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 E₂ (PGE₂), 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 described¹. 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. Cells below passage 8 were used in the experiments. All cells were cultured in a moist tissue culture incubator at 37 °C in 5% CO2-95% 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 PGE2 (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 PGE2 for 24 h. For human aortic VSMC culture, DMEM was used for starvation. To inhibit endogenous PGE2 production by VSMCs, indomethacin was administered at 100 μmol/L for 1 h before and during stimulation with PGE2 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.

**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. 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′-GGA CGA TAG TCA ATT CCA GAA ACC-3′ and 5′-CAT TTC CAC GAT TTC CCA GA-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 (CaCl₂) treatment**

AAA was induced in Non-Tg and EP4-Tg mice by periaortic application of 0.5M CaCl₂ 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 excised 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 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. 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.
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<sup>fl/+;ApoE<sup>−</sup> and EP4<sup>fl/+;SM22-Cre;ApoE<sup>−</sup></sup> 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. 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. 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. EP4 overexpression in VSMCs promoted true AAA after periaortic CaCl₂ application. (A) Representative images of aortas of Non-Tg and EP4-Tg mice after periaortic CaCl₂ 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 CaCl₂ application. n = 5-8. (D) Elastica van Gieson-stained sections of the abdominal aorta for Non-Tg and EP4-Tg mice after periaortic CaCl₂ application. Scale bars; 500 μm. (E) Representative images of aortas of
EP4\textsuperscript{+/+};SM22-Cre and EP4\textsuperscript{+/+};SM22-Cre mice after periaortic CaCl\textsubscript{2} application. (F, G) Maximum aortic diameter and elastin degradation grade of the aorta in EP4\textsuperscript{+/+};SM22-Cre and EP4\textsuperscript{fl/fl};SM22-Cre mice after periaortic CaCl\textsubscript{2} application. \( n = 10-11 \). (H) Elastica van Gieson-stained sections of the abdominal aorta for EP4\textsuperscript{+/+};SM22-Cre and EP4\textsuperscript{fl/fl};SM22-Cre mice after periaortic CaCl\textsubscript{2} application. Scale bars; 500 \( \mu \text{m} \). * \( p < 0.05 \); ** \( p < 0.01 \); *** \( p < 0.001 \); NS, not significant.
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 PGE2 (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 IκBα expression in EP4-Tg VSMCs. n = 5-9. *p < 0.05; **p < 0.01; NS, not significant.
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. 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\textsuperscript{fl/fl};ApoE\textsuperscript{-/-} and EP4\textsuperscript{fl/fl};SM22-Cre;ApoE\textsuperscript{-/-} mice

|                         | EP4\textsuperscript{fl/fl};ApoE\textsuperscript{-/-} (n = 5-6) | EP4\textsuperscript{fl/fl};SM22-Cre;ApoE\textsuperscript{-/-} (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<sup>+/+</sup>;SM22-Cre and EP4<sup>fl/fl</sup>;SM22-Cre mice

