

## **Protease-Activated Receptor-2 Plays a Critical Role in Vascular Inflammation and Atherosclerosis in Apolipoprotein E-deficient Mice**

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**First author's surname and short title:** Hara and Roles of PAR-2 in atherogenesis

**Total word count:** 6908 words

**Total number of figure and table:** 8 figures and 4 tables

**Total number of supplementary figure and table:** 2 figures and 1 table

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## Abstract

**Background:** The coagulation system is closely linked with vascular inflammation, although the underlying mechanisms are still obscure. Recent studies show that protease-activated receptor (PAR)-2, a major receptor of activated factor X (FXa), are expressed in both vascular cells and leukocytes, suggesting that PAR-2 may contribute to the pathogenesis of inflammatory diseases. Here we investigated the role of PAR-2 in vascular inflammation and atherogenesis.

**Methods:** We generated apolipoprotein E-deficient (*ApoE*<sup>-/-</sup>) mice lacking systemic PAR-2 expression (*PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup>). *ApoE*<sup>-/-</sup> mice which lack or express PAR-2 only in bone-marrow (BM) cells were also generated by BM transplantation. Atherosclerotic lesions were investigated after 20 weeks on a western-type diet (WTD) by histological analyses, quantitative RT-PCR, and western blotting. In vitro experiments using BM-derived macrophages were performed to confirm pro-inflammatory roles of PAR-2. The association between plasma FXa level and the severity of coronary atherosclerosis was also examined in humans who underwent coronary intervention.

**Results:** *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice showed reduced atherosclerotic lesions in the aortic arch ( $P < 0.05$ ) along with features of stabilized atherosclerotic plaques such as less lipid deposition ( $P < 0.05$ ), collagen loss ( $P < 0.01$ ), macrophage accumulation ( $P < 0.05$ ), and inflammatory molecule expression ( $P < 0.05$ ) compared with *ApoE*<sup>-/-</sup> mice. Systemic PAR2 deletion in *ApoE*<sup>-/-</sup> mice significantly decreased the expression of inflammatory molecules in the aorta. The results of BM transplantation experiments demonstrated that PAR-2 in hematopoietic cells contributed to atherogenesis in *ApoE*<sup>-/-</sup> mice. PAR-2 deletion did not alter metabolic parameters. In vitro experiments demonstrated that FXa or a specific peptide agonist of PAR-2 significantly increased expression of inflammatory molecules and lipid uptake in BM-derived macrophages from wild-type

mice compared with those from PAR-2-deficient mice. Activation of NF- $\kappa$ B signaling was involved in PAR-2-associated vascular inflammation and macrophage activation. In humans who underwent coronary intervention, plasma FXa level independently correlated with the severity of coronary atherosclerosis as determined by Gensini score ( $P<0.05$ ) and plaque volume ( $P<0.01$ ).

**Conclusions:** PAR-2 signaling activates macrophages and promotes vascular inflammation, increasing atherosclerosis in *ApoE*<sup>-/-</sup> mice. This signaling pathway may also participate in atherogenesis in humans.

**Keywords:** macrophage, atherosclerosis, inflammation, protease-activated receptor-2

## **Clinical Perspective**

### **(1) What is new?**

- Systemic deletion or hematopoietic deletion of protease-activated receptor-2 (PAR-2) attenuates vascular inflammation and atherogenesis in atherosclerotic mouse model.
- The activation of PAR-2 promotes pro-inflammatory activation of macrophages as determined by inflammatory molecule expression and foam cell formation.
- In humans, plasma FXa level positively correlates with the severity of coronary artery disease such as Gensini score and plaque volume determined by intravascular ultrasound analysis.
- In addition to classical coronary risk factors, plasma FXa level predicts the severity of coronary artery disease.

### **(2) What are the clinical implications?**

- The PAR-2 signaling plays an important role in the development of vascular inflammation and atherosclerosis.
- The PAR-2 signaling pathway may provide a novel mechanism of atherogenesis and serve as a potential therapeutic target for atherosclerosis.

## Introduction

It is a widely accepted view that atherosclerosis is a chronic inflammatory disease<sup>1,2</sup>. Many cellular and molecular pathways cause vascular inflammation<sup>3,4</sup>. Previous reports have suggested a link between the blood coagulation system and inflammatory diseases<sup>5,6</sup>. Several coagulation proteases and protease-activated receptor (PAR) pathways serve as underlying mechanisms<sup>7-11</sup>. PARs are a family of G protein-coupled, seven transmembrane domain receptors that are activated by proteolytic cleavage of the receptor N-terminus by several proteases, generating a novel tethered ligand, which subsequently activates the receptor via intramolecular binding. Among the four subtypes of the PAR family, recent studies demonstrated that PAR-2, one of the major receptors of activated factor X (FXa), is expressed in vascular cells (i.e., endothelial cells and smooth muscle cells) and leukocytes, but not in platelets<sup>7,10,11</sup>, and contributes to the development of inflammatory diseases, such as insulin resistance<sup>12</sup> and neointima formation after vascular injury<sup>13</sup>. However, the role of PAR-2 signaling in the development of atherosclerosis is not fully understood. Several studies have reported that PAR-2 in endothelial cells plays an important role in the pathophysiology of inflammatory diseases<sup>14-16</sup>; however, little is known about the role of PAR-2 in macrophage activation, which is a key player in atherogenesis.

