SUPPLEMENTAL MATERIAL

ADAR1-mediated RNA editing, a novel mechanism controlling phenotypic modulation of vascular smooth muscle cells

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Detailed Methods

Animals: Male Sprague-Dawley rats weighing 450-500 g were purchased from Harlan. Male ADAR1+/- mice (B6.129(Cg)-ADAR$^{tm1.1phs}$, Stock # 034620-JAX) were purchased from Mutant Mouse Regional Resource Centers (MMRRC, USA). The mice have been backcrossed with C57BL6 mice more than 10 passage. The control littermates were obtained by crossing ADAR+/- male with female mice. Animals were housed under conventional conditions in the animal care facilities. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia.

Cell culture and transfection: Rat primary aortic SMCs (SMCs) were cultured by enzyme digestion method from rat thoracic aorta as described previously.[1] RASMCs were confirmed by expression of smooth muscle α-actin and SM22α. The SMC was maintained in DMEM with 10% FBS and cultured up to 6 passages. For testing the effect of ADAR1 editing function on SMC pre-mRNA splicing, SMC at passage 2 were cultured to 40% confluence and then transfected with pDONR-221 (empty vector control)), pDONR-221-ADAR1, or pDONR-221-ADAR1 with editase motif mutation (pDONR-221-ADAR1$^{\Delta editase}$ using Lipofectamine LTX reagents (Life Technology) followed by starvation for 48 h and then analysis of mature and pre-mRNA levels or protein expression of SMC markers.

Construction of adenoviral vector: Adenovirus expressing ADAR1 shRNA was generated as described previously.[2] ADAR1 shRNA (shADAR1) coding sequences were: 5'- CGC GTC GAT ACA ATC CTC AGT GTT CTC TGT ATG TAT TCA AGA GAT ACA TAC AGA GAA CAC TGA GGA TTG TAT CTT TTT TCC AAA-3' (top strand) and 5'-AGC CAG GAT TTG GCG GAC CTT CTC GAA CCA CAA GTT CTC TGT GGT TCG AGA AGG TCC GCC AAA TCC TGG AAA AAA GTT TTC GA-3' (bottom strand). Both strands were annealed into pRNAT-H1.1/Adeno (Genscript corporation) digested with Mlu I and Hind III. Recombinant adenoviral vector was produced by homologous recombination in AD-1 competent cells following manufacturer's instruction (Agilent). The resultant recombinant vector was digested with Pac I and transfected into AD-293 cells with Lipofectamine LTX and Plus (Invitrogen) in order to package viral particles expressing shADAR1 (Ad-shADAR1). The adenovirus was purified with gradient density ultracentrifugation of cesium chloride and dialyzed in a dialysis buffer (135 mM NaCl, 1 mM MgCl2, 10 mM Tris-HCl, pH 7.5, 10% glycerol). Green fluorescent protein (GFP)-expressing adenovirus (Ad-GFP) was used as a control.

Reverse transcription (RT)-PCR: Total RNA was isolated from SMCs or artery tissues homogenized in Trizol reagents (Life Technologies). Reverse transcription was performed using iScript Select cDNA synthesis Kit (Bio-Rad). Pre- and mature- mRNA of genes interested was amplified using specific primers

and Advantage Long polymerase mix (Clontech). The primer sequences were listed in Online Table 1 below. The same pair of primers was used to amplify both pre- and mature RNA of SMMHC intron #12. The mature mRNA was distinguished from its pre-mRNA by the size of the PCR product in the agarose gel based on the predicted length of mRNA fragments. For the large-sized intron regions (α-SMA intron #1, SMMHC intron #25, #28, and #32), the primers were designed based on the intron sequences and the mature mRNA and pre-mRNA were amplified by using different sets of primers. The pre- and mature mRNA levels were quantified by normalizing to the expression of GAPDH. To prevent the potential genomic DNA contamination during PCR reaction, RNA samples were treated with DNase I followed by enzyme inactivation at 65℃ for 15 min prior to the reverse transcription. To ensure a quantitative analysis, the reverse transcription reaction were performed using the identical amount of starting RNA template for different samples, and the PCR cycle was controlled in the exponential phase. In addition, an internal control whose expression was not altered by the treatments was amplified in parallel with the target genes. Moreover, using the same primers to amplify both mature- and pre-mRNA in the same reaction allowed a more accurately correlation for their changes.