|                          | EP4<sup>+/+</sup>;SM22-Cre (n = 5-9) | EP4<sup>fl/fl</sup>;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 PGE2 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_TRANSFERRINGACYL_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_TRANSMEMBRANETRANSFERER_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_TRANSFERER_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        |
| Function Description                                                                 | Count | p-value | E-value |
|-------------------------------------------------------------------------------------|-------|---------|---------|
| GO_ATPASE_ACTIVITY                                                                 | 392   | 1.632   | 0.045   |
| GO_TRANSFERASE_ACTIVITY_TRANSFERRING_ONE Carbon Groups                                 | 186   | 1.620   | 0.050   |
| GO_PROTEIN_HETERODIMERIZATION_ACTIVITY                                               | 443   | 1.612   | 0.054   |
| GO_CHROMATIN_BINDING                                                                | 494   | 1.604   | 0.057   |
| GO_DNA_BINDING_TRANScription_FACTOR_Binding                                         | 322   | 1.592   | 0.062   |
| GO_PROTEIN_N_TERMINUS_BINDING                                                        | 102   | 1.589   | 0.063   |
| GO_GUANYL_NUCLEOTIDE_BINDING                                                        | 336   | 1.583   | 0.063   |
| GO_NUCLEAR_HORMONE_RECEPTOR_BINDING                                                 | 145   | 1.577   | 0.066   |
| GO_NUCLEASE_ACTIVITY                                                                | 178   | 1.575   | 0.066   |
| GO_ATPASE_ACTIVITY_COUPLED_TO_MOVEMENT_OF_SUBSTANCES                                 | 109   | 1.570   | 0.068   |
| GO_PHOSPHATASE_ACTIVITY                                                             | 240   | 1.564   | 0.069   |
| GO_UNFOLDED_PROTEIN_BINDING                                                          | 112   | 1.563   | 0.067   |
| GO_ENDONUCLEASE_ACTIVITY                                                             | 108   | 1.546   | 0.075   |
| GO_NUCLEAR_RECEPTOR_BINDING                                                          | 103   | 1.541   | 0.077   |
| GO_PROTEASE_BINDING                                                                 | 103   | 1.537   | 0.078   |
| GO_CARBOXYLIC_ESTER_HYDROLASE_ACTIVITY                                              | 116   | 1.534   | 0.079   |
| GO_UBIQUITIN_LIKE_PROTEIN_LIGASE_ACTIVITY                                            | 206   | 1.533   | 0.079   |
| GO_UDP_GLUCOSYLTRANSFERASE_ACTIVITY                                                 | 120   | 1.514   | 0.088   |
| GO_CATALYTIC_ACTIVITY_ACTING_ON_DNA                                                 | 172   | 1.510   | 0.090   |
| GO_HORMONE_RECEPTOR_BINDING                                                         | 172   | 1.507   | 0.090   |
| GO_TRANSCRIPTION_COREPRESSOR_ACTIVITY                                               | 223   | 1.493   | 0.095   |
| GO_ORGANIC_ACID_BINDING                                                             | 174   | 1.481   | 0.100   |
| GO_PROTEIN_SERINE_THREONINE_KINASE_ACTIVITY                                         | 414   | 1.479   | 0.101   |
| GO_GTPASE_ACTIVITY                                                                 | 273   | 1.479   | 0.101   |
| GO_COFACOR_Binding                                                                 | 425   | 1.460   | 0.110   |
| GO_TRANSLATION_REGULATOR_ACTIVITY                                                   | 121   | 1.460   | 0.109   |
| GO_UBIQUITIN_LIKE_PROTEIN_LIGASE_BINDING                                             | 281   | 1.449   | 0.116   |
| GO_SH3_DOMAIN_BINDING                                                              | 121   | 1.449   | 0.116   |
| GO_PHOSPHOPROTEIN_PHOSPHATASE_ACTIVITY                                              | 172   | 1.445   | 0.119   |
| GO_HYDROLASE_ACTIVITY_ACTING_ON_CARBON_NITROGEN_BUT_NOT_PEP TIDE_BINDING            | 115   | 1.428   | 0.130   |
| GO_COENZYME_BINDING                                                                | 267   | 1.424   | 0.132   |
| GO term | Count | Ratio | P-value | ID |
|---------|-------|-------|---------|----|
| GO:0042596 | 462 | 1.407 | 0.143 | 4634 |
| GO:0005585 | 182 | 1.405 | 0.144 | 6537 |
| GO:0004129 | 100 | 1.401 | 0.147 | 3091 |
| GO:0004873 | 319 | 1.400 | 0.148 | 4497 |
| GO:0004219 | 147 | 1.400 | 0.147 | 8553 |
| GO:0003735 | 254 | 1.382 | 0.161 | 6667 |
| GO:0005585 | 213 | 1.355 | 0.185 | 2615 |
| GO:0005585 | 156 | 1.342 | 0.195 | 8299 |
| GO:0005585 | 395 | 1.339 | 0.197 | 3983 |
| GO:0005585 | 149 | 1.336 | 0.201 | 4626 |
| GO:0005585 | 138 | 1.333 | 0.203 | 5833 |
| GO:0005585 | 235 | 1.322 | 0.215 | 3695 |
| GO:0005585 | 121 | 1.315 | 0.219 | 6598 |
| GO:0005585 | 208 | 1.313 | 0.221 | 6851 |
| GO:0005585 | 166 | 1.307 | 0.225 | 3315 |
| GO:0005585 | 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 | Gene Symbol | 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 1                                  | 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 1                                                           | 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

| Parent | Vendor or Source   | Breeding Strategy | Other Information |
|--------|-------------------|-------------------|-------------------|
| In house | EP4-Tg            | C57BL/6J          | Lab generated (from Drs. Richard M. Breyer & Matthew D. Breyer) |
| The Jackson Laboratory | Tg(Tagln-cre)1Her | C57BL/6J          | Tg(Tagln-cre)1Her |

#### EP4^fl/+;SM22-Cre;ApoE^/- and EP4^fl/+;ApoE^/- breeding

| Parent | Vendor or Source   | Breeding Strategy | Other Information |
|--------|-------------------|-------------------|-------------------|
| In house | EP4^fl/+;ApoE^/-  | C57BL/6N          | Lab generated (from Drs. Richard M. Breyer & Matthew D. Breyer) |
| In house | EP4^fl/+;SM22-Cre;ApoE^/- | C57BL/6N          | Lab generated (from Drs. Richard M. Breyer & Matthew D. Breyer) |

#### EP4^fl/+;SM22-Cre and EP4^+/+;SM22-Cre breeding

| Parent | Vendor or Source   | Breeding Strategy | Other Information |
|--------|-------------------|-------------------|-------------------|
| Lab generated (from Drs. Richard M. Breyer & Matthew D. Breyer) | EP4^fl/+;SM22-Cre;ApoE^/- | C57BL/6N          | Lab generated (from Drs. Richard M. Breyer & Matthew D. Breyer) |
| The Jackson Laboratory | Tg(Tagln-cre)1Her | C57BL/6J          | Tg(Tagln-cre)1Her |
### 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    |
| IκBα            | 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 |

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