Recently, we reported that a direct FXa inhibitor, rivaroxaban, attenuates atherosclerotic plaque progression and destabilization in apolipoprotein E-deficient (*ApoE*<sup>-/-</sup>) mice<sup>17</sup>. Consistent with the results of our animal study, recent clinical trials demonstrated that patients with acute coronary syndrome treated with rivaroxaban experienced fewer cardiovascular events compared with the control<sup>18-20</sup>. Furthermore, the result of the Cardiovascular Outcomes for People Using Anticoagulation Strategies (COMPASS) trial demonstrated that the addition of low-dose

rivaroxaban to aspirin reduced major adverse cardiovascular events in patients with coronary or peripheral artery disease compared with traditional aspirin alone group <sup>21</sup>. The anti-coagulation effects of FXa inhibitors might play a role in these results, although these studies also suggest that the inhibition of FXa-PAR-2 signaling by a FXa inhibitor might have anti-atherosclerotic effects through attenuation of vascular inflammation.

In this study, to address the hypothesis that FXa-PAR-2 signaling, especially in macrophages, promotes vascular inflammation and atherosclerosis, we performed in vivo studies using *ApoE*<sup>-/-</sup> mice that genetically lack PAR-2 (*PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice) and bone marrow (BM) chimeric *ApoE*<sup>-/-</sup> mice which lack or express PAR-2 only in hematopoietic cells. In vitro experiments using macrophages were also performed to investigate the role of PAR-2 in pro-inflammatory activation of this cell-type. Furthermore, we examined the relationship between plasma FXa level and the severity of coronary atherosclerosis in human to obtain clinically translatable evidence that FXa-PAR-2 signaling is involved in the development of atherosclerosis. The results of our study suggest a novel mechanism of atherogenesis that could serve as a potential therapeutic target.

## Methods

All data and supporting materials have been provided with the published article.

## Animals

Male *ApoE*<sup>-/-</sup> mice and PAR-2-deficient (*PAR-2*<sup>-/-</sup>) mice (C57BL/6J background), originally purchased from Jackson Laboratory, were used in this study. We generated *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice

by cross-breeding *PAR-2*<sup>-/-</sup> mice and *ApoE*<sup>-/-</sup> mice. Sex-age-matched *ApoE*<sup>-/-</sup> mice served as the control. We administered a western-type diet (WTD) for 20 weeks from 8 weeks of age. Some of 8-week-old male *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice received a WTD supplemented with rivaroxaban (5 mg/kg body weight/day) for 20 weeks<sup>17</sup>. Sex-age-matched non-treated *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice were served as the control. Rivaroxaban was supplied by Bayer Health Care. The mice were housed in a room in which lighting was controlled (12 h on/12 h off), and room temperature was kept at 25 °C. All experimental procedures conformed to the guidelines for animal experimentation of Tokushima University. The protocol was reviewed and approved by our institutional ethics committee.

### **Bone marrow transplantation**

To investigate the role of PAR-2 in hematopoietic cells, we performed bone marrow transplantation (BMT) as described previously<sup>22</sup>. For generating *ApoE*<sup>-/-</sup> mice which lack PAR-2 in hematopoietic cells, BM of *ApoE*<sup>-/-</sup> mice was reconstituted with that of *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice. For generating *ApoE*<sup>-/-</sup> mice which express PAR-2 only in hematopoietic cells, BM of *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice was reconstituted with that of *ApoE*<sup>-/-</sup> mice. *ApoE*<sup>-/-</sup> mice which BM was replaced with that of *ApoE*<sup>-/-</sup> mice and *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice which BM was replaced with that of *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice were used for the control respectively. BM cells were harvested from the femurs and tibias of female donor mice. Eight-week-old male recipient mice were lethally X-irradiated with a total dose of 9.0 Gy. One day later, recipient mice received unfractionated BM cells by injection into the tail vein. At 8 weeks after BMT, all animals were started on a WTD. The replacement rate of BM cells was determined by fluorescence in-situ hybridization (FISH) of the X and Y chromosomes in male recipient mice repopulated with female BM<sup>23</sup>. Interphase nucleic slides were prepared from spleen using an air-



drying method. FISH analysis was performed using mouse X and Y chromosome probes (Chromosome Science Labo) following the manufacturer's protocol, and the signals were observed under an all-in-one fluorescence microscope BZ-9000 (KEYENCE Japan). We used only BM chimeric mice in which more than 90% of BM had been replaced by donor BM.