Western blot: ADAR1 antibody was purchased from Santa Cruz. SMMHC, α-SMA, and α-tubulin antibody was purchased from Abcam or Sigma. To detect their protein expression, 20 μg of proteins were separated on 10% SDS-PAGE and were transferred to PVDF membrane (Bio-Rad). The immunoblots were then analyzed using chemiluminescence method (Millipore). The protein expression was quantified by normalizing to α-tubulin.

Cloning and sequencing of pre-mRNA cDNA: SMMHC and α-SMA pre-mRNAs were reverse transcribed, amplified, and purified using Gel Extraction kit (Qiagen). The purified fragments were cloned into pGEM-T easy vector (Promega) followed by sequencing to detect A-I editing. The I was recognized as G in reverse transcribed cDNA. The A-I editing was identified by comparing the pre-mRNA reverse-transcribed cDNA sequence with the genomic DNA sequence in control cells, or the splicing inhibitor-treated contractile SMC.

RNA secondary structure prediction: Secondary structure of normal or editing pre-mRNA fragments was predicted based on the sequencing data using mfold web servers (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form). Free energy of thermodynamic ensemble of the normal or edited pre-mRNA was analyzed using Vienna RNA web servers (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

Contractility assay: SMCs were transduced with adenovirus expressing control or ADAR1 shRNA followed by PDGF-BB treatment for 2 days. Cells were then washed once with PBS followed by stimulation with 75 mM KCl in the Krebs' solution as described previously.[3] Contractility of the cells was observed with an inverted microscope for up to 10 min. Images of the same field before and after KCl treatment were snapped and compared. Cellular contraction was quantified by measuring the 2-dimensional cell perimeter using Image J software. The experiments were repeated three times with triplicates, and the fields in each sample were randomly selected. The cell perimeters of 12 cells each treatment were used for quantification.

Rat carotid artery injury model: Rat carotid artery balloon injury and adenovirus transduction into injured artery were performed as described previously[2]. Briefly, rats were anesthetized, and a 2F Fogarty arterial embolectomy balloon catheter (Baxter Edwards Healthcare) was introduced through left external carotid artery and advanced 4 cm toward the thoracic aorta. The balloon was inflated and withdrawn through the common carotid artery to the carotid bifurcation with constant rotation during denudation of the endothelium. For introducing adenovirus, the injured vessel segment from the proximal edge of omohyoid muscle to the carotid bifurcation was washed with saline, and incubated with 100 μl saline or adenovirus ($5x10^9$ pfu) expressing GFP or shADAR1 for 20 minutes. 14 days later, the balloon-injured and adenovirus-dwelled segment was perfused with saline and removed. The vessel segments were then fixed with 4%

paraformaldehyde and embedded in paraffin. Subsequent morphometric analyses were performed in a double-blinded manner.

Mouse wire injury model: ADAR1+/- mice and its littermates were anesthetized, and carotid artery injury was performed using 0.15 inches straight tipped wire catheter (Cook Medical Inc) with the similar procedure as for rat balloon-injury model. 28 days later, the injured arteries were excised and fixed with 4% paraformaldehyde followed by morphometric analyses in a double-blinded manner.

Histomorphometric analysis and immunohistochemistry (IHC) staining: Artery segments were cut by serial sectioning (5 μm). Ten sections that were evenly distributed in the vessel segment were collected for analysis[2]. The sections were stained with modified hematoxylin and eosin or Elastica van Gieson staining. Cross-section images were captured with a Nikon microscope. The areas of the lumen, internal elastic lamina, and external elastic lamina were measured using Image-pro Plus Software. For immunohistochemistry, sections were rehydrated, blocked with 5% goat serum and permeabilized with 0.01% Triton X-100 in PBS, and incubated with ADAR1 antibody (Santa Cruz) overnight at 4 ºC followed by incubation with HRP-conjugated secondary antibody. The sections were counterstained with hematoxylin.

Site-directed mutagenesis of ADAR1 editase domain: pDONR-221-ADAR1 expression plasmid was purchased from the Biodesign Institute/Arizona State University and used as template and wild type control for editase motif mutant ADAR1, which was generated by converting Lycine to Arginine at the amino acid 258 through replacing the A with G at nucleotide 776 in ADAR1 cDNA as described previously using Quick Change Mutagenesis Kit (Aglient) [4]. The primer sequences used for constructing the mutant ADAR1 were listed in Supplemental Table S1. The nucleotide substitution was confirmed by sequencing.

