SUPPLEMENTAL MATERIALS

Title: Excessive EP4 signaling in smooth muscle cells induces abdominal aortic aneurysm by amplifying inflammation

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SUPPLEMENTAL MATERIAL

Detailed Methods

Reagents

EP4 agonist (ONO-AE1-329) and EP4 antagonist (ONO-AE3-208) were kindly provided by Ono Pharmaceutical Company (Osaka, Japan). An anti-IL-6R antibody (MR16-1) was kindly provided by Chugai Pharmaceutical Company (Tokyo, Japan). Indomethacin and BAY 11-10782 were purchased from Tokyo Chemical Industry (Tokyo, Japan). Prostaglandin E_2 (PGE_2), U0126, and 5Z-7-oxozeaenol were purchased from Calbiochem (Billerica, MA, USA). SB203580 and SP600125 were purchased from Cell Signaling Technology (Danvers, MA, USA). IKK16, SN50, and SB225002 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). An antibody for GAPDH (#sc-25778) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies for phospho-TAK1 (Ser412) (#9339), phospho-TAK1 (Thr187) (#4536), TAK1 (#4505), phospho-p38 (#9211), p38 (#9212), phospho-JNK (#9251), JNK (#9252), and phospho-IKKα/β (#2697) were purchased from Cell Signaling Technology. Antibodies for IL-6 (AB-206-NA for human samples, AB-406-NA for mouse samples) were purchased from R&D Systems (Minneapolis, MN, USA). Antibody for lysyl oxidase (#ab31238), and CD68 (#ab125212) were purchased from Abcam (Cambridge, UK). Antibody for CD45.2 (#109822), CD11b (#101245), Ly-6G (#127607), and Ly-6C (#128008) were purchased from BioLegend (SanDiego, CA, USA). Antibody for F4/80 (#MCA497) and CD68 (#MCA1957T) were purchased from Bio-Rad (Hercules, CA, USA). An antibody for smooth muscle actin (#A2547) was purchased from Sigma-Aldrich (St Louis, MO, USA). Rat IgG was purchased from BioX Cell (West Lebanon, NH, USA). Antibody for mouse IgG, Alexa Fluor 594 (#A11005), rabbit IgG, Alexa Fluor 488 and 546 (#A11008 and A10040), and goat IgG, Alexa Fluor488 (#A11055) were purchased from Invitrogen (Carlsbad, CA, USA). An antibody for mouse CXCL1 was purchased from NOVUS (Centennial, CO, USA).

Measurement of blood pressure

Blood pressure of mice was measured by the tail-cuff method using a BP-98A-L (Softron, Tokyo, Japan) in a quiet room at 7–10 AM. Blood pressure was calculated as the average of ten measurements taken on the same day for each mouse.

Cell isolation and culture

Adult mouse aortic smooth muscle cells were obtained by the explant method as previously described[1]. Mice were euthanized with pentobarbital (13 mg, i.p.), and the descending aortas were collected. Aortic tissues were digested with collagenase II (Worthington) solution at 37 °C for 3 minutes. Adventitial tissue was removed with forceps, and the tunica media was cut into 1-mm-square pieces. The pieces were put on a 60-mm dish coated with fibronectin (Sigma-Aldrich, 10 g/ml × 3 ml) and cultured in DMEM (Sigma-Aldrich) containing 10% FBS for three weeks until the

cells migrated onto the dish. Human aortic smooth muscle cells derived from AAA were obtained as described elsewhere[3]. Cells below passage 8 were used in the experiments. All cells were cultured in a moist tissue culture incubator at 37 °C in 5% CO_2-95% ambient air.

In-vitro assays

VSMCs were plated on 96-well plates at 1×10^4 cells/well for analysis of IL-6 expression in culture media, on 12-well plates at 8×10^4 cells/well for RNA or protein extraction from cell lysates, or on 6-well plates at 1.5×10^5 cells/well for analysis of LOX expression in culture media. Cells were serum-starved for 24 h and then stimulated with PGE_2 (1 μmol/L) or EP4 agonist (ONO-AE1-329, 1 μmol/L). For microarray analysis, aortic VSMCs isolated from two individual EP4-Tg mice were stimulated with PGE_2 for 24 h. For human aortic VSMC culture, DMEM was used for starvation. To inhibit endogenous PGE_2 production by VSMCs, indomethacin was administered at 100 μmol/L for 1 h before and during stimulation with PGE_2 or EP4 agonist. ONO-AE3-208 (1 μmol/L), H89 (10 μmol/L), 5Z-7-oxozeaenol (0.5 μmol/L), U0126 (10 μmol/L), SB206580 (10 μmol/L), SP600125 (50 μmol/L), IKK16 (3 μmol/L) and SN50 (20 μmol/L) were administered in the same manner as indomethacin.

Measurement of intracellular cAMP concentration

After aortic VSMCs derived from EP4-Tg mice were cultured on 24-well plates with 10%FBS/DMEM, VSMCs were serum-starved for 24 h. VSMCs were treated with AE1-329 for 10 min followed by indomethacin for 1 h. According to the manufacturer's instructions, ASMCs were lysed with 120 μl of 0.25% solution of dodecyltrimethylammonium bromide, and 100 μl of the lysate was used for the measurement of cAMP using an enzyme linked immunosorbent assay (ELISA) (RPN225, GE Healthcare Life Sciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Cell viability assay

After EP4-Tg VSMCs were cultured on 96-well plates with 10%FBS/DMEM. The VSMCs were treated with AE1-329 for 24 h. According to the manufacturer's instructions, EP4-Tg VSMCs were incubated reagents for XTT assay (#20-300-1000, Biological Industries, CT, USA) for 2 h.

Tissue and section staining

For evaluation of elastic fiber formation, aorta tissue sections were subjected to Elastica van Gieson staining (Muto Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions. Immunohistochemical analysis and immunofluorescent imaging were performed as described[2].

Elastin degradation grade

To assess the severity of elastin layer destruction, elastin degradation grade was evaluated in EVG-stained tissue sections from the aorta. Elastin degradation grade ranged from Grade 1-4. Grade 1 represented a normal elastin layer, grade 2 represented minor breakdown of the elastin

layer, grade 3 represented some elastin layer breakdown and grade 4 represented loss or rupture of elastin layer. Each aortic section was separated into six equal parts. Each part was assessed for elastin degradation grade and the average was calculated and used as the overall elastin degradation grade.

Gelatin zymography

MMP-9 activities were evaluated by gelatin zymography as described elsewhere[3]. Murine aorta tissue was freed of connective tissues and lysed in neutral lysis buffer. Total proteins (5 μg) were assayed.

Quantification of protein expression

Protein expression was determined by ELISA (IL-6) according to the manufacturer's instructions (R&D Systems). Abundance of IL-6 protein in aortic tissues was normalized by total protein concentration determined by Bradford assay. Abundance of LOX protein in culture media was determined by Western Blotting. Medium for LOX expression level analysis was condensed using Centrifugal Filter Units (UFC5010, MERCK Milipore, Burlington, MA, USA).

Collection of human aorta specimens

Tissues from AAA (*n* = 7) walls were collected from patients undergoing open-repair surgery at Yokohama City University and Yokohama City University Medical Center. Excised tissues were put in ice-cold physiological salt solution and immediately taken to the Cardiovascular Research Institute for analysis within 3 h after excision. Tissues were either fixed in 4% paraformaldehyde for histological analysis or were subjected to primary culture. Non aneurysmal abdominal aortic control samples (*n* = 6) were collected at autopsies. All specimens from human samples were approved by Institutional Review Board at Yokohama City University (B130307001).

Gene-Set Enrichment Analysis (GSEA)

Microarray was performed using SurePrint G3 Mouse GE 8x60K Microarray (Order number 252800515849, Agilent, Santa Clara, CA, USA). The data of this microarray were deposited to public database (accession number: GSE146758). GSEA was performed using the Broad Institute algorithm v2.2.2 on all the probe sets with a gene name. The data were classified and tested based on molecular function derived from Gene Ontology (GO) terms (c5.mf.v5.1.symbols.gmt).

