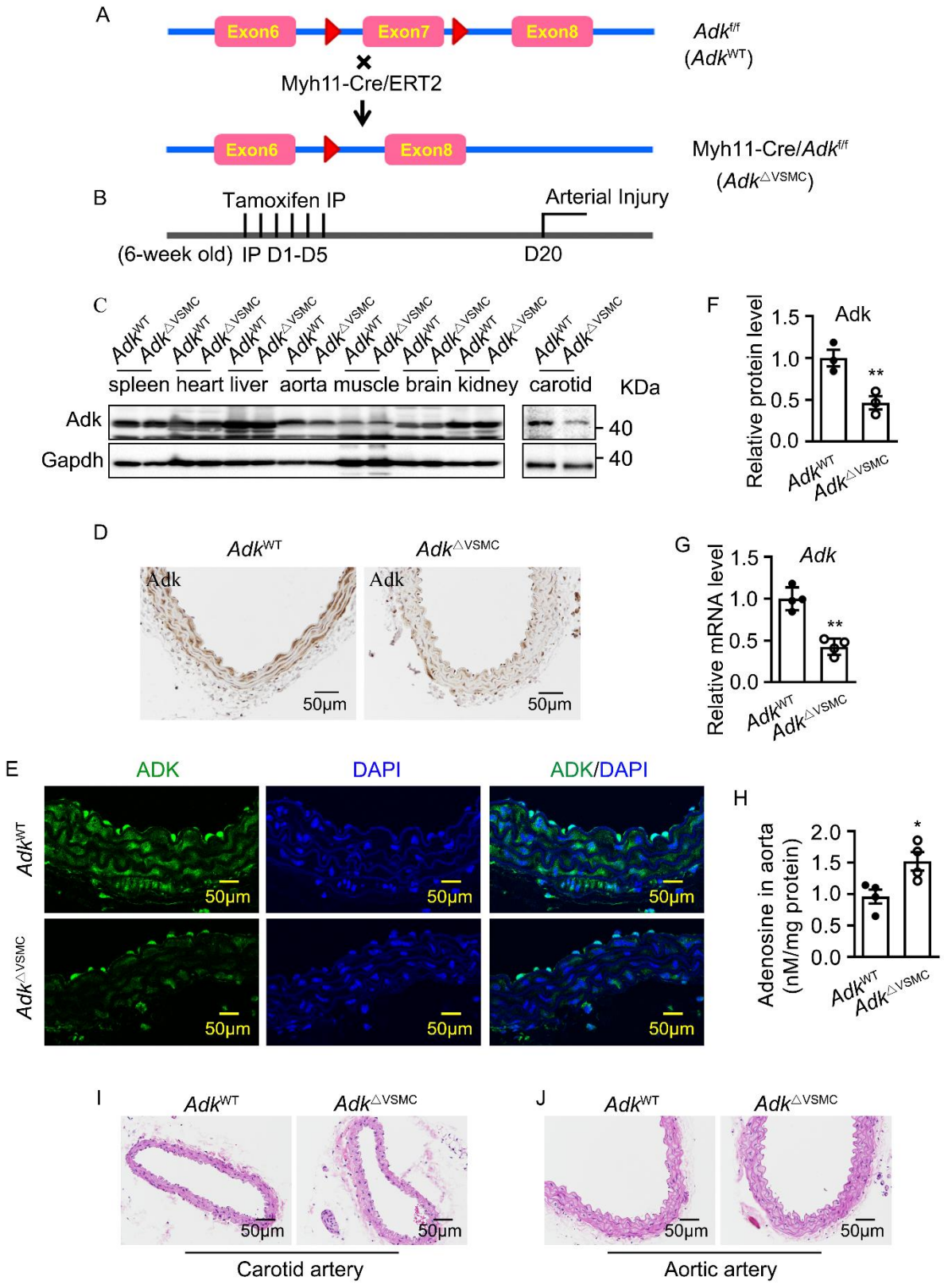
Supplemental Figures



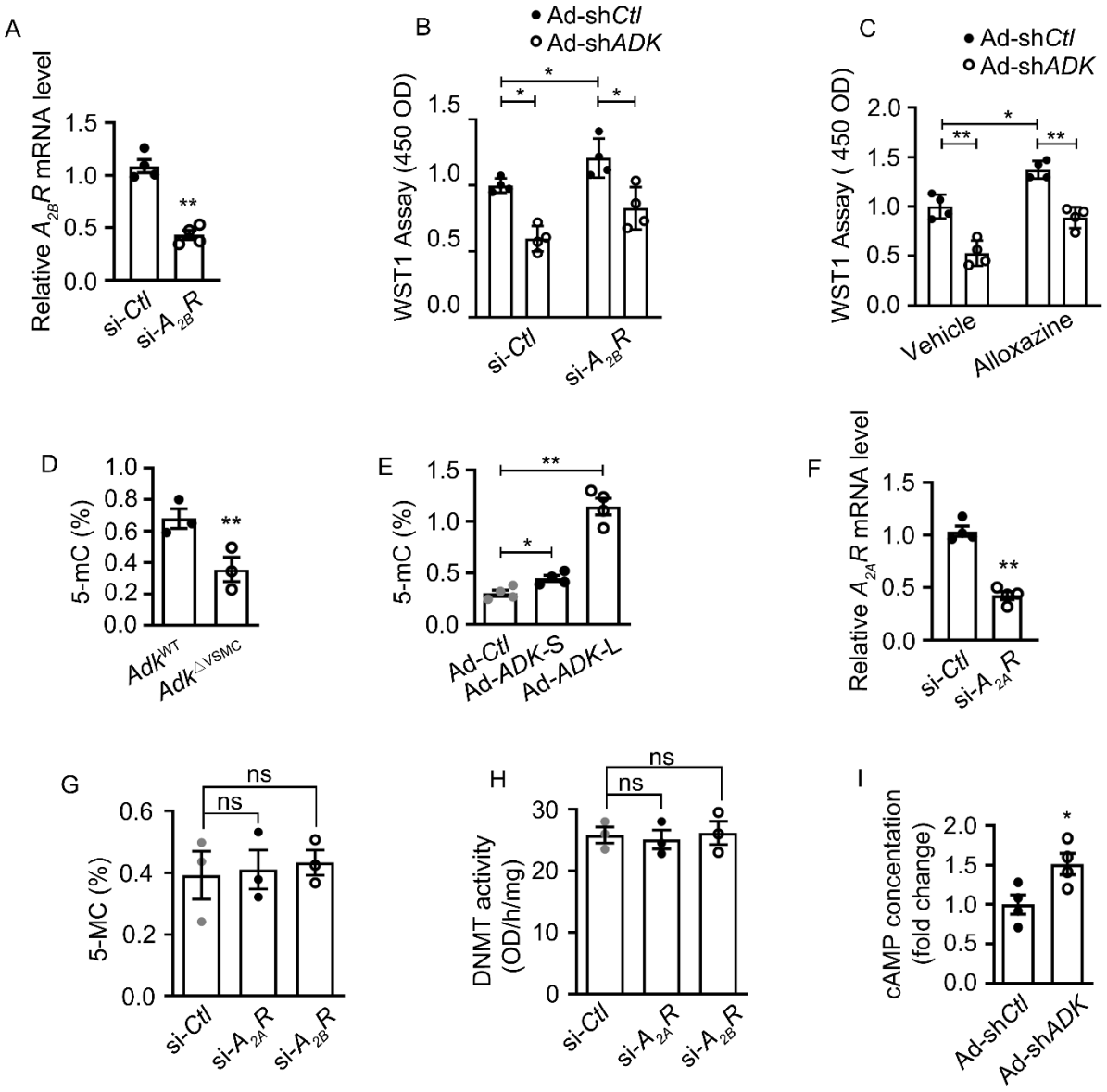
Supplemental Figure 1. Metabolic pathways of adenosine and ADK expression upon PDGF treatment.

(A) Intracellular adenosine is generated by both stepwise dephosphorylation of adenosine triphosphate (ATP) and hydrolysis of S-adenosylhomocysteine (SAH) by SAH hydrolase (SAHH). The intracellular adenosine is metabolized to 5'-adenosine monophosphate (AMP) via adenosine kinase (ADK) or to inosine via adenosine deaminase (ADA). Adenosine in intracellular and extracellular compartments can be exchanged via nucleoside transporters. The transmethylation pathway is regulated by mass action and the reversibility of the SAHH-mediated reaction. When metabolic clearance of adenosine through ADK is impaired, the level of SAH rises and in turn, suppresses methyltransferase activity, reducing methylation of genes and proteins. **(B)** Immunofluorescent staining was performed to evaluate the expression of ADK (red) and β-tubulin (green) in VSMCs. 5×10^3 human coronary artery VSMCs were seeded into an 8-well culture slide, cultured in basal medium for 12 hours, and treated with 20ng/ml PDGF-BB for 12 hours. **(C-E)** Quantitative RT-PCR (C) and Western blot (D and E) analyses of ADK expression at both the protein and mRNA levels in control and *ADK* KD HCSMCs (n=3 or 6 independent cultures). For all bar graphs, data are the means ± SEM, * $P < 0.05$ (Unpaired, two-tailed Student's t-test).



Supplemental Figure 2. Generation of VSMC-specific *Adk* inducible knockout mice.