### **Blood pressure and plasma lipid level measurement**

The blood pressure of each mouse was measured using a tail-cuff system (BP-98A, Softron) in conscious animals. The average value of three measurements was used for comparison. At the time of sacrifice, blood was collected from the heart into K2-EDTA-containing tubes. After centrifuged, plasma was stored at -80°C until required. Plasma lipid levels were measured using commercially available kits (Wako Diagnostics).

### **Preparation of aortas and atherosclerotic lesion analysis**

Atherosclerotic lesion analysis was performed as described previously<sup>17</sup>. Mice were sacrificed by administration of an overdose of pentobarbital, and perfused with 0.9% sodium chloride solution at a constant pressure via the left ventricle. Both the heart and whole aorta were immediately removed. The thoracic aorta was excised, opened longitudinally, and fixed with 10% neutral buffered formalin. To quantify atherosclerotic lesions in the aortic arch, en face Sudan IV staining was performed. The percentage of the Sudan IV-positive area was measured. The abdominal aorta was removed and snap-frozen in liquid nitrogen for gene or protein expression analysis.

### **Histology and immunohistochemical analyses**

Histological and immunohistochemical staining were performed as described previously<sup>17</sup>. Each heart was cut along a horizontal plane between the lower tips of the left and right atria. The upper portion was snap-frozen in OCT compound (TissueTeck). Then, the aortic root was sectioned serially (5  $\mu$ m intervals) from the point where the aortic valves appeared to the ascending aorta, until the valve cusps were no longer visible. Lipid and collagen content within atherosclerotic plaques were determined by Oil red O staining and picrosirius red staining, respectively. Macrophage antigen-3 (Mac-3), monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinase (MMP)-9 expression were detected using anti-macrophage antigen-3 (BD Biosciences), anti-MCP-1 (Santa-Cruz) or anti-MMP-9 (R&D Systems Inc.) antibody followed by the avidin-biotin complex technique, and stained using a Vector Red substrate kit (Vector). Each section was counterstained with hematoxylin.

### **Cell culture**

BM-derived macrophages were used in this study. BM cells were obtained from femurs and tibias of 8-week-old wild-type mice and *PAR-2*<sup>-/-</sup> mice. Cells were cultured in DMEM supplemented with 10 ng/ml macrophage colony-stimulating factor (R&D Systems) for 7 days. To assess the role of PAR-2 in macrophage activation, BM-derived macrophages from wild-type mice and *PAR-2*<sup>-/-</sup> mice were treated with 50 nM activated factor X (FXa) (Haematologic Technologies, Inc.) or 10 nM PAR-2 specific agonist peptide (AP-2; Sigma-Aldrich) for 24 h after 24-h serum starvation. In addition, to assess the role of FXa-PAR-2 signaling in foam cell formation, BM-derived macrophages from wild-type mice and *PAR-2*<sup>-/-</sup> mice were pre-treated with 50 nM FXa or 10 nM AP-2 for 24 h, and then cultured with 100  $\mu$ g/mL oxidized LDL (oxLDL, Biomedical Technologies Inc.) for 48 h.

Intracellular lipids were stained by Oil red O staining after fixation with 10% neutral buffered formalin. The ratio of Oil red O-positive area/cellular area (%) was calculated in 100 cells and averaged. All cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in a 5% CO<sub>2</sub> humidified atmosphere.

### **Reverse transcription, real-time polymerase chain reaction**

Total RNA was extracted from tissues and cells using an illustra RNAspin RNA Isolation Kit (GE Healthcare). Reverse transcription was performed using a QuantiTect Reverse Transcription kit (Qiagen) from 1 µg of the extracted total RNA. Quantitative real-time PCR (qPCR) was performed on an Mx3000P (Agilent Technologies) using gene-specific primers (Supplemental Table) and Power SYBR Green PCR Master Mix (Applied Biosystems). Data are expressed in arbitrary units that were normalized by β-actin.

### **Western blot**

Protein were isolated from cells or tissue, and separated by SDS–polyacrylamide gel electrophoresis gels as we described previously<sup>23</sup>. The following antibodies were used: IκBα, and β-actin (Sigma). β-actin was used as an internal control. Antibody distribution was visualized with enhanced chemiluminescence-plus reagent (GE Healthcare) using a luminescent image analyzer (LAS-1000, Fuji Film).

### **Clinical research**

We included patients with stable angina who had been receiving a statin for at least 6 months. All

patients underwent elective percutaneous coronary intervention (PCI) and intravascular ultrasound (IVUS) study of the vessel undergoing PCI. We excluded patients with acute coronary syndrome, renal insufficiency, history of PCI or coronary artery bypass surgery, and those with inadequate IVUS imaging. Blood samples were collected into Na-EDTA-containing tubes just before PCI. After blood samples were centrifuged, plasma was stored at -80°C until required. Plasma FXa level was measured using a commercially available kit (Human Coagulation factor Xa ELISA Kit; MBS721605, MyBioSource). All experimental procedures were approved by the Institutional Review Board of Tokushima University Hospital and all subjects were gave informed consent.