Quantitative reverse transcriptase-PCR

Reverse transcription was performed using a PrimeScript RT reagent kit (TaKaRa Bio, Shiga, Japan) and quantitative reverse transcriptase-PCR (RT-PCR) was performed using either SYBR Premix Ex Taq Tli RNaseH Plus (TaKaRa Bio) or Taqman gene expression assay (Applied Biosystems, Waltham, MA, USA). The expression of each gene was calculated as the abundance relative to that of 18S ribosomal RNA using the ΔΔCT method. The sequences of primers used in

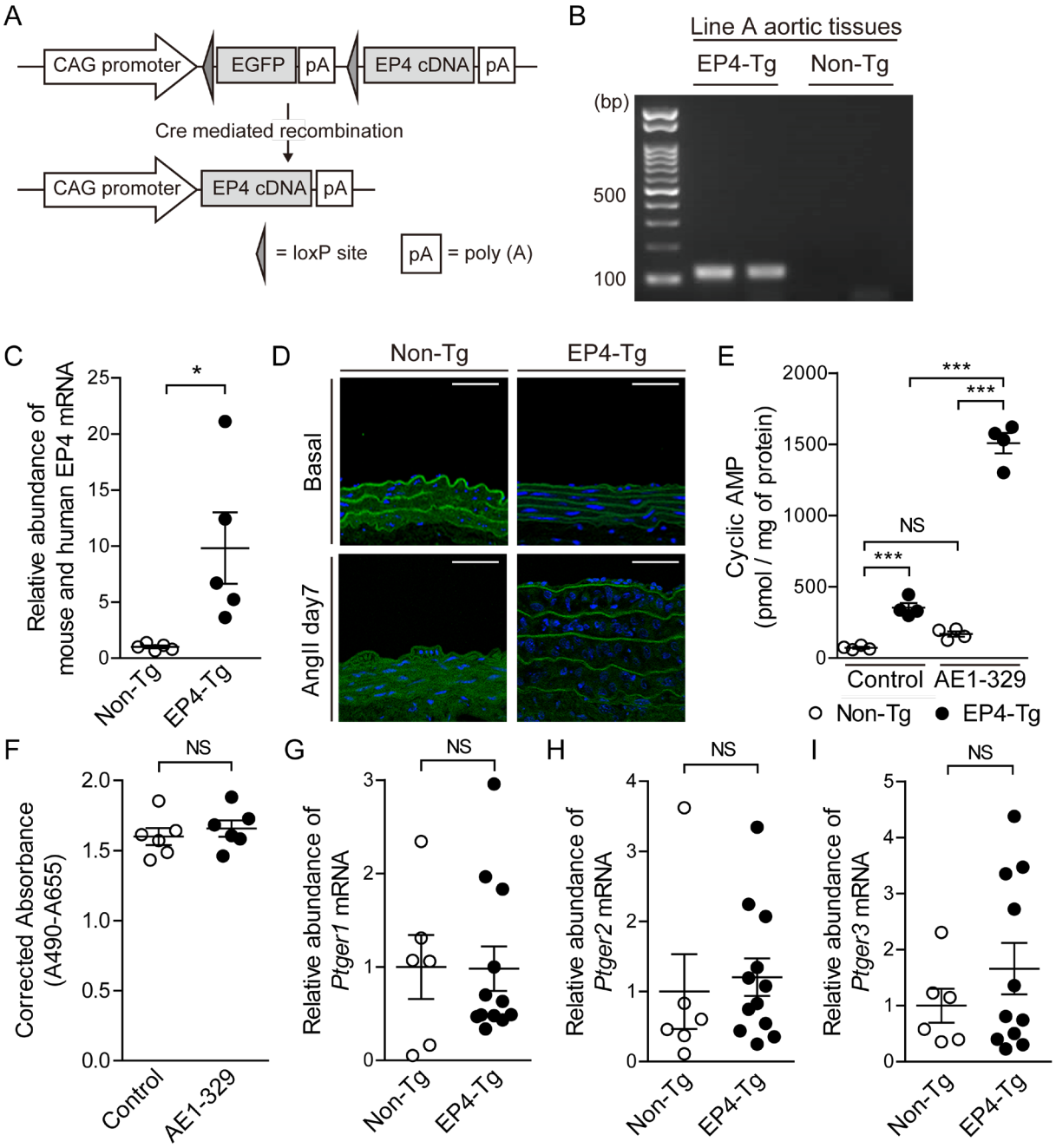
SYBR Green assay were as follows: mouse *Ptgs2* (NM_011198.3, 5′–GCA CTA CAT CCT GAC CCA CTT C–3′ and 5′–GCT CCT TAT TTC CCT TCA CAC C–3′), mouse *Il6* (NM_031168, 5′ -GAA CGA TAG TCA ATT CCA GAA ACC-3′ and 5′ -CAT TTC CAC GAT TTC CCA GA-3′), *Lox* (NM_010728, 5′ -TCT TCT GCT GCG TGA CAA CC-3′ and 5′ - GAG AAA CCA GCT TGG AAC CAG-3′), mouse *Cxcl1* (NM_008176.3, 5′ - GCA CCC AAA CCG AAG TCA-3′ and 5′ -AAG CCA GCG TTC ACC AGA-3′) and 18S ribosomal RNA (5′ -GTA ACC CGT TGA ACC CCA TT-3′ and 5′ -CCA TCC AAT CGG TAG TAG CG-3′). The assay numbers of TaqMan probes used in the study were: mouse *Ptger1*, Mm00443097_m1; mouse *Ptger2*, Mm00436051_m1; mouse *Ptger3*, Mm01316856_m1; mouse *Ptger4*, Mm00436052_g1; and mouse + human *Ptger4*, Mm00436053_m1.

Calcium chloride ($CaCl_2$) treatment

AAA was induced in Non-Tg and EP4-Tg mice by periaortic application of 0.5M $CaCl_2$ between the renal arteries and bifurcation of the iliac arteries. After 10 minutes of treatment, the aorta was rinsed once with 0.9% sterile saline. During laparotomy, mice were anesthetized by 1.5% isoflurane with an airflow of 200 mL/h. Fourteen days after the procedure, mice were euthanized with pentobarbital (13 mg, i.p.) and their aortas were excited after formalin perfusion. Luminal aortic diameter and external adventitial diameter were determined at short axis of Elastica van Gieson-stained cross section in the maximally dilated region of the abdominal aorta using Image J software.