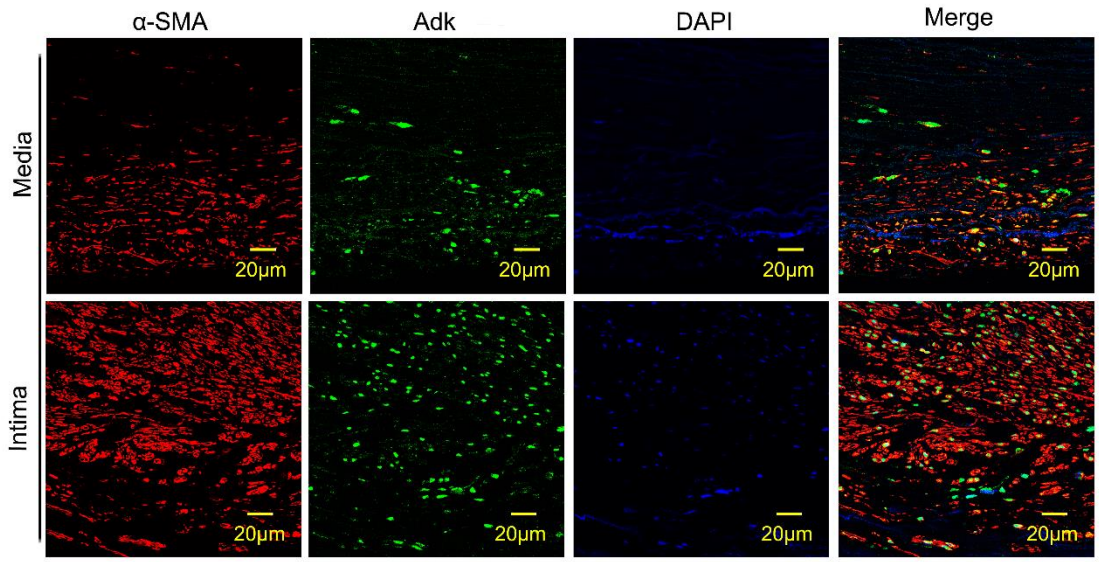
(A) Schematic of Cre-mediated *Adk* exon-7 deletion in VSMCs. **(B)** Timelines of tamoxifen IP treatment and carotid artery injury. **(C)** Representative Western blots showing Adk expression in different organs of Adk^{WT} and $Adk^{\triangle VSMC}$ mice after tamoxifen peritoneal injection. **(D)** Representative immunohistochemical staining of Adk in aortas of Adk^{WT} and $Adk^{\triangle VSMC}$ mice. **(E)** Representative immunofluorescent staining of Adk in carotid arteries of Adk^{WT} and $Adk^{\triangle VSMC}$ mice. **(F)** Quantitation of Adk expression in mouse aortic smooth muscle cells isolated from Adk^{WT} and $Adk^{\triangle VSMC}$ mice (n= 3 independent experiments). **(G)** Quantitative RT-PCR analysis of ADK mRNA level in mouse aortic smooth muscle cells isolated from Adk^{WT} and $Adk^{\triangle VSMC}$ mice (n= 4 independent experiments). **(H)** Quantification of adenosine levels in aortas from Adk^{WT} and $Adk^{\triangle VSMC}$ mice (n = 4 mice per group). **(I and J)** Hematoxylin and eosin (H&E) staining of common carotid arteries **(I)** and thoracic arteries **(J)** from Adk^{WT} and $Adk^{\triangle VSMC}$ mice. For all bar graphs, data are the means ± SEM, * $P < 0.05$ and ** $P < 0.01$(Unpaired, two-tailed Student's t-test).



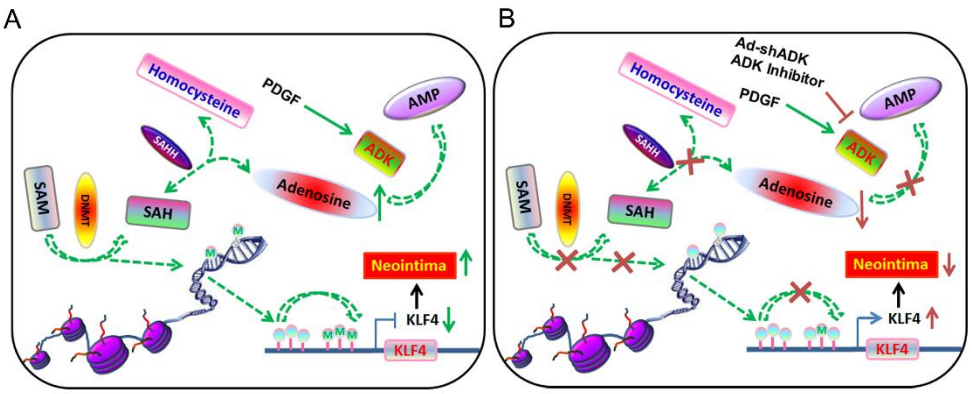
Supplemental Figure 3. The effects of adenosine receptor A_{2A} or A_{2B} on ADK KD-induced inhibition of VSMC proliferation and on DNA methylation.

(A) Quantification of the levels of $A_{2B}R$ in HCSMCs transfected with control or $A_{2B}R$ siRNA (n = 3). **(B)** Analysis of proliferation by WST-1 assay in HCSMCs that were first transfected with siRNA targeting $A_{2B}R$ or control siRNA, and then infected 6 h later with adenovirus encoding shRNA targeting *ADK* or control shRNA (n=4 independent experiments). **(C)** Analysis of proliferation by WST-1 assay in HCSMCs that were first pretreated with $A_{2B}R$ antagonist alloxazine at 1μM for 30 min, and then infected 6 h later with adenovirus encoding shRNA targeting *ADK* or control shRNA (n=4 independent experiments). **(D)** Quantification of 5-mC content in mouse aortic SMCs isolated from Adk^{WT} or $Adk^{\Delta VSMC}$ mice (n=3 independent experiments). **(E)** Quantification of 5-mC content in HCSMCs

transfected with the ADK-S and ADK-L (n=4 independent experiments). **(F)** Quantification of the levels of $A_{2A}R$ in HCSMCs transfected with control or $A_{2A}R$ siRNA (n = 3). **(G)** Quantification of 5-mC content in HCSMCs transfected with control siRNA (si-Ctl), $A_{2A}R$ siRNA (si-$A_{2A}R$) or $A_{2B}R$ siRNA (si-$A_{2B}R$) for 30 hours (n=3 independent experiments). **(H)** Quantification of DNMT activity in HCSMCs transfected with control siRNA (si-Ctl), $A_{2A}R$ siRNA (si-$A_{2A}R$) or $A_{2B}R$ siRNA (si-$A_{2B}R$) for 30 hours (n=3 independent experiments). **(I)** Quantification of the intracellular cAMP level in control and *ADK* KD VSMCs (n=4 independent experiments). For all bar graphs, data are the means ± SEM, * $P < 0.05$ and ** $P < 0.01$ (One-way ANOVA with Tukey's post hoc test for C, D, F, G, and H; Unpaired, two-tailed Student's t-test for A, B, E and I).