Statistical analysis: Pre-mRNA, mature mRNA, and protein expression results were expressed as MEAN±SEM. One way ANOVA was used for comparison among groups. Significance was confirmed by post-hoc analysis using Fisher's Least Significant Difference (Fisher's LSD) test. $P<0.05$ was considered statistical significant.

Reference:

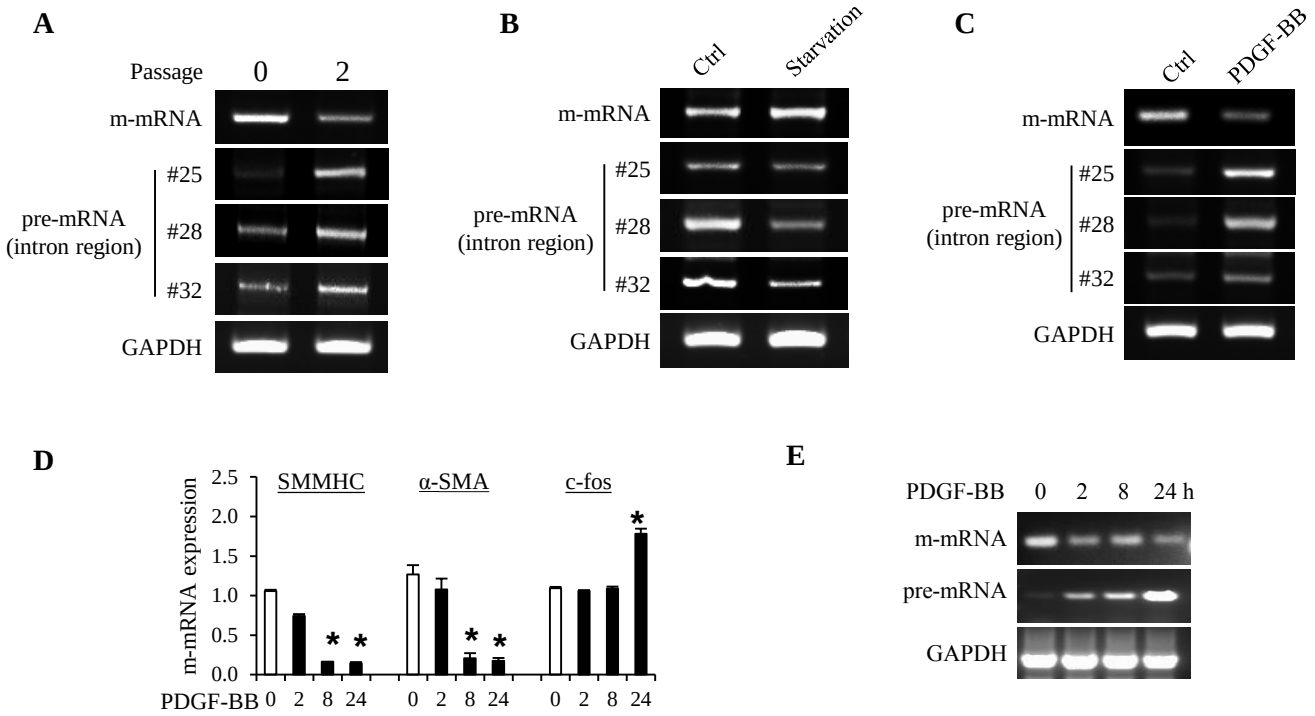
1. Christen T, Bochaton-Piallat ML, Neuville P, Rensen S, Redard M, van Eys G and Gabbiani G. Cultured porcine coronary artery smooth muscle cells. A new model with advanced differentiation. *Circ Res*. 1999;85:99-107.
2. Wang JN, Shi N, Xie WB, Guo X and Chen SY. Response gene to complement 32 promotes vascular lesion formation through stimulation of smooth muscle cell proliferation and migration. *Arteriosclerosis, thrombosis, and vascular biology*. 2011;31:e19-26.
3. Guo X, Stice SL, Boyd NL and Chen SY. A novel in vitro model system for smooth muscle differentiation from human embryonic stem cell-derived mesenchymal cells. *Am J Physiol Cell Physiol*. 2013;304:C289-98.
4. Kim U, Wang Y, Sanford T, Zeng Y and Nishikura K. Molecular cloning of cDNA for double-stranded RNA adenosine deaminase, a candidate enzyme for nuclear RNA editing. *Proc Natl Acad Sci U S A*. 1994;91:11457-61.