Supplemental Figures

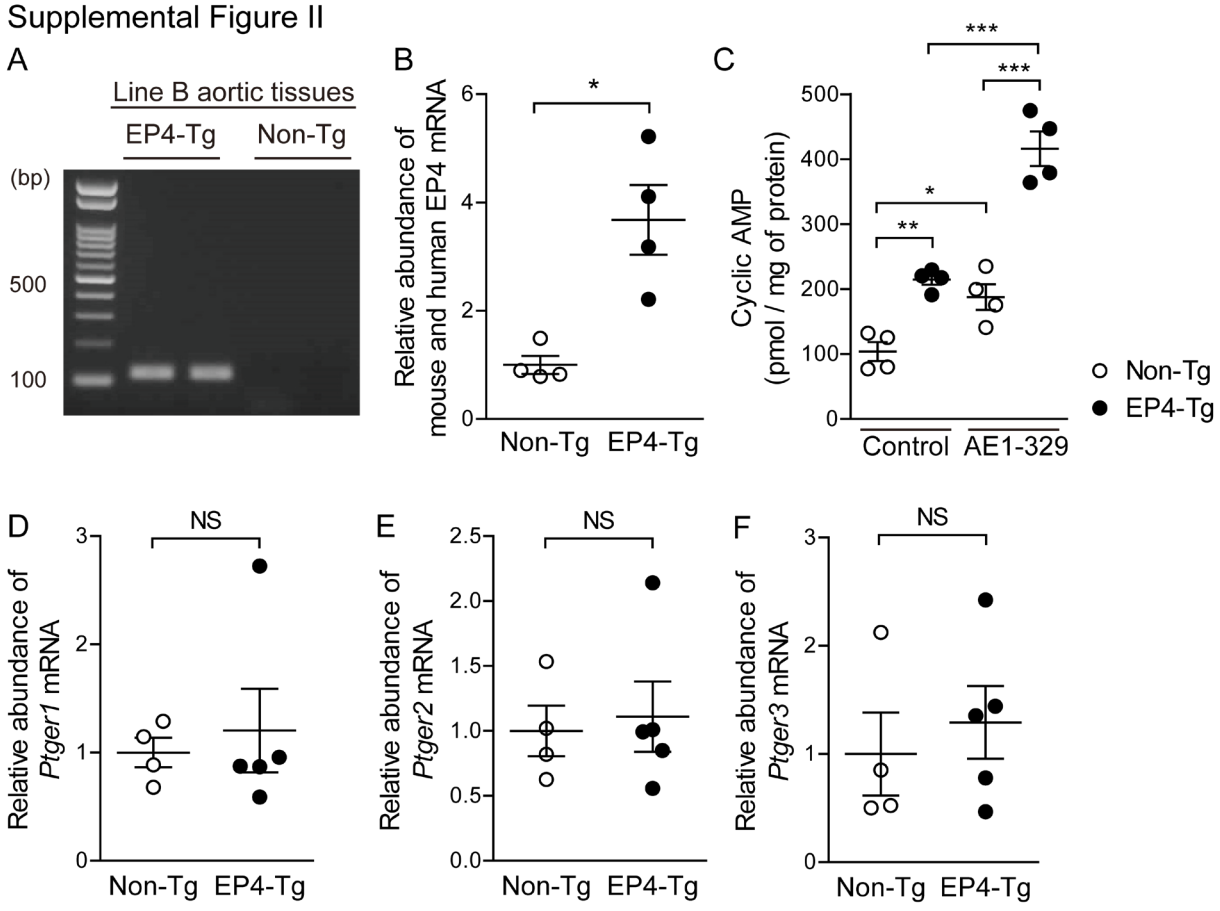
Supplemental Figure I



Supplemental Figure I. VSMC-selective EP4 overexpression in EP4-Tg (line A). (A) Generation of conditional EP4 overexpression using the Cre-loxP system. **(B)** Human EP4 (*PTGER4)* mRNA expression in EP4-Tg and Non-Tg aorta. **(C)** Total EP4 mRNA expression (mouse endogenous *Ptger4* and overexpressed human *PTGER4*) in EP4-Tg and Non-Tg aortic tissues. *n* = 5 from 5 individual mice. **(D)** GFP fluorescence images of aorta sections of Non-Tg and EP4-Tg before and after AngII infusion. Scale bars; 50μm. **(E)** Intracellular cyclic AMP level in VSMCs of Non-Tg and EP4-Tg before and 10 min after ONO-AE1-329 (AE1-329, EP4 agonist, 1 mol/L) administration. *n* = 4. **(F)** A XTT assay in EP4-Tg VSMCs with or without 24 h of AE1-329 administration. *n* = 6. **(G-I)**

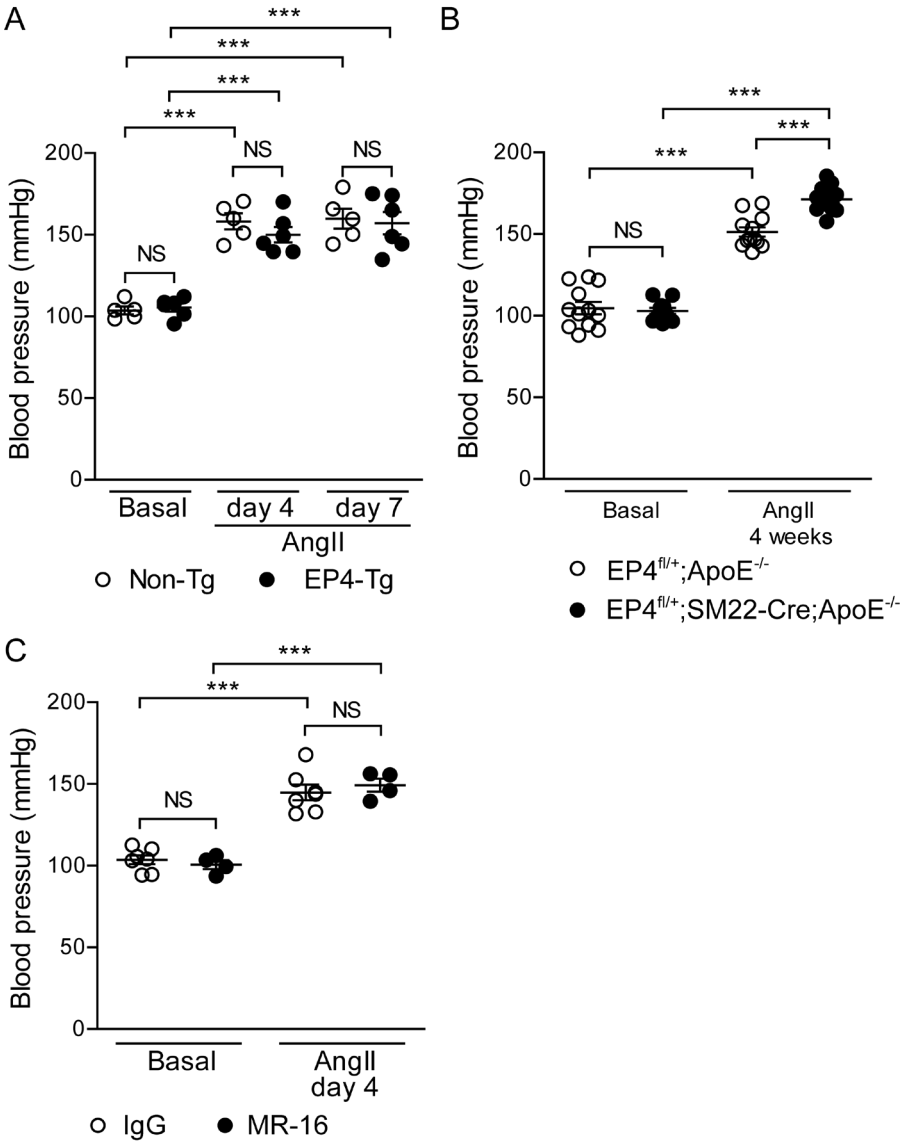
Expression levels of mouse *Ptger1* (EP1), *Ptger2* (EP2) and *Ptger3* (EP3) mRNAs in Non-Tg and EP4-Tg aorta. n = 6-12 from 6-12 individual mice. *$p < 0.05$; *** $p < 0.001$; NS, not significant.

Supplemental Figure II



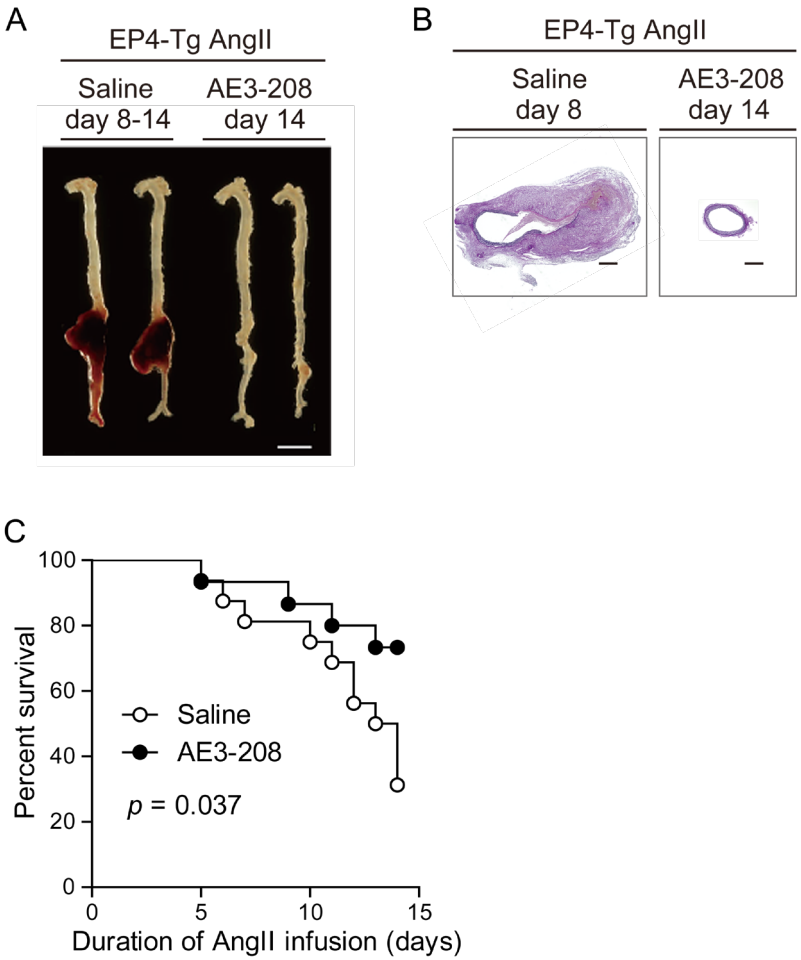
Supplemental Figure II. VSMC-selective EP4 overexpression in EP4-Tg (line B).
(A) Human *PTGER4* mRNA expression in EP4-Tg and Non-Tg aorta. **(B)** Total EP4 mRNA expression (mouse endogenous *Ptger4* and overexpressed human *PTGER4*) in EP4-Tg and Non-Tg aortic tissues. *n* = 4 from 4 individual mice. **(C)** Intracellular cyclic AMP level in VSMCs of Non-Tg and EP4-Tg before and 10 min after ONO-AE1-329 (AE1-329, EP4 agonist, 1 mol/L) administration. *n* = 4. **(D-F)** Expression levels of mouse *Ptger1* (EP1), *Ptger2* (EP2) and *Ptger3* (EP3) mRNAs in Non-Tg and EP4-Tg aorta. *n* = 4-5 from 4-5 individual mice. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; NS, not significant.