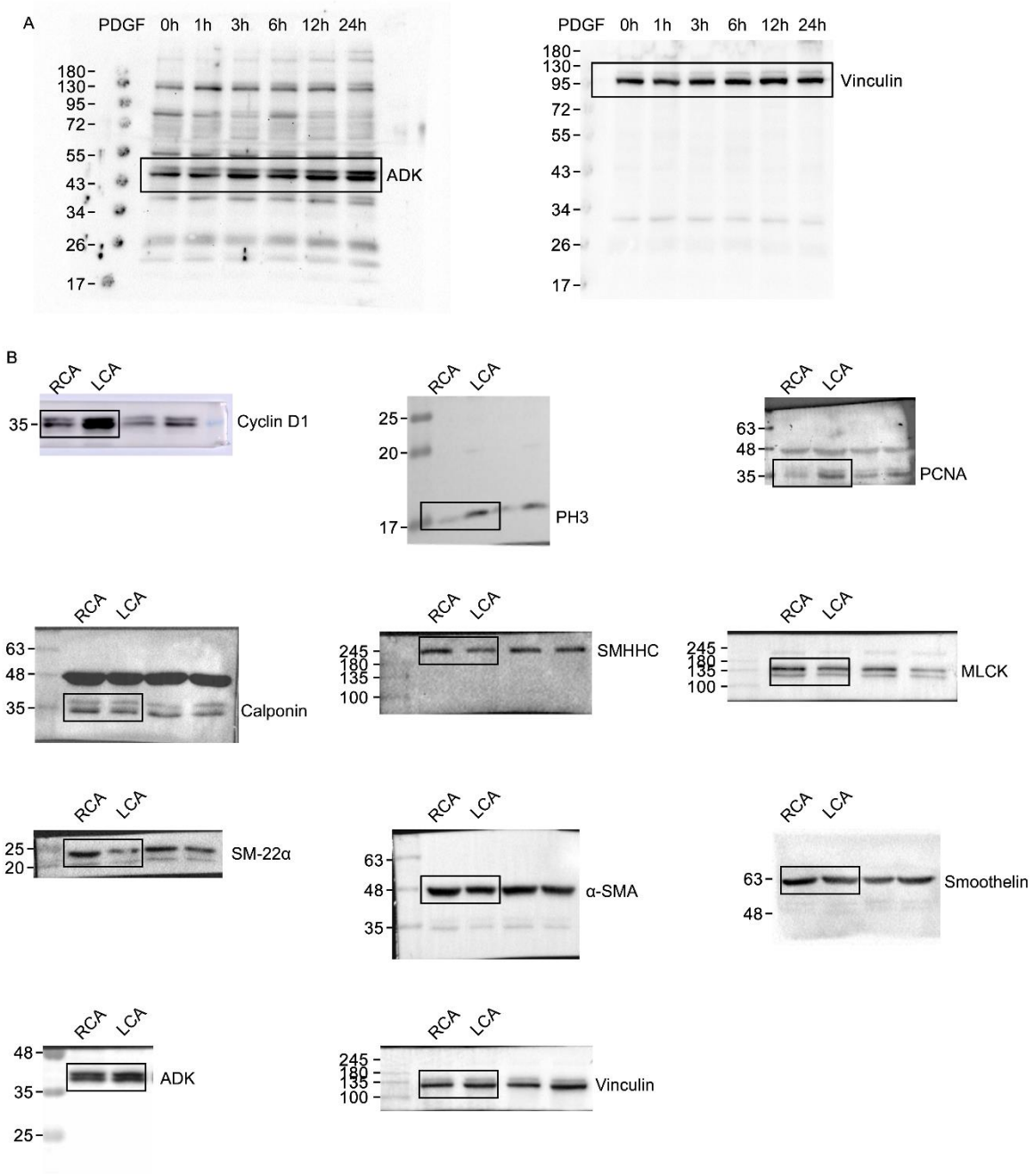


Supplemental Figure 4. ADK expression in human femoral arterial neointima.
Representative IF staining of ADK (green) on sections of human stenotic femoral artery. Top panels are images of media layer smooth muscle while bottom panels are images of neointima layer smooth muscle.