We calculated Gensini scores<sup>24</sup> from initial angiograms as a global severity marker of coronary atherosclerosis. Our IVUS imaging protocol was described previously<sup>25</sup>. In brief, we selected plaques of intermediate stenotic lesions (25% < percent diameter stenosis < 50% on quantitative coronary angiography) > 5 mm from the intervention site. We used imaging catheters (Atlantis; Boston Scientific/Cardiovascular System) and a console (Galaxy; Boston Scientific/Cardiovascular System). We captured 10 continuous slice images (total length 5 mm) at intervals of 0.5 mm using an auto-pullback system in each plaque. Cross-sectional lumen area, cross-sectional vessel area within the external elastic membrane, and plaque area were determined using software attached to the IVUS system. Plaque volume was calculated using integration.

### **Statistical analysis**

Numerical values are expressed as mean ± SEM. Comparison of parameters between two groups was performed with unpaired Student's t-test when data followed a normal distribution or with

Mann-Whitney U test when data did not follow a normal distribution. Univariate analysis between the severity of coronary atherosclerosis (Gensini score and plaque volume) and plasma FXa level was performed by Spearman's rank correlation coefficient. Multiple regression analysis was performed for prediction of the Gensini score and coronary plaque volume using metabolic parameters and plasma FXa level. A value of  $P < 0.05$  was considered significant.

## Results

### **Genetic deletion of PAR-2 attenuated plaque progression and destabilization in *ApoE*<sup>-/-</sup> mice**

After 20 weeks of WTD feeding, en face Sudan IV staining of the aortic arch showed a significant reduction of atherosclerotic lesions in *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice compared with *ApoE*<sup>-/-</sup> mice ( $49.0 \pm 3.1$  vs.  $38.9 \pm 3.7\%$ ,  $P < 0.05$ ) (Figure 1). Genetic deletion of PAR-2 demonstrated a significant reduction of lipid deposition ( $6.98 \pm 0.21$  vs.  $6.23 \pm 0.21\%$ ,  $P < 0.05$ ) (Figure 2A) and an increase in collagen content ( $66.0 \pm 1.3$  vs.  $72.3 \pm 1.3\%$ ,  $P < 0.01$ ) (Figure 2B) in atherosclerotic plaques in *ApoE*<sup>-/-</sup> mice. Also, genetic deletion of PAR-2 in *ApoE*<sup>-/-</sup> mice significantly reduced the accumulation of macrophages as determined by Mac-3 staining ( $5.35 \pm 0.58$  vs.  $3.02 \pm 0.58\%$ ,  $P < 0.05$ ) (Figure 2C) and the expression of MCP-1 ( $2.17 \pm 0.34$  vs.  $1.00 \pm 0.34\%$ ,  $P < 0.05$ ) (Figure 2D) and MMP-9 ( $2.60 \pm 0.24$  vs.  $1.39 \pm 0.27\%$ ,  $P < 0.05$ ) (Figure 2E) in atherosclerotic plaques compared with control animals. There were no differences in body weight gain, blood pressure, plasma glucose level, and plasma lipid levels between *ApoE*<sup>-/-</sup> mice and *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice (Table 1). PAR-2 deletion did not affect plasma FXa level. These results indicated that genetic deletion of PAR-2 in *ApoE*<sup>-/-</sup> mice attenuates plaque progression and destabilization without alteration of metabolic parameters.

### **Genetic deletion of PAR-2 attenuated vascular inflammation in *ApoE*<sup>-/-</sup> mice**

We also compared the expression of inflammatory molecules in the atherosclerotic aorta using qPCR between *ApoE*<sup>-/-</sup> mice and *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice. Consistent with the results of immunohistochemical study, *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice demonstrated reduced expression of the macrophage marker F4/80 ( $P=0.05$ ) compared with *ApoE*<sup>-/-</sup> mice. *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice also demonstrated a reduction of inflammatory molecules, such as MCP-1, macrophage inflammatory protein (MIP)-1 $\alpha$ , interleukin (IL)-6 and MMP-9 ( $P<0.05$ , respectively) (Figure 3A). PAR-2 deletion attenuated the degradation of I $\kappa$ B $\alpha$  in the atherosclerotic aorta, suggesting inhibition of NF- $\kappa$ B signaling (Figure 3B). Genetic deletion of PAR-2 did not change RNA expression of PAR-1, PAR-3, and PAR-4 (Figure 3C). These results indicated that genetic deletion of PAR-2 in *ApoE*<sup>-/-</sup> mice reduces vascular inflammation.