Supplemental Figure III



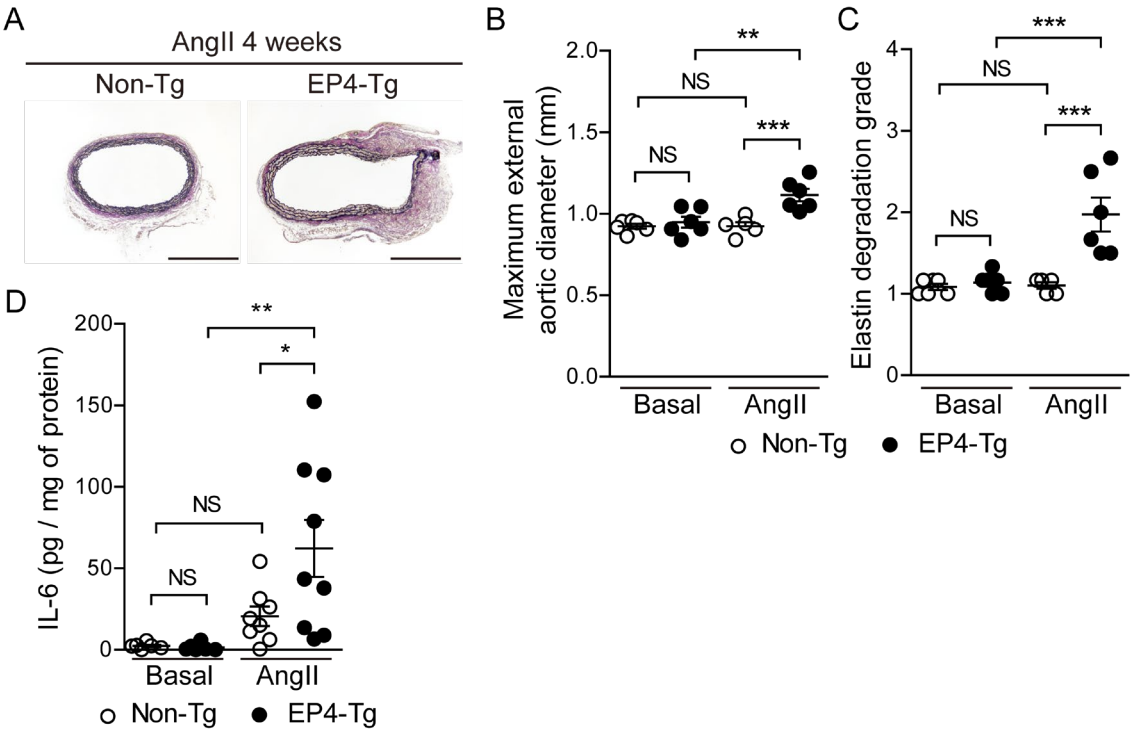
Supplement Figure III. Blood pressure changes after AngII infusion. (A) Systolic blood pressure of Non-Tg and EP4-Tg mice before and after AngII infusion (1.0 μg/kg/min). *n* = 5-6. **(B)** Systolic blood pressure of EP4$^{fl/+}$;ApoE$^{-/-}$ and EP4$^{fl/+}$;SM22-Cre; ApoE$^{-/-}$ mice before and 4 weeks after AngII infusion (1.0 μg/kg/min). *n* = 11-12. **(C)** Systolic blood pressure for AngII-infused (1.0 μg/kg/min) Non-Tg and EP4-Tg mice with MR-16 or control rat IgG administration. *n* = 4-7. *** $p <$ 0.001; NS, not significant.

Supplemental Figure IV



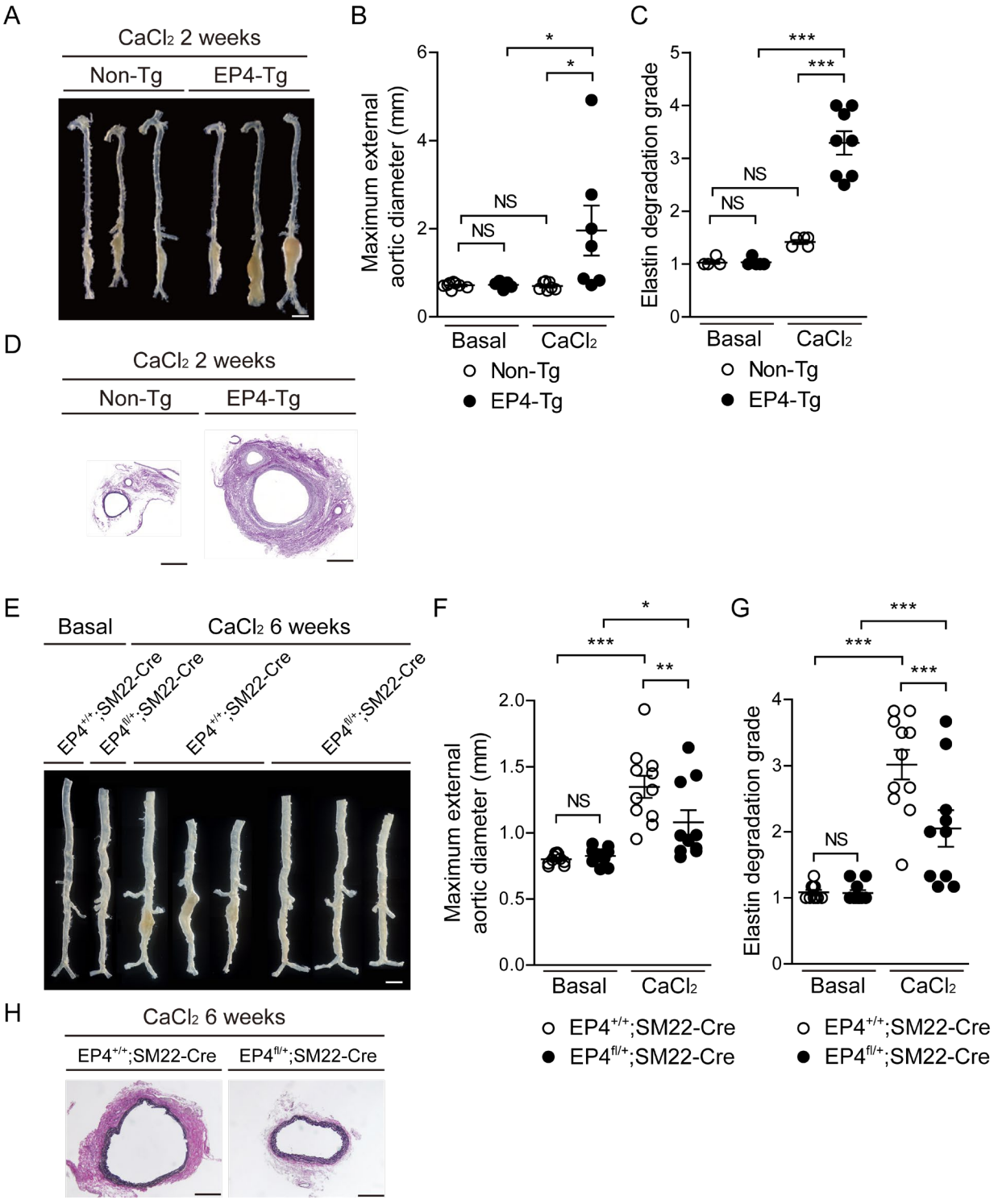
Supplemental Figure IV. EP4 antagonist inhibited AngII-induced AAA in EP4-Tg mice.
(A) Representative image of the aorta of AngII-infused EP4-Tg mice with ONO-AE3-208 or saline administration. Scale bar; 5 mm. **(B)** Elastica van Gieson-stained sections of (A). Scale bars; 500 μm. **(C)** Survival rates of AngII-infused EP4-Tg mice with ONO-AE3-208 or saline administration. *n* = 15-16.

Supplemental Figure V



Supplemental Figure V. VSMC-selective EP4-Tg (line B) mice exhibited dissecting AAA after AngII infusion. (A) Elastica van Gieson-stained sections of the abdominal aorta of Non-Tg and EP4-Tg mice infused with AngII (3.0 μg/kg/min). Scale bars, 500 μm. **(B-C)** Maximum aortic diameter and elastin degradation grade of the aorta in Non-Tg and EP4-Tg mice infused with AngII for 4 weeks. *n* = 5-6. **(D)** IL-6 protein expression in abdominal aorta from EP4-Tg before and after 4 weeks of AngII infusion. *n* = 6-8. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; NS, not significant.