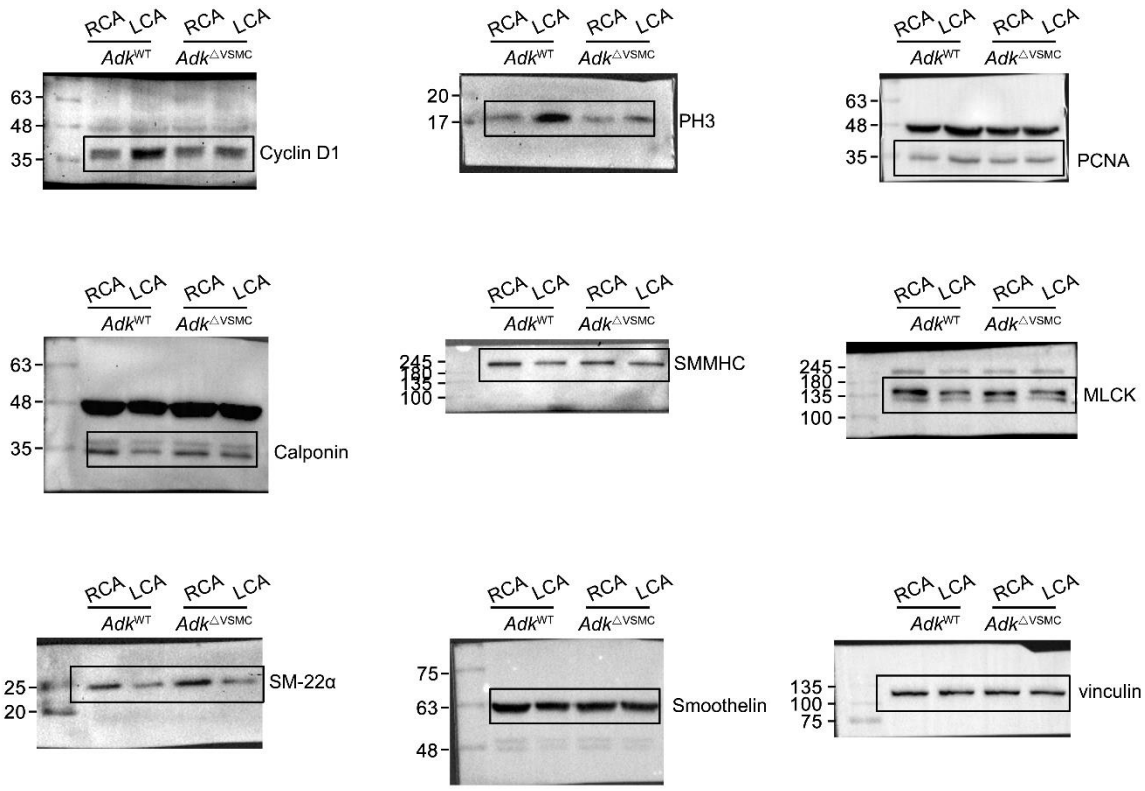


Supplemental Figure 5. Schematic of the proposed epigenetic mechanism of ADK-mediated facilitation of DNA hypermethylation for neointima formation.

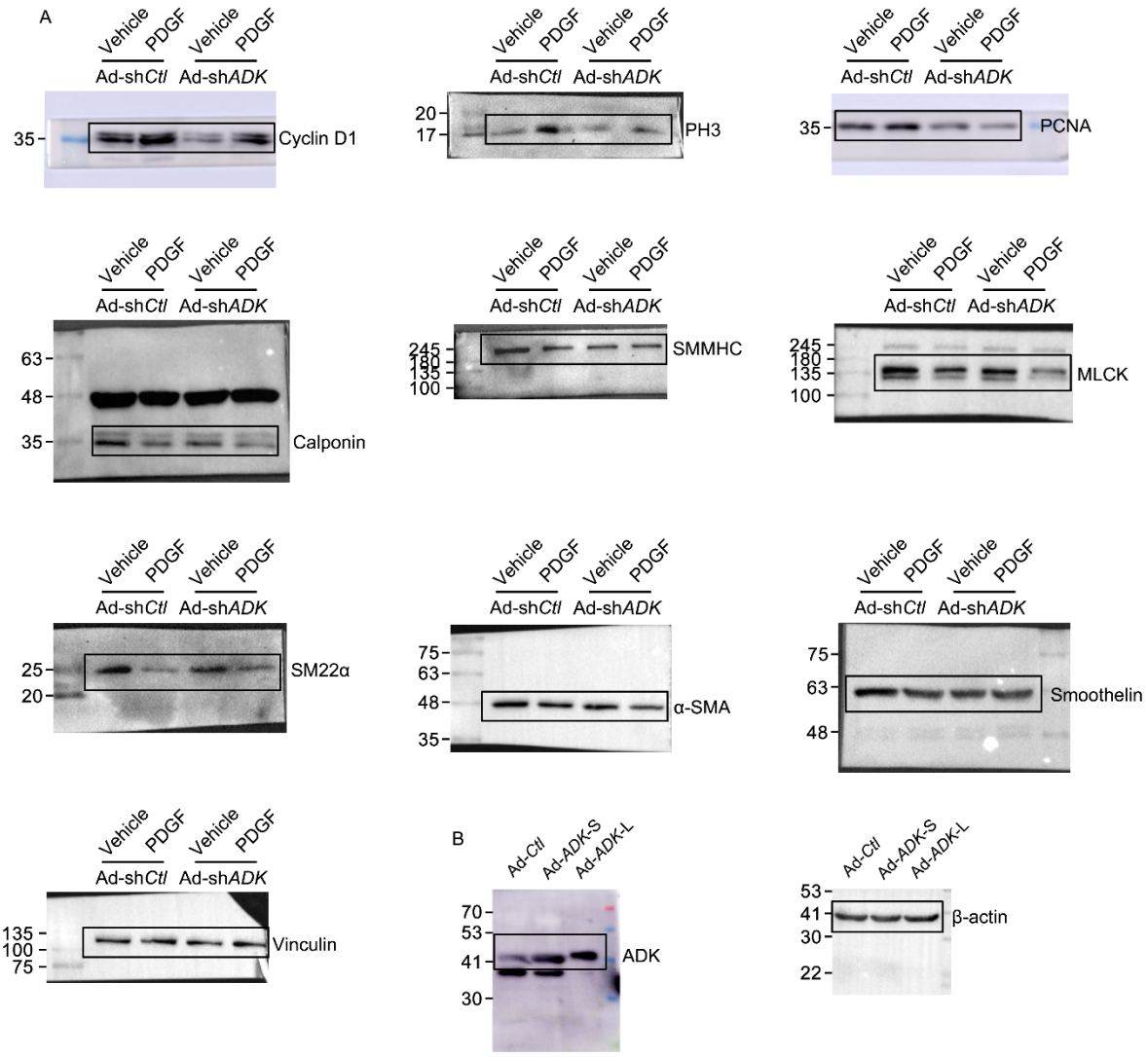
Under prolonged PDGF-BB treatment or pathological conditions for neointima formation **(A)**, the increased level of ADK removes the adenosine and facilitates the SAHH-mediated hydrolysis of SAH, which also accelerates the SAM-dependent hypermethylation of *KLF4* in its promoter. Hypermethylation results in inhibition of *KLF4* transcription and drives VSMC proliferation and neointima formation. Upon genetic or pharmacological inhibition of ADK **(B)**, the elevated level of adenosine inhibits SAHH-mediated hydrolysis of SAH and results in accumulation of SAH, which suppresses PDGF-induced SAM-dependent hypermethylation of *KLF4* in its promoter through a mechanism of product inhibition. Hypomethylation results in the increase of *KLF4* transcription and suppresses excessive VSMC proliferation and neointima formation.