### **FXa inhibition did not affect atherogenesis in *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice**

FXa activates both PAR-1 and PAR-2. Previous studies reported the expression of PAR-1 and PAR-2 in the vasculature and contribution to the development of vascular inflammation and atherogenesis<sup>7, 10, 11</sup>. Therefore, to examine the role of FXa-PAR-2 signaling in atherogenesis, we administered a specific FXa inhibitor, rivaroxaban, to *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice for 20 weeks. Rivaroxaban attenuated atherogenesis in *ApoE*<sup>-/-</sup> mice as demonstrated in our previous study<sup>17</sup>, although it did not inhibit the progression of atherosclerotic lesions in the aortic arch ( $35.1\pm 2.3$  vs.  $31.4\pm 3.5\%$ ) and the expression of inflammatory molecules, except for IL-1 $\beta$ , in atherosclerotic aorta in *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice, suggesting the pivotal role of FXa-PAR-2 signaling in atherogenesis (Supplemental Figure 1).

## **PAR-2 in hematopoietic cells contributed to plaque progression and destabilization in *ApoE*<sup>-/-</sup> mice**

To further investigate the role of PAR-2 in hematopoietic cells in the development of atherosclerosis, we generated BM specific PAR-2 deleted or expressing *ApoE*<sup>-/-</sup> mice. The replacement rate of BM cells determined by fluorescence in-situ hybridization was more than 90% in all BM chimeric mice as shown in Supplemental Figure 2.

PAR-2 deletion in BM cells reduced the development of atherosclerotic lesions in the aortic arch ( $10.8\pm 1.1$  vs.  $5.8\pm 1.0\%$ ,  $P<0.01$ ) (Figure 4A) and lipid deposition in atherosclerotic plaques in the aortic root ( $5.71\pm 0.66$  vs.  $3.08\pm 0.63\%$ ,  $P<0.01$ ) (Figure 5A). In contrast, PAR-2 deletion in BM cells significantly increased collagen content in plaques ( $15.3\pm 2.1$  vs.  $24.2\pm 2.0\%$ ,  $P<0.01$ ) (Figure 5B). Furthermore, the results of immunostaining of atherosclerotic lesions revealed that PAR-2 deletion in BM cells in *ApoE*<sup>-/-</sup> mice reduced the accumulation of macrophages as determined by Mac-3 staining ( $7.43\pm 0.29$  vs.  $4.03\pm 0.35\%$ ,  $P<0.01$ ) (Figure 5C), and the expression of MMP-9 ( $6.13\pm 0.18$  vs.  $4.25\pm 0.48\%$ ,  $P<0.01$ ) (Figure 5E) compared with control chimeric mice. The expression of MCP-1 was also lower in plaques in *ApoE*<sup>-/-</sup> mice lacking PAR-2 in BM cells compared with that in control mice (Figure 5D). On the other hand, BM specific PAR-2 expression promoted atherosclerotic lesion progression (Figure 4B). Lipid deposition and collagen loss in atherosclerotic lesions were greater in BM specific PAR-2 expressing mice compared to the control (Supplementary Figure 3).

There were no differences in body weight gain, blood pressure, plasma glucose level, and plasma lipid levels despite the presence or absence of PAR-2 in BM (Table 2 and Supplementary

Table 2). These results indicated that PAR-2 in hematopoietic cells contributes to plaque progression and destabilization in *ApoE*<sup>-/-</sup> mice. Also, these results suggested that PAR-2 signaling in macrophages contributes, at least partially, to atherogenesis.

### **PAR-2 in hematopoietic cells promoted vascular inflammation in *ApoE*<sup>-/-</sup> mice**

We also examined the role of PAR-2 in hematopoietic cells in the expression of inflammatory molecules in the atherosclerotic aorta using qPCR. PAR-2 deletion in hematopoietic cells reduced the expression of the macrophage marker F4/80 ( $P<0.05$ ). PAR-2 deletion in BM cells also reduced the expression of inflammatory molecules, such as MCP-1 ( $P<0.05$ ), MIP-1 $\alpha$  ( $P<0.05$ ), IL-1 $\beta$  ( $P<0.05$ ), IL-6 ( $P<0.01$ ), tumor necrosis factor (TNF)- $\alpha$  ( $P<0.05$ ), and MMP-9 ( $P<0.01$ ) (Figure 6A). On the other hand, PAR-2 expression in BM increased the expression of these inflammatory molecules (Figure 6B). These results indicated that PAR-2 in hematopoietic cells promotes the expression of inflammatory molecules in atherosclerotic aorta, leading to the development of vascular inflammation.