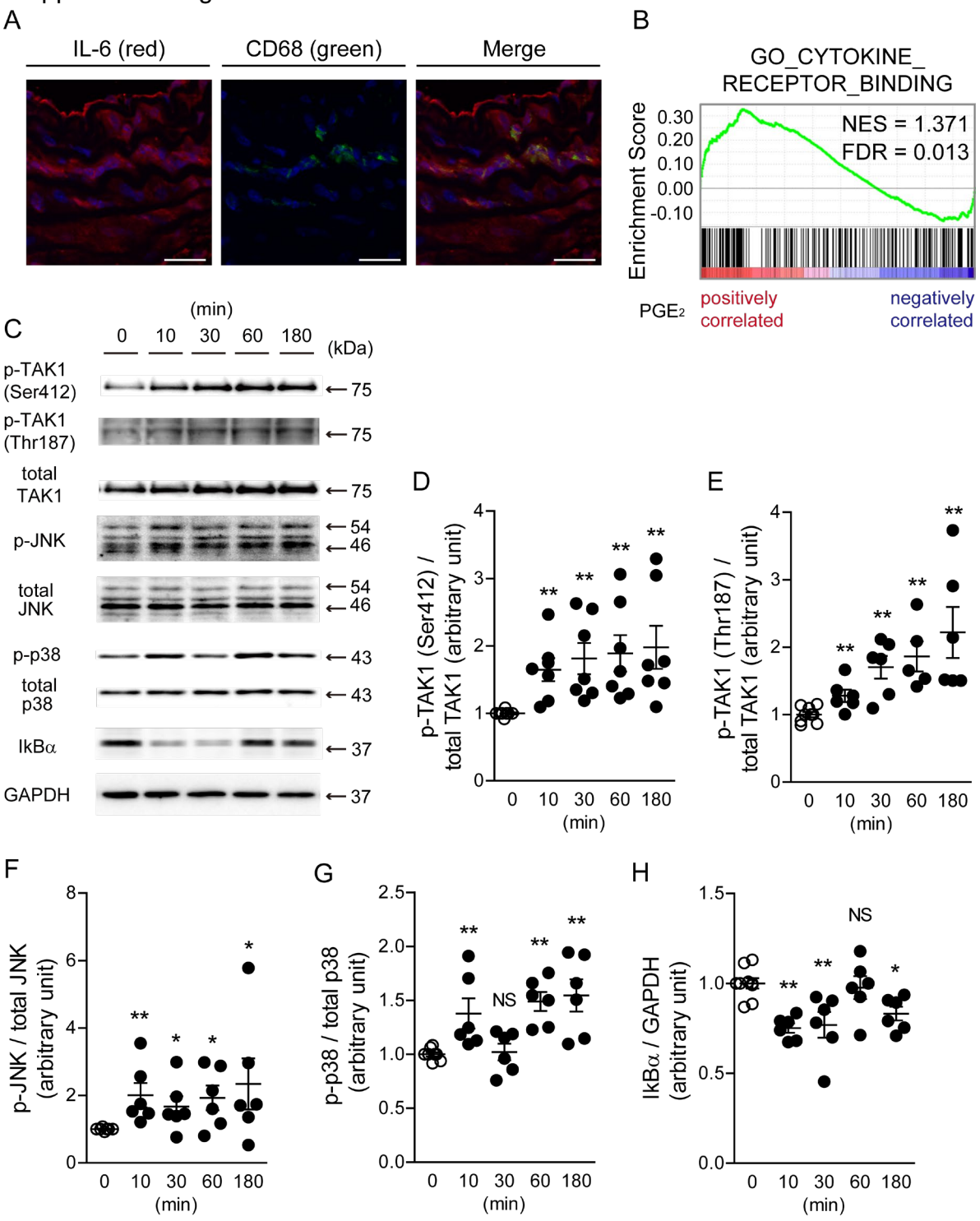
Supplemental Figure VI



Supplemental Figure VI. EP4 overexpression in VSMCs promoted true AAA after periaortic $CaCl_2$ application. (A) Representative images of aortas of Non-Tg and EP4-Tg mice after periaortic $CaCl_2$ application. Scale bars; 1 mm. **(B, C)** Maximum aortic diameter and elastin degradation grade of the aorta in Non-Tg and EP4-Tg mice with periaortic $CaCl_2$ application. *n* = 5-8. **(D)** Elastica van Gieson-stained sections of the abdominal aorta for Non-Tg and EP4-Tg mice after periaortic $CaCl_2$ application. Scale bars; 500 μm. **(E)** Representative images of aortas of

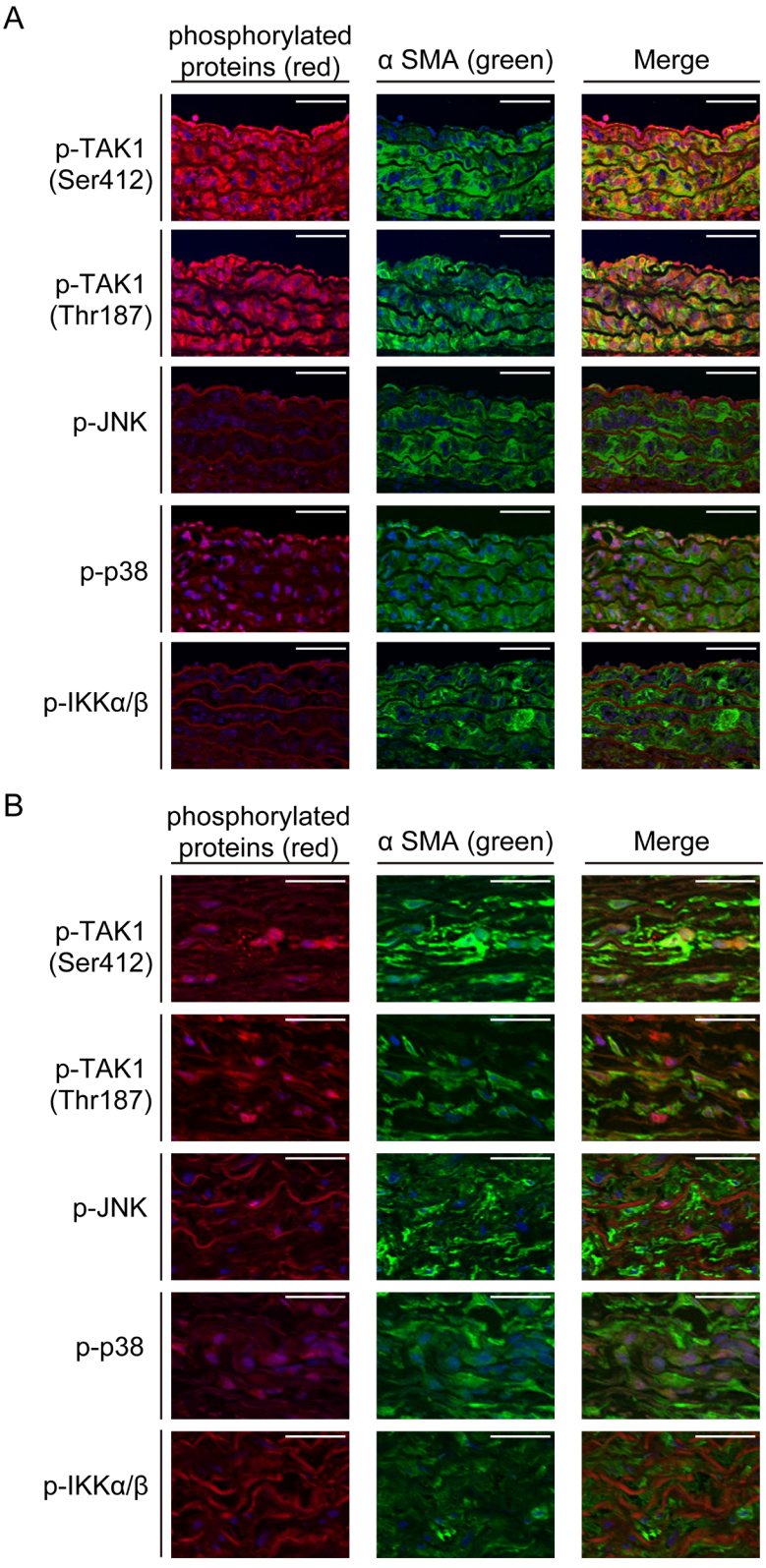
$EP4^{+/+}$;SM22-Cre and $EP4^{fl/+}$;SM22-Cre mice after periaortic $CaCl_2$ application. **(F, G)** Maximum aortic diameter and elastin degradation grade of the aorta in $EP4^{+/+}$;SM22-Cre and $EP4^{fl/+}$;SM22-Cre mice after periaortic $CaCl_2$ application. *n* = 10-11. **(H)** Elastica van Gieson-stained sections of the abdominal aorta for $EP4^{+/+}$;SM22-Cre and $EP4^{fl/+}$;SM22-Cre mice after periaortic $CaCl_2$ application. Scale bars; 500 μm. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; NS, not significant.