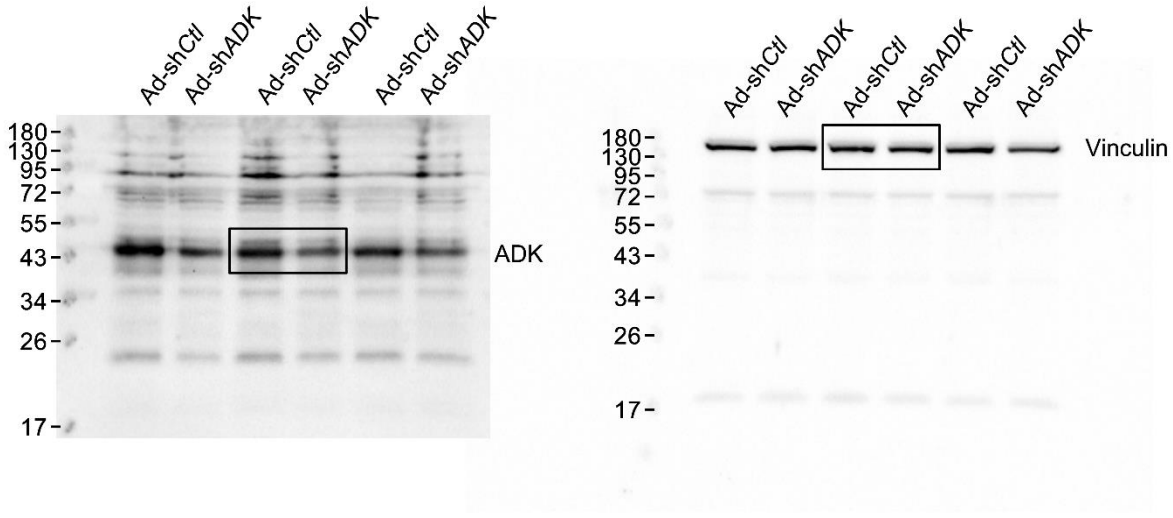
Supplemental Figure 6. Full gel scans for Fig. 1B and 1E. A, Gel scans for Fig. 1B. B, Gel scans for Fig. 1E.



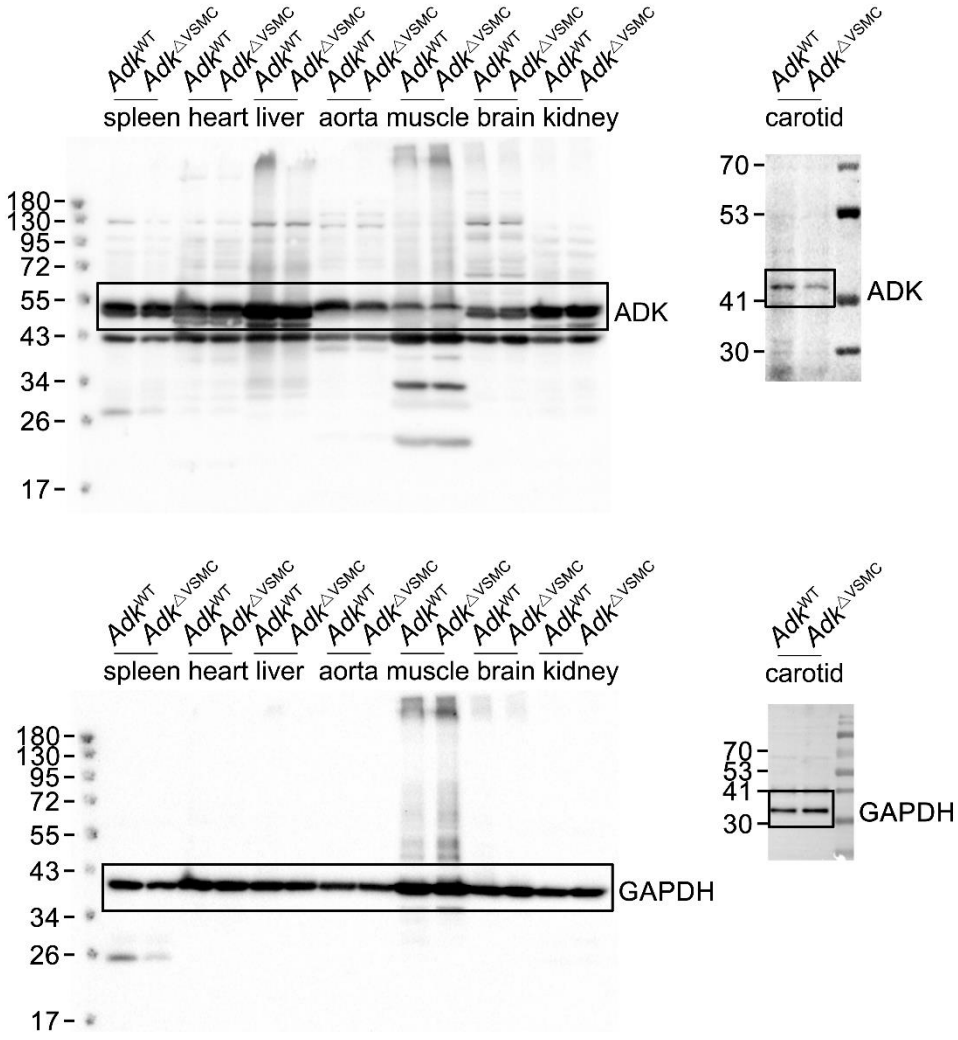
Supplemental Figure 7. Full gel scans for Fig. 2G.