Online Table I: Primers used in RT-PCR and site-mutagenesis.

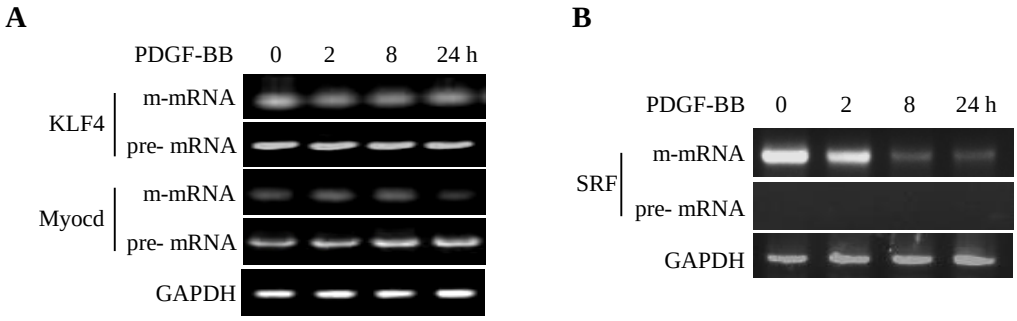
Domain	Sequence*
α-SMA (intron #1)	F: CAGTCGCCATCAGGGTAAGT
	R: CGAGGTTCTGCAGAGACAGA
α-SMA m-mRNA	F: GGAGAAGCCCAGCCAGTC
	R: CACGATGGATGGGAAAACAG
SMMHC (intron #12)	F: ATCATCCGTCCTTCAGCTTG
	R: TCTGAGCTTTCTGCACCACA
SMMHC (intron #25)	F: AGGTCCAGTTGGGTCTGATG
	R: GGTGGTACCTGCTCCAAGAA
SMMHC (intron #28)	F: CCATTAAACTGGCCAAGGAA
	R: TGGACACATTGAGCTTCTGC
SMMHC (intron #32)	F: GGGATGAGCAGAATGAGGAG
	R: CAACACAAGCATCACCAACC
SMMHC m-mRNA	F: CTACTCGGGCCTCTTCTGTG
(for pre-mRNA #25, #28, #32)	R: TTCCAGCTCCAGACTCACCT
SRF (intron #2)	F: ACAGGGACACAGGTGCTGTT
	R: ACTGGGGCTGACACTAGCAG
KLF4 (intron #2)	F: GCGAGTCTGACATGGCTGT
	R: TGGGCTTCCTTTGCTAACAC
Myocardin (intron #3)	F: AGTTCCATGACCCGAGGAA
	R: CGGTCCGATCTGTTTCTGAC
c-fos (intron #2)	F: CGCAGATCTGTCCGTCTCTA
	R: GCTGCAGCCATCTTATTCCT
ADAR1	F: CTTAGAAAGGCAAGGCGATG
	R: GAGGTGCTTTGAGTGGCTTC
ADAR2	F: CAAGTACCGCCTGAAGAAGC
	R: AAAGACCTGCCCGTTTACCT
GAPDH	F: AGACAGCCGCATCTTCTTGT
	R: CTTGCCGTGGGTAGAGTCAT
$ADAR1^{\Delta editase}$ (A776G)	F: CAAACCCCAGGAGGAAAGGAACTTTTATCTCTGCC
	R: GGCAGAGATAAAAGTTCCTTTCCTCCTGGGGTTTG

*F: Forward primer; R: Reverse primer.