Supplemental Figure VII



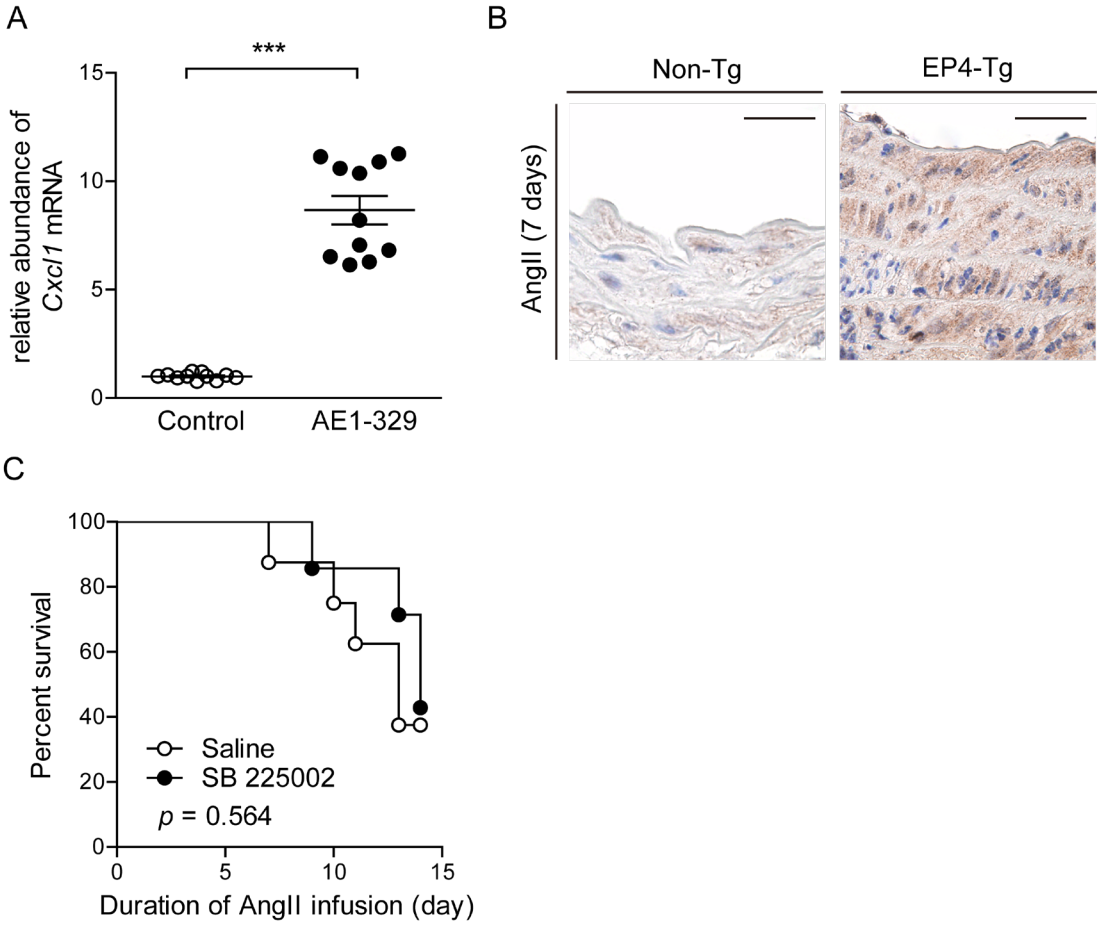
Supplemental Figure VII. EP4 signaling promoted TAK1 and MAPK phosphorylation and NF-κB activation in EP4-Tg VSMCs. (A) Immunofluorescence staining sections of the abdominal aorta of EP4-Tg after AngII infusion. Nuclei were stained by Hoechst 33342. Scale bars; 25 μm. **(B)** Gene set enrichment analysis (GSEA) of the microarray data using EP4-Tg VSMCs stimulated with PGE_2 (1 μmol/L) for 24 h. **(C-H)** Representative images and time-dependent changes in ONO-AE1-329 (EP4 agonist, 1 μmol/L) induced phosphorylation of TAK1 (Ser412 and Thr187), JNK, p38, and IκB expression in EP4-Tg VSMCs. *n* = 5-9. **p* < 0.05; ***p* < 0.01; NS, not significant.

Supplemental Figure VIII



Supplemental Figure VIII. EP4 down signaling pathways were activated in VSMCs of AngII-induced AAA in EP4-Tg and human AAA. (A) Immunofluorescence staining of the abdominal aorta of EP4-Tg infused with AngII for 7 days. Nuclei were stained by Hoechst 33342. Scale bars; 50 μm. **(B)** Immunofluorescence staining of tissues of human AAA. Nuclei were stained by Hoechst 33342. Scale bars; 25 μm.

Supplemental Figure IX



Supplemental Figure IX. CXCR2 antagonist did not inhibit AngII-induced AAA. (A) Chemokine (C-X-C motif) ligand 1 (*Cxcl1*) mRNA expression in EP4-Tg VSMCs stimulated with ONO-AE1-329 (EP4 agonist, 1 μmol/L). n = 7-8; ***$p < 0.001$.

(B) Immunohistochemically-stained sections of the abdominal aortas of Non-Tg and EP4-Tg mice after AngII infusion. Scale bars; 25 μm. **(C)** Survival rates of AngII-infused (1.0 μg/kg/min) EP4-Tg mice with and without SB225002 [C-X-C Motif Chemokine Receptor 2 (CXCR2) antagonist] administration. n = 7-8.

Supplemental Tables

Supplemental Table I. Basal characteristics of Non-Tg and EP4-Tg mice (line A)

	Non-Tg (*n* = 6-7)	EP4-Tg (*n* = 5-6)	*p* value
Body weight (g)	29.0 ± 1.5	30.4 ± 2.2	0.20
Cardiac function			
HR (beats per min)	479 ± 6.08	476 ± 4.5	0.66
LVDd (mm)	3.4 ± 0.03	3.3 ± 0.04	0.13
LVDS (mm)	2.1 ± 0.02	2.1 ± 0.03	0.14
LVEF (%)	75.0 ± 0.4	73 ± 0.7	0.13
LVFS (%)	36.5 ± 0.4	35.3 ± 0.6	0.25
Lipid profile			
Total cholesterol (mg/dl)	141.0 ± 8.1	136.2 ± 19.54	0.81
LDL cholesterol (mg/dl)	13.8 ± 1.5	14.5 ± 5.7	0.80
HDL cholesterol (mg/dl)	114.8 ± 11.5	110.7 ± 18.3	0.75
Triglyceride (mg/dl)	107.8 ± 26.9	101.5 ± 56.0	0.57
Free fatty acid (mEq/l)	2.5 ± 1.1	2.2 ± 0.9	0.69

BW, body weight; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; HR, heart rate; LVDd, left ventricular diastolic diameter; LVDs, left ventricular systolic diameter; LDL, low-density lipoprotein; HDL, high-density lipoprotein

Supplemental Table II. Basal characteristics of Non-Tg and EP4-Tg mice (line B)

	Non-Tg (*n* = 4-8)	EP4-Tg (*n* = 4-8)	*p* value
Body weight (g)	26.1 ± 0.95	27.3 ± 0.83	0.35
Cardiac function			
HR (beats per min)	475 ± 11.8	496 ± 8.4	0.16
LVDd (mm)	4.2 ± 0.14	4.0 ± 0.1	0.47
LVDS (mm)	2.84 ± 0.12	2.74 ± 0.11	0.54
LVEF (%)	68 ± 1.6	68.6 ± 2.1	0.80
LVFS (%)	31.8 ± 1.1	32.1 ± 1.4	0.84
Lipid profile			
Total cholesterol (mg/dl)	106.00 ± 3.03	106.17 ± 3.16	0.97
LDL cholesterol (mg/dl)	14.50 ± 0.87	14.83 ± 0.79	0.78
HDL cholesterol (mg/dl)	77.00 ± 2.8	78.83 ± 1.87	0.61
Triglyceride (mg/dl)	29.75 ± 7.63	32.50 ± 4.79	0.77
Free fatty acid (mEq/l)	0.64 ± 0.09	0.61 ± 0.07	0.81

Supplemental Table III. Basal characteristics of $EP4^{fl/+}$;$ApoE^{-/-}$ and $EP4^{fl/+}$;SM22-Cre;$ApoE^{-/-}$ mice