Supplemental Figure 8. Full gel scans for Fig. 3E and 3G. A, Gel scans for Fig. 3E. B, Gel scans for Fig. 3G.



Supplemental Figure 9. Full gel scans for Supp. Fig. 1D.



Supplemental Figure 10. Full gel scans for Supp. Fig. 2C.

Supplemental Tables

Supplementary Table 1: Methylation of SMC proliferation-negative regulated genes in human SMCs

UCSC gene	Gene name	Accession number	probes	M increase	M decrease	M Increase (%)	M Decrease (%)
ESR2	Estrogen receptor beta	NM_001437	18	11	7	61	39
IL15	**Interleukin-15**	**NR_037840**	**20**	**10**	**10**	**50**	**50**
NPR3	Atrial natriuretic peptide receptor 3	NM_000908	16	11	5	68	32
SF1	Splicing factor 1	NM_001178030	14	9	5	64	36
VIPR2	**Vasoactive intestinal polypeptide receptor 2**	**NM_003382**	**7**	**2**	**5**	**28**	**72**
ESR1	**Estrogen receptor**	**NM_001291241**	**50**	**1**	**49**	**2**	**98**
CTNNBIP1	Beta-catenin-interacting protein 1	NM_020248	23	16	7	69	31
PPARD	Peroxisome proliferator-activated receptor delta	NM_001171819	17	9	8	52	48
IGFBP3	Insulin-like growth factor-binding protein 3	NM_000598	32	25	7	78	22
NDRG2	Protein NDRG2 isoform a	NM_201535	14	10	4	71	29
MFN2	Mitofusin-2	NM_001127660	14	8	6	57	43
TRIB1	**Tribbles homolog 1**	**NM_025195**	**16**	**7**	**9**	**43**	**57**
PPARG	Peroxisome proliferator-activated receptor gamma	NM_015869	19	14	5	73	27
PTGIR	**Prostacyclin receptor**	**NM_000960**	**4**	**0**	**4**	**0**	**100**
NDRG4	Protein NDRG4	NR_040072	25	19	6	76	24
KLF4	**Krüppel-like factor 4**	**NM_004235**	**4**	**1**	**3**	**25**	**75**
COMT	**Catechol O-methyltransferase**	**NM_001135161**	**29**	**7**	**22**	**24**	**76**
IL12A	Interleukin-12 subunit alpha	NM_000882	10	8	2	80	20
IGFBP5	Insulin-like growth factor binding protein 5	NM_000599	12	9	3	75	25

APOD	Apolipoprotein D	NM_001647	6	4	2	66	24
ANG	**Angiogenin**	**NM_001145**	**13**	**5**	**8**	**38**	**62**
IFNG	**Interferon gamma**	**NM_000619**	**5**	**0**	**5**	**0**	**100**
NPR1	Atrial natriuretic peptide receptor 1	NM_000906	16	9	7	56	44
ADIPOQ	**Adiponectin**	**NM_001177800**	**8**	**2**	**6**	**25**	**75**
ILK	Integrin-linked protein kinase	NM_004517	19	13	6	68	32
TPM1	**Tropomyosin alpha-1 chain**	**NM_000366**	**13**	**5**	**8**	**38**	**62**
TNFAIP3	Tumor necrosis factor alpha-induced protein 3	NM_001270507	15	10	5	66	34
CAV1	Caveolin-1	NM_001753	18	16	2	88	22
VIP	**VIP peptides**	**NM_003381**	**3**	**1**	**2**	**33**	**67**
IL12B	Interleukin-12 subunit beta	NM_002187	10	7	3	70	30