### **PAR-2 signal promoted pro-inflammatory activation of macrophages**

To investigate the mechanism by which inhibition of PAR-2 signaling prevented the development and destabilization of atherosclerotic lesions without improvement of metabolic parameters, we performed in vitro experiments using BM-derived macrophages. We treated BM-derived macrophages with FXa, a major ligand of PAR-2, or a specific agonist peptide of PAR-2 (AP-2). The results of qPCR analysis demonstrated that the expression of inflammatory molecules (e.g., MCP-1, IL-6 and TNF- $\alpha$ ) was significantly higher in BM-derived macrophages from wild-type mice



compared with those from *PAR-2*<sup>-/-</sup> mice after FXa or AP-2 treatment (Figure 7A). The result of western blotting demonstrated that the activation of PAR-2 signaling by FXa increased the degradation of I $\kappa$ B $\alpha$ , suggesting the activation of NF- $\kappa$ B in macrophages (Figure 7B). We further examined the role of PAR-2 signaling in lipid uptake by macrophages in vitro. The activation of PAR-2 signaling by FXa or AP-2 significantly increased uptake of oxidized low-density lipoprotein (oxLDL) by BM-derived macrophages from wild-type mice compared with those from *PAR-2*<sup>-/-</sup> mice (Figure 7C). These results suggested that PAR-2 signaling promotes pro-inflammatory activation of macrophages.

### **Relationship between FXa-PAR-2 signaling and atherosclerosis in clinical samples**

To investigate the role of the FXa-PAR-2 signaling pathway in the development of atherosclerosis in humans, we investigated the link between plasma FXa level and the severity of coronary atherosclerosis. Blood samples, Gensini score and intravascular ultrasound (IVUS) data were obtained from 32 patients who underwent percutaneous coronary intervention. Patient characteristics at enrollment are shown in Table 3. Mean age of the participants was 69.6 $\pm$ 1.1 years; 63% were men. Plasma FXa level positively correlated with the Gensini score (Spearman  $\rho=0.36$ ,  $P<0.05$ ) (Figure 8A). Plasma FXa level tended to correlate positively with coronary plaque volume determined by intravascular ultrasound study (Spearman  $\rho=0.42$ ,  $P=0.06$ ) (Figure 8B). Multiple regression analysis revealed that plasma FXa level remained as an independent predictor of the Gensini score (corrected  $R^2=0.29$ ,  $P<0.05$ ). Multiple regression analysis revealed that plasma FXa level, as well as male sex and HbA1c level, remained as independent predictors of coronary plaque volume (corrected  $R^2=0.44$ ,  $P<0.01$ ) (Table 4). These results indicated that FXa

promotes vascular inflammation through PAR-2 and is associated with the severity of atherosclerosis in coronary arteries in humans.

## Discussion

Accumulating evidence suggests that PAR-2 signaling promotes pro-inflammatory responses in many cell types <sup>7, 10, 11</sup>, contributing to the pathogenesis of inflammatory diseases <sup>12-16</sup>, although little is known about the role of PAR-2, especially in hematopoietic cells, in the development of vascular inflammation and atherosclerosis. In this study, in vivo experiments using PAR-2 deficient *ApoE*<sup>-/-</sup> mice and BM-specific PAR-2 expression or deletion animals demonstrated that PAR-2 deletion attenuated vascular inflammation and reduced the development and destabilization of atherosclerotic lesions without the alteration of metabolic parameters including blood lipid levels. The results of in vitro experiments using peritoneal macrophages demonstrated that PAR-2 signaling participates in pro-inflammatory activation of macrophages through NF- $\kappa$ B pathway. Our findings suggest that the PAR-2 signaling promotes vascular inflammation and atherogenesis, and that pro-inflammatory activation of macrophages through the PAR-2 signaling plays a pivotal role as an underlying mechanism.

Previous reports demonstrated that several proteases including trypsin, tryptase and FXa, but not thrombin, activate PAR-2. Different from other members of the PAR family, platelets do not express PAR-2, whereas vascular cells, such as endothelial cells and smooth muscle cells, and leukocytes express PAR-2 <sup>11</sup>. Because of its unique expression pattern, previous studies investigated the role of PAR-2 in vascular functions. In fact, the contribution of PAR-2 to vasodilation

<sup>26, 27</sup> and inflammatory responses <sup>15, 16</sup> has been reported. However, the role of PAR-2 signaling in macrophage activation is not fully investigated. Therefore, in this study, we generated BM-chimeric mice which express or lack PAR-2 in BM. PAR-2 deletion in hematopoietic cells attenuated vascular inflammation and atherogenesis. On the contrary, PAR-2 expression only in hematopoietic cells promoted vascular inflammation and atherogenesis. Furthermore, our in vitro experiments demonstrated that treatment of macrophages with FXa, the major endogenous ligand of PAR-2, or AP-2, a specific agonist for PAR-2, increases inflammatory gene expression and lipid uptake, both of which are critical for atherosclerotic lesion progression and destabilization. These results suggested that PAR-2 plays a pivotal role in pro-inflammatory activation of macrophages, contributing to the development of vascular inflammation and atherogenesis.