	$EP4^{fl/+}$;$ApoE^{-/-}$ (*n* = 5-6)	$EP4^{fl/+}$;SM22-Cre;$ApoE^{-/-}$ (*n* = 5-7)	*p* value
Ptger4 mRNA expression	1.00 ± 0.15	0.53 ± 0.09	0.01
Body weight (g)	30.1 ± 0.8	29.1 ± 0.5	0.35
Cardiac function			
HR (beats per min)	482 ± 4.18	500 ± 5.11	0.43
LVDd (mm)	3.4 ± 0.12	3.5 ± 0.11	0.61
LVDS (mm)	2.0 ± 0.07	2.2 ± 0.09	0.50
LVEF (%)	77.1 ± 0.6	77.2 ± 1.0	0.61
LVFS (%)	39.1 ± 0.5	38.9 ± 0.9	0.38
Lipid profile			
Total cholesterol (mg/dl)	654.3 ± 183.8	855.8 ± 164.3	0.18
LDL cholesterol (mg/dl)	372.7 ± 111.5	476.7 ± 122.2	0.18
HDL cholesterol (mg/dl)	49.2 ± 0.8	47.2 ± 10.9	0.94
Triglyceride (mg/dl)	121.0 ± 80.3	156.2 ± 45.1	0.40
Free fatty acid (mEq/l)	1.1 ± 0.4	1.6 ± 0.7	0.31

Supplemental Table IV. Basal characteristics of $EP4^{+/+}$;SM22-Cre and $EP4^{fl/+}$;SM22-Cre mice

	$EP4^{+/+}$;SM22-Cre (*n* = 5-9)	$EP4^{fl/+}$;SM22-Cre (*n* = 5-7)	*p* value
Ptger4 mRNA expression	1.00 ± 0.15	0.57 ± 0.09	0.03
Body weight (g)	24.3 ± 0.8	29.8 ± 0.9	0.002
Cardiac function			
HR (beats per min)	471.6 ± 5.2	480.6 ± 9.4	0.44
LVDd (mm)	3.75 ± 0.15	3.67 ± 0.03	0.62
LVDS (mm)	2.41 ± 0.09	2.35 ± 0.02	0.45
LVEF (%)	74.6 ± 0.7	73.4 ± 0.9	0.61
LVFS (%)	36.8 ± 0.6	36.0 ± 0.7	0.38

Supplemental Table V. Gene Ontology molecular function terms (Size>100) upregulated significantly (FDR<0.25) by PGE_2 in EP4-Tg VSMCs.

Gene set name	Size	NES	FDR q-value	Rank at MAX
GO_CATALYTIC_ACTIVITY_ACTING_ON _RNA	317	2.039	0.004	6822
GO_RIBONUCLEOPROTEIN_COMPLEX _BINDING	118	1.941	0.011	9155
GO_NUCLEOTIDYLTRANSFERASE _ACTIVITY	117	1.938	0.010	8341
GO_TRANSFERASE_ACTIVITY _TRANSFERRING_ACYL_GROUPS	230	1.925	0.011	5958
GO_TRANSCRIPTION_COACTIVATOR _ACTIVITY	308	1.821	0.017	7323
GO_HISTONE_BINDING	174	1.818	0.017	8093
GO_S_ADENOSYLMETHIONINE _DEPENDENT_METHYLTRANSFERASE _ACTIVITY	129	1.803	0.018	8190
GO_ENHANCER_BINDING	127	1.798	0.017	5455
GO_HELICASE_ACTIVITY	138	1.798	0.017	8224
GO_PRIMARY_ACTIVE_TRANSMEMBRANE _TRANSPORTER_ACTIVITY	101	1.781	0.017	5079
GO_UBIQUITIN_LIKE_PROTEIN_TRANSFERASE _ACTIVITY	371	1.779	0.017	8716
GO_MRNA_BINDING	215	1.771	0.017	7492
GO_MAGNESIUM_ION_BINDING	198	1.766	0.018	4126
GO_KINASE_REGULATOR_ACTIVITY	189	1.754	0.020	5805
GO_PROTON_TRANSMEMBRANE _TRANSPORTER_ACTIVITY	104	1.730	0.024	6875
GO_ATPASE_ACTIVITY_COUPLED	323	1.706	0.028	6871
GO_TRANSFERASE_ACTIVITY_TRANSFERRING _HEXOSYL_GROUPS	176	1.696	0.031	5810
GO_HEAT_SHOCK_PROTEIN_BINDING	112	1.696	0.030	5847
GO_TRANSFERASE_ACTIVITY_TRANSFERRING _GLYCOSYL_GROUPS	236	1.676	0.033	5810
GO_CATALYTIC_ACTIVITY_ACTING_ON_A_TRNA	111	1.675	0.033	7094
GO_PHOSPHORIC_ESTER_HYDROLASE _ACTIVITY	333	1.655	0.040	4297
GO_MODIFICATION_DEPENDENT_PROTEIN _BINDING	126	1.641	0.044	7222
GO_CHROMATIN_DNA_BINDING	106	1.640	0.044	6653
GO_ISOMERASE_ACTIVITY	132	1.639	0.044	5817

GO_ATPASE_ACTIVITY	392	1.632	0.045	7046
GO_TRANSFERASE_ACTIVITY_TRANSFERRING _ONE_CARBON_GROUPS	186	1.620	0.050	7950
GO_PROTEIN_HETERODIMERIZATION_ACTIVITY	443	1.612	0.054	5459
GO_CHROMATIN_BINDING	494	1.604	0.057	6246
GO_DNA_BINDING_TRANSCRIPTION_FACTOR _BINDING	322	1.592	0.062	6667
GO_PROTEIN_N_TERMINUS_BINDING	102	1.589	0.063	5054
GO_GUANYL_NUCLEOTIDE_BINDING	336	1.583	0.063	4048
GO_NUCLEAR_HORMONE_RECEPTOR_BINDING	145	1.577	0.066	8616
GO_NUCLEASE_ACTIVITY	178	1.575	0.066	6806
GO_ATPASE_ACTIVITY_COUPLED_TO _MOVEMENT_OF_SUBSTANCES	109	1.570	0.068	5079
GO_PHOSPHATASE_ACTIVITY	240	1.564	0.069	4143
GO_UNFOLDED_PROTEIN_BINDING	112	1.563	0.067	6216
GO_ENDONUCLEASE_ACTIVITY	108	1.546	0.075	6789
GO_NUCLEAR_RECEPTOR_BINDING	103	1.541	0.077	7834
GO_PROTEASE_BINDING	103	1.537	0.078	3855
GO_CARBOXYLIC_ESTER_HYDROLASE_ACTIVITY	116	1.534	0.079	6977
GO_UBIQUITIN_LIKE_PROTEIN_LIGASE _ACTIVITY	206	1.533	0.079	6949
GO_UDP_GLYCOSYLTRANSFERASE_ACTIVITY	120	1.514	0.088	4563
GO_CATALYTIC_ACTIVITY_ACTING_ON_DNA	172	1.510	0.090	8274
GO_HORMONE_RECEPTOR_BINDING	172	1.507	0.090	7834
GO_TRANSCRIPTION_COREPRESSOR_ACTIVITY	223	1.493	0.095	6949
GO_ORGANIC_ACID_BINDING	174	1.481	0.100	2615
GO_PROTEIN_SERINE_THREONINE_KINASE _ACTIVITY	414	1.479	0.101	5680
GO_GTPASE_ACTIVITY	273	1.479	0.101	6300
GO_COFACTOR_BINDING	425	1.460	0.110	5642
GO_TRANSLATION_REGULATOR_ACTIVITY	121	1.460	0.109	8561
GO_UBIQUITIN_LIKE_PROTEIN_LIGASE_BINDING	281	1.449	0.116	6586
GO_SH3_DOMAIN_BINDING	121	1.449	0.116	4432
GO_PHOSPHOPROTEIN_PHOSPHATASE _ACTIVITY	172	1.445	0.119	6211
GO_HYDROLASE_ACTIVITY_ACTING_ON _CARBON_NITROGEN_BUT_NOT_PEPTIDE _BONDS	115	1.428	0.130	5541
GO_COENZYME_BINDING	267	1.424	0.132	4783

GO_ENZYME_ACTIVATOR_ACTIVITY	462	1.407	0.143	4634
GO_PROTEIN_C_TERMINUS_BINDING	182	1.405	0.144	6537
GO_HYDROLASE_ACTIVITY_ACTING_ON _GLYCOSYL_BONDS	100	1.401	0.147	3091
GO_ACTIVE_TRANSMEMBRANE_TRANSPORTER_A CTIVITY	319	1.400	0.148	4497
GO_CYSTEINE_TYPE_PEPTIDASE_ACTIVITY	147	1.400	0.147	8553
GO_RNA_POLYMERASE_II_SPECIFIC_DNA_BINDIN G_TRANSCRIPTION_FACTOR_BINDING	254	1.382	0.161	6667
GO_CARBOHYDRATE_BINDING	213	1.355	0.185	2615
GO_RAB_GTPASE_BINDING	156	1.342	0.195	8299
GO_DNA_BINDING_TRANSCRIPTION_ACTIVATOR_ ACTIVITY	395	1.339	0.197	3983
GO_LYASE_ACTIVITY	149	1.336	0.201	4626
GO_LIGASE_ACTIVITY	138	1.333	0.203	5833
GO_CYTOKINE_RECEPTOR_BINDING	235	1.322	0.215	3695
GO_VITAMIN_BINDING	121	1.315	0.219	6598
GO_DNA_BINDING_TRANSCRIPTION_REPRESSOR _ACTIVITY_RNA_POLYMERASE_II_SPECIFIC	208	1.313	0.221	6851
GO_METALLOPEPTIDASE_ACTIVITY	166	1.307	0.225	3315
GO_CADHERIN_BINDING	304	1.296	0.237	4183