Supplementary Table 2 Primer sequences used in the study

A. Primers for quantitative RT-PCR

Gene Name	*Species*	*Sequence*
A_1R	Mouse	F: 5’- CTGGGGCACATCTTCGCAGGG- 3’
	Mouse	R: 5’- GCCAGCACCTGTGGTCAGGAAG- 3’
$A_{2A}R$	Mouse	F: 5’- CCGGGTGACCTGTCTGTTTGAGGA- 3’
	Mouse	R: 5’- GCTGTCTCCGGGCTGCCAAAAA- 3’
$A_{2B}R$	Mouse	F: 5’- GAGCTCCATCTTTAGCCTCTTG- 3’
	Mouse	R: 5’- TGTCCCAGTGACCAAACCTT- 3’
A_3R	Mouse	F: 5'- CTCGTCCCCCTGGTTGTCATGTGT- 3'
	Mouse	R: 5'- GGACTTAGCTGTCTTGAACTCCCGTCC- 3'
A_1R	Human	F: 5’- TGCGAGTTCGAGAAGGTCATC- 3’
	Human	R: 5’- AGCTGCTTGCGGATTAGGTA- 3’
$A_{2A}R$	Human	F: 5’- CGAGGGCTAAGGGCATCATTG- 3’
	Human	R: 5’- CTCCTTTGGCTGACCGCAGTT- 3’
$A_{2B}R$	Human	F: 5’- CTCTTCCTCGCCTGCTTCGTG- 3’
	Human	R: 5’- TTATACCTGAGCGGGACACAG- 3’
A_3R	Human	F: 5’- TACATCATTCGGAACAAACTC- 3’
	Human	R: 5’- GTCTTGAACTCCCGTCCATAA- 3’
KLF4	Human	F: 5’- TTCCCATCTCAAGGCACAC- 3’
	Human	R: 5’- GGTCGCATTTTTGGCACT- 3’
PCNA	Human	F: 5’- TCCGCCACCATGTTCGA- 3’
	Human	R: 5’- TATCCCAGCAGGCCTCGTT- 3’
P14arf	Human	F: 5’- ATGGTGCGCAGGTTCTTGG- 3’
	Human	R: 5’- TGCGGGCATGGTTACTGCCTC- 3’
P15ink4b	Human	F: 5’- CGCAGACCCTGCCACTCT- 3’
	Human	R: 5’- AGGCATCGCGCACGTC- 3’
P16ink4a	Human	F: 5’- CCCAACGCACCGAATAGTTA- 3’
	Human	R: 5’- ACCAGCGTGTCCAGGAAG- 3’
P18ink4c	Human	F: 5’- CATCATGCAGCCTGGTTAGG- 3’
	Human	R: 5’- GCTGGCCGTGTGCTTCAC- 3’
P19arf	Human	F: 5’- CCCTCGTGCTGATGCTACTGA- 3’
	Human	R: 5’- ACCACCAGCGTGTCCAGGAA- 3’
P27kip1	Human	F: 5’- TGGAGAAGCACTGCAGAGAC- 3’
	Human	R: 5’- GCGTGTCCTCAGAGTTAGCC- 3’
Cyclin D1	Human	F: 5’- GTGCTGCGAAGTGGAAACC- 3’
	Human	R: 5’- ATCCAGGTGGCGACGATCT- 3’
ADK	Human	F: 5’- TGCCCTAATTGCTTCCTGAG - 3’
	Human	R: 5’- TTGGCATTTAAGTGGCACTATC- 3’
MKLF4	Mouse	F: 5’- GTGCCCCGACTAACCGTTG - 3’
	Mouse	R: 5’- GTCGTTGAACTCCTCGGTCT- 3’
HPRT	Mouse	F: 5’-TGGCCCTCTGTGTGCTCAA- 3’
	Mouse	R: 5’-TGATCATTACAGTAGCTCTTCAGTCTGA- 3’
Hβ-actin	Human	F: 5’- CGAGGCCCAGAGCAAGAGAG- 3’
	Human	R: 5’- CTCGTAGATGGGCACAGTGTG- 3’

B. Primers for evaluation of mRNA expression for genes screened from 450K methylation assay

Gene Name	*Species*	*Sequence*
iL15	Human	F: 5'- AGCCAGCCCATACAAGATCG- 3'
	Human	R: 5'- CACGGTAAATCCTTAAGTAT- 3'
VIPR2	Human	F: 5'- GTTTCCCATCAGCATCTCCT- 3'
	Human	R: 5'- CTGCACCTCACTGTTCAGG- 3'
ESR1	Human	F: 5'- CTGTGAGGGCTGCAAGGCCT- 3'
	Human	R: 5'- CTTCGTAGCATTTGCGGAGC- 3'
TRIB1	Human	F: 5'- GGAGGAGAGAACCCAGCTTA- 3'
	Human	R: 5'- GGAGTAGGTCCCAGTGGTGT- 3'
PTGIR	Human	F: 5'- CTGACACACAGACCGACACA- 3'
	Human	R: 5'- GTCTGGGCTCTCCAGTCTTG- 3'
COMT	Human	F: 5'- AGAAGGAGTGGGCCATGAAC- 3'
	Human	R: 5'- GCACAGCTGAGTAGCCACAG- 3'
ANG	Human	F: 5'- GCCCGTTTCTGCGGACTTGT- 3'
	Human	R: 5'- GCACGAAGACCAACAACAAA- 3'
IFNG	Human	F: 5'- TGTTACTGCCAGGACCCATA- 3'
	Human	R: 5'- TTCTGTCACTCTCCTCTTTCCA- 3'
ADIOQ	Human	F: 5'- CATACCAGAGGAGACGGGAT- 3'
	Human	R: 5'- CATCCTGAGCCCTGATGTC- 3'
TPM1	Human	F: 5'- AGATGAGCTGGTGTCACTGC- 3'
	Human	R: 5'- CTCCTGGGCATCTTTGAGAG- 3'
VIP	Human	F: 5'- AGCTCCTTGTGCTCCTGACT- 3'
	Human	R: 5'- GGGTATTCTGTCACCCAACC- 3'

C. Primers used for human *KLF4* gene MS sequence

Gene Name	*Species*	*Sequence*
KLF4	Human	F: 5'- GAGTYGATAATGGYGGTGAGTA- 3'
	Human	R: 5'- CRCCAAATAAAACTAACTACCR- 3'

D. Human *KLF4* si-RNA sequence

Gene Name	*Species*	*Sequence*
KLF4 S1	Human	Sense: 5'- UGACCAGGCACUACCGUAATT- 3'
		Antisense: 5'- UUACGGUAGUGCCUGGUCAGT- 3'
KLF4 S2	Human	Sense: 5'- GCAGCUUCACCUAUCCGAUTT- 3'
		Antisense: 5'- AUCGGAUAGGUGAAGCUGCAG- 3'