Several previous studies have already reported a role of PAR-2 in non-hematopoietic cells in the development of vascular inflammation in different models <sup>28-31</sup>. Therefore, it is plausible that PAR-2 in non-hematopoietic cells such as endothelial cells may participate in atherogenesis. In fact, in our BMT experiments, expression of PAR-2 in the vasculature enhanced lesion development despite of the presence or absence of PAR-2 in hematopoietic cells. These results indicated pivotal roles of PAR-2 of both hematopoietic cells, especially macrophages, and non-hematopoietic cells in atherogenesis, and suggested PAR-2 can be a potential therapeutic target against atherosclerosis.

Since clinical approval as an anti-coagulant, a direct oral FXa inhibitor, rivaroxaban, attracts much attention because of its beneficial effects on cardiovascular events proved by several clinical trials <sup>18-21</sup>. In our clinical study, plasma FXa level predicts the severity of coronary atherosclerosis such as Gensini score and coronary plaque volume. PAR-2 is activated by FXa

and upregulates inflammation through NF- $\kappa$ B pathway<sup>32-34</sup>. Therefore, the results of in vivo animal studies and in vitro studies of the present study may provide one of the possible mechanisms for recent clinical studies. Interestingly, recent studies have demonstrated that several coagulation factors such as tissue factor and factor X exist not only in the circulating blood but also in atherosclerotic plaques in human autopsy samples<sup>35</sup>. In our present study, plasma FXa level between *ApoE*<sup>-/-</sup> mice and *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice did not differ, although, in our previous study, we reported higher expression of PAR-2 in atherosclerotic aorta of *ApoE*<sup>-/-</sup> mice compared with the aorta in wild-type mice<sup>17</sup>. These results suggested that the role of FXa-PAR-2 signaling in vascular inflammation is enhanced in the atherosclerotic plaques. In addition, in this study, we could not observe the inhibitory effect of rivaroxaban on atherogenesis in *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice. This result also may give support to the role of FXa-PAR-2 signaling in the development of atherosclerosis at least partially.

This study has several limitations. First, to investigate the role of PAR-2 in macrophages, a key cell-type in atherogenesis, we performed BMT experiments. Previous studies demonstrated that other hematopoietic cells such as neutrophils express PAR-2. Although our in vitro studies using peritoneal macrophages showed that FXa-PAR-2 signaling participates in pro-inflammatory activation of macrophages, other hematopoietic cells activated by FXa-PAR-2 signaling might have contributed to the results of in vivo studies. Second, we only examined the role of FXa-PAR-2 signaling in atherogenesis, however, FXa activates PAR-1 as well. Some of previous studies reported that contribution of PAR-1 to inflammation<sup>36, 37</sup>. Therefore, it might also participate in the development of atherosclerosis. Further studies are needed to clarify the role of FXa-PARs signaling in vascular inflammation and atherogenesis. Third our patient population in our clinical

study was small, and a large scale study is required to confirm our results. Lastly, in our clinical study, total cholesterol level did not predict the coronary atherosclerosis determined by IVUS. We speculate that because we included only patients receiving statins for at least 6 months, lipid levels were well controlled in this study population.

Our present study demonstrated proof of the concept that PAR-2 signaling in macrophages plays a pivotal role in the development of atherosclerosis. Further studies are needed to elucidate the participation of FXa-PARs signaling in the development of vascular inflammation and atherosclerosis. In conclusion, PAR-2 deletion attenuates vascular inflammation and atherogenesis in *ApoE*<sup>-/-</sup> mice. PAR-2 in hematopoietic cells may contribute, at least in part, to the development of atherosclerosis. Combined with the results of our in vitro studies, these results indicated that PAR-2 signaling in macrophages plays a pivotal role in vascular inflammation. The PAR-2 signaling pathway may provide a novel mechanism of atherogenesis and serve as a potential therapeutic target for atherosclerosis.

### **Acknowledgements**

The authors thank Yumiko Saga and Etsuko Uematsu for their technical assistance. The authors also thank Dr. Yoshihiro Okayama, University Hospital of Tokushima Clinical Trial Center for Developmental Therapeutics, for his significant advice for statistical analyses.

### **Funding**

This work was partially supported by Japan Society for the Promotion of Science KAKENHI Grants (Number 26860565 to T.H., Number 16K09517 to D.F., and Number 16H05299 and 26248050 to

M. Sata), a Sakakibara Memorial Research Grant from The Japan Research Promotion Society for Cardiovascular Diseases (D.F.), a Grant from Japan Cardiovascular Research Foundation (D.F.), a grant-in-aid from the Cardiovascular Research Fund, Tokyo, Japan (D.F.), the Takeda Science Foundation (D.F. and M.Sata), the Fugaku Trust for Medical Research (M.S.), the Vehicle Racing Commemorative Foundation (M.S.), and a Bayer Scholarship for Cardiovascular Research (D.F). M. Sata received research funding from Bayer Yakuhin, Ltd. The funders had no role in the study design, data collection, and analysis, or preparation of the manuscript.