Supplemental Table VI. The genes increased by PGE_2 in EP4Tg VSMCs within the gene set related to “GO-cytokine receptor binding”

Genbank Accession	GeneSymbol	Description	Fold change PGE_2/control
NM_031168	*Il6*	interleukin 6	46.6
NM_029796	*Lrg1*	leucine-rich alpha-2-glycoprotein 1	37.4
NM_011824	*Grem1*	gremlin 1	24.9
NM_019568	*Cxcl14*	chemokine (C-X-C motif) ligand 14	15.3
NM_008176	*Cxcl1*	chemokine (C-X-C motif) ligand 1	9.7
NM_009141	*Cxcl5*	chemokine (C-X-C motif) ligand 5	8.6
NM_177371	*Tnfsf15*	tumor necrosis factor (ligand) superfamily, member 15	7.8
NM_008109	*Gdf5*	growth differentiation factor 5	4.2
NM_007899	*Ecm1*	extracellular matrix protein 1, transcript variant 1	3.7
NM_008091	*Gata3*	GATA binding protein 3	3.5
NM_019952	*Clcf1*	cardiotrophin-like cytokine factor 1	3.1
NM_009370	*Tgfbr1*	transforming growth factor, beta receptor I	2.6
NM_213659	*Stat3*	signal transducer and activator of transcription 3, transcript variant 1	2.5
NM_018827	*Crlf1*	cytokine receptor-like factor 1	2.5
NM_029646	*Il34*	interleukin 34, transcript variant 2	2.4
NM_010272	*Gdf11*	growth differentiation factor 11	2.4
NM_009404	*Tnfsf9*	tumor necrosis factor (ligand) superfamily, member 9	2.4
NM_007540	*Bdnf*	brain derived neurotrophic factor, transcript variant 1	2.3
NM_011577	*Tgfb1*	transforming growth factor, beta 1	2.2
NM_013654	*Ccl7*	chemokine (C-C motif) ligand 7	2.2
NM_011333	*Ccl2*	chemokine (C-C motif) ligand 2	2.1
NM_001098227	*Sdcbp*	syndecan binding protein, transcript variant 1	2.1
NM_206975	*Ifna14*	interferon alpha 14	2.0

Supplementary References

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2. Aoki R, Yokoyama U, Ichikawa Y, Taguri M, Kumagaya S, Ishiwata R, Yanai C, Fujita S, Umemura M, Fujita T, Okumura S, Sato M, Minamisawa S, Asou T, Masuda M, Iwasaki S, Nishimaki S, Seki K, Yokota S and Ishikawa Y. Decreased serum osmolality promotes ductus arteriosus constriction. *Cardiovascular Research*. 2014;104:326-36.

3. Yokoyama U, Ishiwata R, Jin MH, Kato Y, Suzuki O, Jin H, Ichikawa Y, Kumagaya S, Katayama Y, Fujita T, Okumura S, Sato M, Sugimoto Y, Aoki H, Suzuki S, Masuda M, Minamisawa S and Ishikawa Y. Inhibition of EP4 signaling attenuates aortic aneurysm formation. *PloS ONE*. 2012;7:e36724.

Major Resources Tables

Mouse model (in vivo studies)

Mouse Models	Vendor or Source	Background Strain	Sex
EP4-Tg	In house breeding	C57BL/6J	Male
Non-Tg	In house breeding	C57BL/6J	Male
$EP4^{fl/+}$;SM22-Cre;$ApoE^{-/-}$	In house breeding	C57BL/6N	Male
$EP4^{fl/+}$;$ApoE^{-/-}$	In house breeding	C57BL/6N	Male
$EP4^{fl/+}$;SM22-Cre	In house breeding	C57BL/6N, C57BL/6J	Male
$EP4^{+/+}$;SM22-Cre	In house breeding	C57BL/6J	Male

EP4-Tg and Non-Tg mouse breeding

	Vendor or Source	Breeding Strategy	Other Information
Parent	In house	EP4-Tg	C57BL/6J
Parent	The Jackson Laboratory	Tg(Tagln-cre)1Her	C57BL/6J

$EP4^{fl/+}$;SM22-Cre;$ApoE^{-/-}$ and $EP4^{fl/+}$;$ApoE^{-/-}$ breeding

	Vendor or Source	Breeding Strategy	Other Information
Parent	In house	$EP4^{fl/fl}$;$ApoE^{-/-}$	C57BL/6N
Parent	In house	$EP4^{+/+}$;SM22-Cre;$ApoE^{-/-}$	C57BL/6N

$EP4^{fl/+}$;SM22-Cre and $EP4^{+/+}$;SM22-Cre breeding

	Vendor or Source	Breeding Strategy	Other Information
Parent	Lab generated (from Drs. Richard M. Breyer & Matthew D. Breyer)	$EP4^{fl/fl}$	C57BL/6N
Parent	The Jackson Laboratory	Tg(Tagln-cre)1Her	C57BL/6J

Antibodies for FACS

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot #
CD45.2	BioLegend	#109822	200ng/mL (1:1000)	B202947
CD11b	BioLegend	#101245	800 ng/mL (1:100)	B05619
Ly6G	BioLegend	#127624	400 ng/mL (1:500)	B209108
Ly6C	BioLegend	#128008	8 ng/mL (1:10000)	B195689

Antibodies for Western Blotting

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot #
p-TAK1 (Ser412)	Cell Signaling Technology	#9339	43ng/mL (1:1000)	2
p-TAK1 (Thr187)	Cell Signaling Technology	#4531	848ng/mL (1:250)	5
total TAK1	Cell Signaling Technology	#4505	460 ng/mL (1:250)	7
p-JNK	Cell Signaling Technology	#9251	146 ng/mL (1:1000)	25
total JNK	Cell Signaling Technology	#9252	50 ng/mL (1:1000)	1
p-p38	Cell Signaling Technology	#9211	20 ng/mL (1:1000)	20
total p38	Cell Signaling Technology	#9212	26 ng/mL (1:1000)	16
IkBα	Cell Signaling Technology	#4814	463 ng/mL (1:1000)	17
GAPDH	Santa Cruz Biotechnology	#sc-25778	200 ng/mL (1:500)	D0621
Lysyl oxidase	Abcam	#ab31238	2 µg/mL (1:500)	GR302344-1

Primary antibodies for Immunohistochemical analysis

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot #
p-TAK1 (Ser412)	Cell Signaling Technology	#9339	mouse 430 ng/mL (1:100) human 143 ng/mL (1:300)	2
p-TAK1 (Thr187)	Cell Signaling Technology	#4531	mouse 2.12 µg/mL (1:100) human 707 ng/mL (1:300)	5
IL-6 (mouse)	R & D Systems	#AB-406-NA	5 µg/mL (1:200)	BF09
IL-6 (human)	R & D Systems	#MAB206	5 µg/mL (1:100)	HD4311011R
αSMA	Abcam	#Ab5694	400 ng/mL (1:500)	GR3183259-12
CD68	Bio-Rad	#MCA1957T	5 µg/mL (1:200)	1708
CXCL1	NOVUS	#NBP1-51188	10 µg/mL (1:100)	CN24151

Primary antibodies for Immunofluorescent analysis

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot #
p-TAK1 (Ser412)	Cell Signaling Technology	#9339	860 ng/mL (1:50)	2
p-TAK1 (Thr187)	Cell Signaling Technology	#4531	4.24 μg/mL (1:50)	5
p-JNK	Cell Signaling Technology	#9251	760 ng/mL (1:50)	27
p-p38	Cell Signaling Technology	#9211	660 ng/mL (1:50)	25
p-IKKα/β	Cell Signaling Technology	#2697	420 ng/mL (1:50)	19
IL-6 (mouse)	R & D Systems	#AB-406-NA	20 μg/mL (1:50)	BF09
αSMA	Sigma Aldrich	#A2547	112 μg/mL (1:50)	084M4795V
CD68	Abcam	#ab125212	10 μg/mL (1:50)	GR3302988-2

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)
aortic VSMCs of EP4-Tg	Founder	Male
hAAA VSMCs	Isolated from human specimens	Female and Male