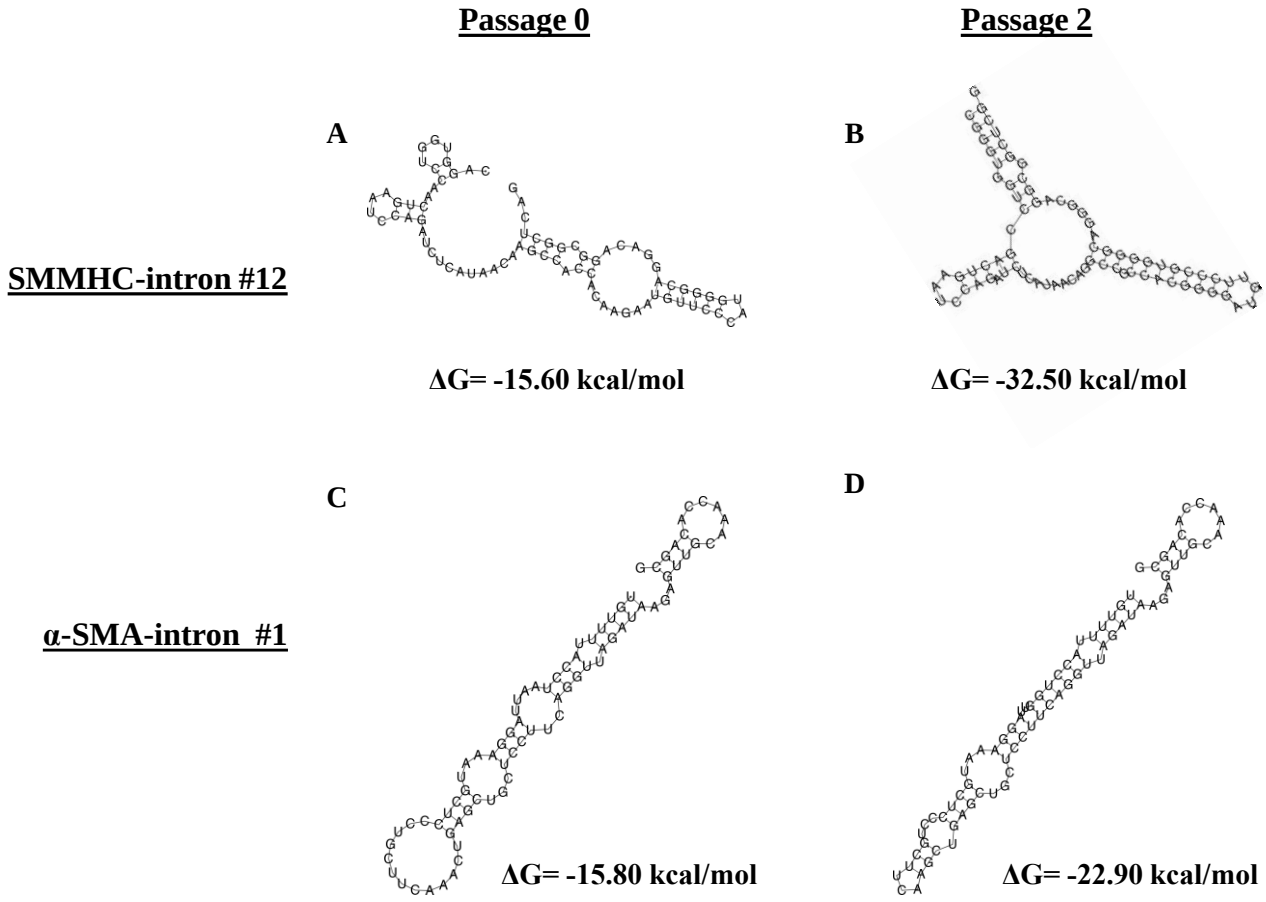
Online Figures



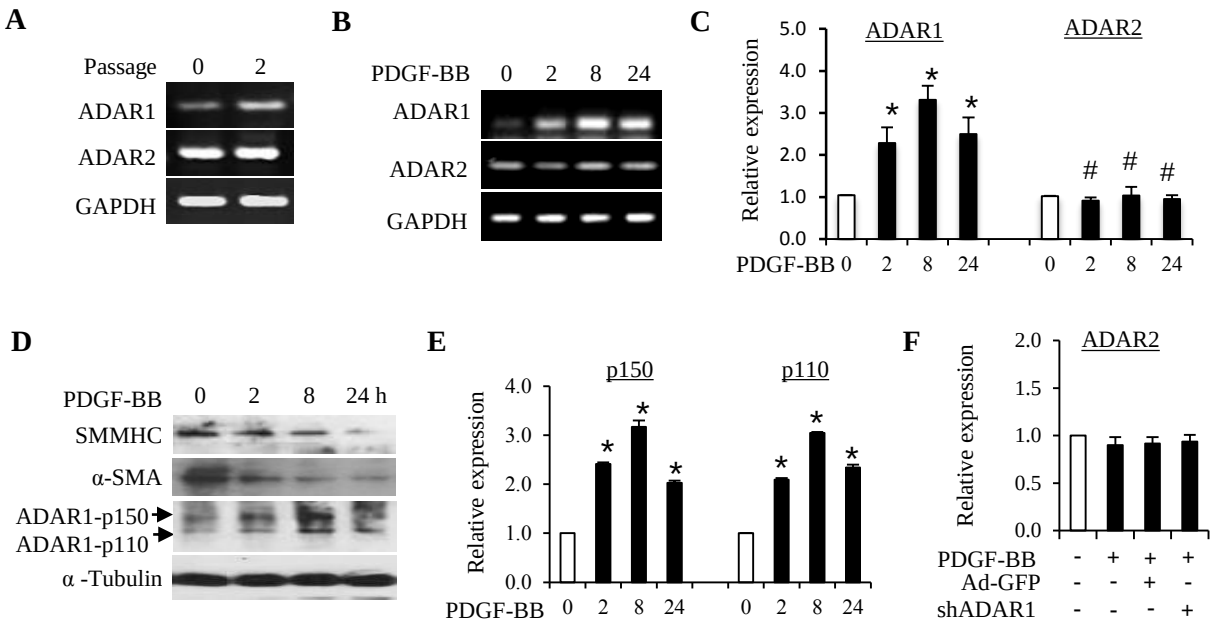
Online Figure I. Pre-mRNA splicing of SMC markers. (A) SMMHC pe-mRNA transcripts from different SMMHC intron regions (as indicated) were increased in rat SMC after passaging. (B) Serum starvation increased mature SMMHC mRNA level while decreasing the levels of pe-mRNA transcripts from different SMMHC intron regions in rat SMC. (C) PDGF-BB induced abnormal splicing of SMMHC pre-mRNA at different intron regions as indicated. (D) Quantification of mature mRNA levels shown in Figure 1F by normalizing to GAPDH level. *P<0.05 compared to vehicle-treated group (0 h) for each corresponding gene (n=4). (E) PDGF-BB induced an abnormal splicing of SMMHC pre-mRNA transcript in the region of intron #5 in human SMCs, similarly as in rat SMC.



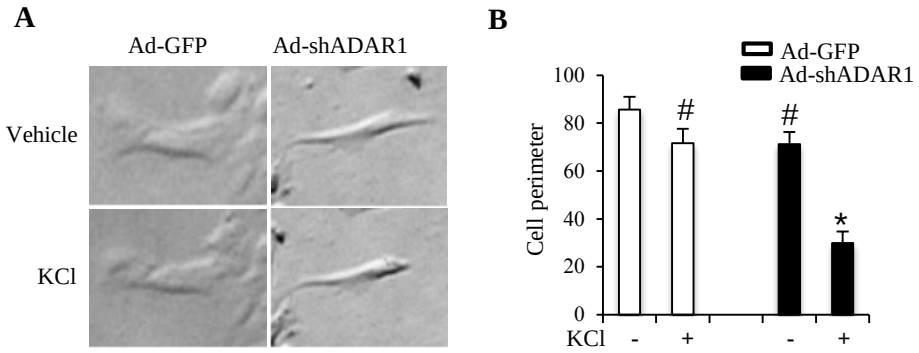
Online Figure II. Pre-mRNA splicing of SMC phenotype-related genes. (A) The pre-mRNA splicing of KLF4 and myocardin (Myocd) was not altered during PDGF-BB-induced SMC phenotypic modulation. (B) The pre-mRNA splicing of serum response factor (SRF) was not altered during PDGF-BB-induced SMC phenotypic modulation.



Online Figure III. RNA editing altered pre-mRNA secondary structures and decreased thermodynamic free energy. (A) Predicted control pre-mRNA secondary structure of a small region in SMMHC intron #12 and its free energy. (B) Predicted pre-mRNA secondary structure of the small region in SMMHC intron #12 with RNA editing and its free energy. (C) Predicted normal pre-mRNA secondary structure of a small region in α-SMA intron #1 and free energy. (D) Predicted pre-mRNA secondary structure of the small region in α-SMA intron #1 with RNA editing and its free energy. The sequences are shown in Fig 2A for A and B, and Fig 2B for C and D.



Online Figure IV. ADAR1 and ADAR2 expression during SMC phenotypic modulation. (A) Arterial SMC culture for 2 passages resulted in ADAR1, but not ADAR2 induction. (B) PDGF-BB induced mRNA expression of ADAR1, but not ADAR2, in a time dependent manner. (C) Quantification of ADAR1 and ADAR2 expression shown in B. *P<0.01, #P>0.05 compared to vehicle-treated cells (0 h). (D) PDGF-BB induced a time-dependent ADAR1 protein expression while down-regulating SMMHC and α-SMA protein levels. (E) Quantification of the protein expression of two ADAR1 isoforms (p150 and p110) shown in D. *P<0.01 compared to the vehicle-treated group (0 h). (F) Quantification of ADAR2 protein expression by normalizing to α-Tubulin as shown in Figure 2C.



Online Figure V. ADAR1 was important for synthetic SMC phenotype. (A) Knockdown of ADAR1 induced contractile SMC phenotype. ADAR1 shRNA (Ad-shADAR1) changed the flatten-shaped SMC morphology to spindle-shaped morphology. Ad-shADAR1-treated cells contracted in response to KCl stimulation. **(B)** Quantification of the contraction by measuring the alteration of cell perimeters before and after KCl treatment. #P>0.05 compared to Ad-GFP-transduced cells with vehicle treatment, *P<0.05 compared to Ad-shADAR1-transduced cells with KCl treatment (-) (n = 12).

A

SMMHC Intron # 12

809831, MYH11 12th IVS

Isoginkgetin: ACACACATATGCACAAATAAAACTAAAACCTTTAAAAAG**A**GATCCTACTATAAAGTGTTATTTCTCTGAAATTCTTGTTG
PDGF-BB: ACACACATATGCACAAATAAAACTAAAACCTTTAAAAAG**G**GATCCTACTATAAAGTGTTATTTCTCTGAAATTCTTGTTG

Isoginkgetin : AACTCAGAACCCTGGTTCCTCTCTGGATTTTCCACCACTGATTCTGGCATCTTTCTTGAA**A**ATACTAAAAATTTCTGTTT
PDGF-BB: AACTCAGAACCCTGGTTCCTCTCTGGATTTTCCACCACTGATTCTGGCATCTTTCTTGAA**G**ATACTAAAAATTTCTGTTT

809981, MYH11 12th IVS

B

α-SMA Intron #1

252549912, α-SMA 1st IVS

Isoginkgetin: **A**GGAGTGAACTGGCGCGGTTGCCTGCGCTCTGGTTTTGGCTGAGTGGACTGCGTTGCCTCTGGGTTTCCGGGGCTCTAACAGTAGA
PDGF-BB: **G**GGAGTGAACTGGCGCGGTTGCCTGCGCTCTGGTTTTGGCTGAGTGGACTGCGTTGCCTCTGGGTTTCCGGGGCTCTAACAGTAGA

Isoginkgetin: CATGTATATCTTGTGCCCTTACGATTCAAACCTATGTCATTGGTCATTTGCAGCAAAGCATAGCTCCTCT**A**CTCTCTGCA**A**A
PDGF-BB: CATGTATATCTTGTGCCCTTACGATTCAAACCTATGTCATTGGTCATTTGCAGCAAAGCATAGCTCCTCT**G**CTCTCTGCA**G**A

252549745, α-SMA 1st IVS

Online Figure VI: SMC marker pre-mRNA editing was observed in phenotypically modulated SMC, but not in contractile SMC. Starved rat SMC was treated with splicing inhibitor Isoginkgetin (33 μM) or PDGF-BB (20 ng/ml) for 24 h. SMMHC and α-SMA pre-mRNA cDNAs were cloned and sequenced. The RNA edits in the pre-mRNAs of SMMHC (A) and α-SMA (B) were observed in SMCs treated with PDGF-BB, but not with isoginkgetin.

SMMHC pre-mRNA

809689, MYH11 12th IVS

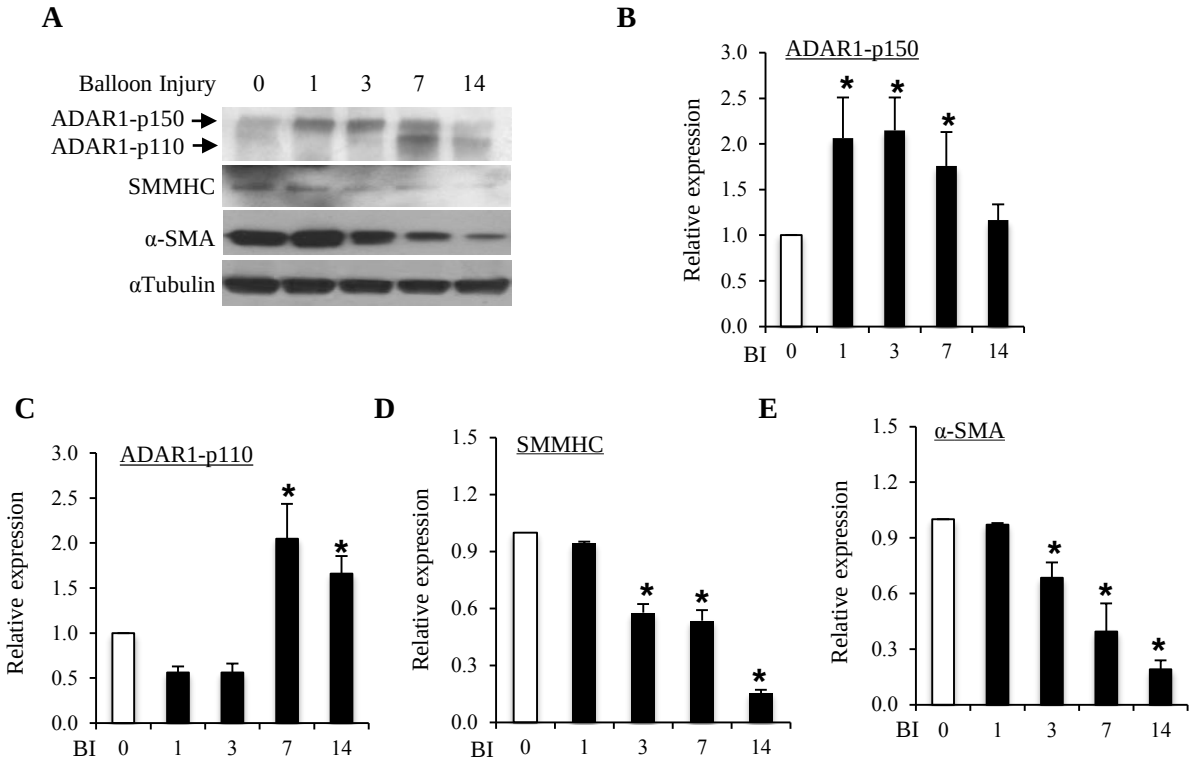
Control: GTGTGATTCTCAG**CACATC**AGTCAGGAATTTCACACCTTCCTGTTAACTTCATCTCTCCA
Injury: GTGTGATTCTCAGCAC**G**TCAGTCAGGAATTTCACACCTTCCTGTTAACTTCATCTCTCCA

Control: GATATCCACCACTCTTTTTAGTACCCACATGCACATGTGCCT**ACACACACACAC**ACACAC
Injury: GATATCCACCACTCTTTTTAGTACCCACATGCACATGTGCCT----------ACACAC

Control: ACACACACACACACACACACACACACACACATATGCACAAATAAAACTAA**A**ACCTTTAAAAA
Injury: ACACACACACACACACACACACACACACACATATGCACAAATAAAACTAA**G**ACCTTTAAAAA

809807, MYH11 12th IVS

Online Figure VII: RNA editing of SMMHC pre-mRNA occurred in balloon-injured rat carotid artery *in vivo*. Pre-mRNA transcript of SMMHC was amplified from RNA samples extracted from control or the injured rat carotid arteries. The transcripts from injured arteries and genomic DNA isolated from the control were cloned and sequenced. A-I (G) edits were observed in SMMHC pre-mRNA. Several CA repeats were deleted in the SMMHC pre-mRNA from the injured artery. Note that the RNA editing in SMMHC pre-mRNA caused a mutation in the binding site (blue rectangle box) of the splicing factor Serine/arginine-rich (SR) proteins SRp55. There was also a deletion in the CA repeats (red fonts) in phenotypically modulated SMC. CA repeats are involved in pre-mRNA splicing.



Online Figure VIII. ADAR1 was induced in balloon-injured arteries along with the down-regulation of SMC contractile proteins. (A) Balloon injury (BI) induced a time-dependent expression of ADAR1 while down-regulated the SMC contractile proteins SMMHC and α-SMA in rat carotid arteries. (B-E) Quantification of the protein expression of two ADAR1 isoforms (B-C), SMMHC (D), and α-SMA (E) shown in A. *P<0.01 compared to the right arteries without injury (0 h) for each individual protein, respectively (n = 7).