#### **Conflict of Interest Disclosures**

The Department of Cardio-Diabetes Medicine, Tokushima University Graduate School, is supported in part by unrestricted research grants from Boehringer Ingelheim, Tanabe-Mitsubishi, Kowa, and Actelion. Dr. Sata received research funding from Bayer Yakuhin, Ltd. Other authors declare that they have no conflict of interest.

## References

1. Aikawa M, Libby P. Atherosclerotic plaque inflammation: the final frontier? *Can J Cardiol.* 2004;20:631-634.
2. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med.* 1999;340:115-126.
3. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med.* 2005;352:1685-1695.
4. Paoletti R, Gotto AM, Jr., Hajjar DP. Inflammation in atherosclerosis and implications for therapy. *Circulation.* 2004;109:III20-26.
5. Esmon CT, Fukudome K, Mather T, Bode W, Regan LM, Stearns-Kurosawa DJ, Kurosawa S. Inflammation, sepsis, and coagulation. *Haematologica.* 1999;84:254-259.
6. Levi M, van der Poll T, Buller HR. Bidirectional relation between inflammation and coagulation. *Circulation.* 2004;109:2698-2704.
7. Borissoff JI, Spronk HM, ten Cate H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med.* 2011;364:1746-1760.
8. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature.* 2000;407:258-264.
9. Croce K, Libby P. Intertwining of thrombosis and inflammation in atherosclerosis. *Curr Opin Hematol.* 2007;14:55-61.
10. Leger AJ, Covic L, Kuliopulos A. Protease-activated receptors in cardiovascular diseases. *Circulation.* 2006;114:1070-1077.
11. Major CD, Santulli RJ, Derian CK, Andrade-Gordon P. Extracellular mediators in atherosclerosis and thrombosis: lessons from thrombin receptor knockout mice.

- Arterioscler Thromb Vasc Biol.* 2003;23:931-939.
12. Badeanlou L, Furlan-Freguia C, Yang G, Ruf W, Samad F. Tissue factor-protease-activated receptor 2 signaling promotes diet-induced obesity and adipose inflammation. *Nat Med.* 2011;17:1490-1497.
  13. Tennant GM, Wadsworth RM, Kennedy S. PAR-2 mediates increased inflammatory cell adhesion and neointima formation following vascular injury in the mouse. *Atherosclerosis.* 2008;198:57-64.
  14. Redecha P, Franzke CW, Ruf W, Mackman N, Girardi G. Neutrophil activation by the tissue factor/Factor VIIa/PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. *J Clin Invest.* 2008;118:3453-3461.
  15. Samad F, Ruf W. Inflammation, obesity, and thrombosis. *Blood.* 2013;122:3415-3422.
  16. Spronk HM, de Jong AM, Crijns HJ, Schotten U, Van Gelder IC, Ten Cate H. Pleiotropic effects of factor Xa and thrombin: what to expect from novel anticoagulants. *Cardiovasc Res.* 2014;101:344-351.
  17. Hara T, Fukuda D, Tanaka K, Higashikuni Y, Hirata Y, Nishimoto S, Yagi S, Yamada H, Soeki T, Wakatsuki T, Shimabukuro M, Sata M. Rivaroxaban, a novel oral anticoagulant, attenuates atherosclerotic plaque progression and destabilization in ApoE-deficient mice. *Atherosclerosis.* 2015;242:639-646.
  18. Mega JL, Braunwald E, Mohanavelu S, Burton P, Poulter R, Misselwitz F, Hricak V, Barnathan ES, Bordes P, Witkowski A, Markov V, Oppenheimer L, Gibson CM. Rivaroxaban versus placebo in patients with acute coronary syndromes (ATLAS ACS-TIMI 46): a randomised, double-blind, phase II trial. *Lancet.* 2009;374:29-38.



19. Mega JL, Braunwald E, Murphy SA, Plotnikov AN, Burton P, Kiss RG, Parkhomenko A, Tendera M, Widimsky P, Gibson CM. Rivaroxaban in patients stabilized after a ST-segment elevation myocardial infarction: results from the ATLAS ACS-2-TIMI-51 trial (Anti-Xa Therapy to Lower Cardiovascular Events in Addition to Standard Therapy in Subjects with Acute Coronary Syndrome-Thrombolysis In Myocardial Infarction-51). *J Am Coll Cardiol.* 2013;61:1853-1859.
20. Mega JL, Braunwald E, Wiviott SD, Bassand JP, Bhatt DL, Bode C, Burton P, Cohen M, Cook-Bruns N, Fox KA, Goto S, Murphy SA, Plotnikov AN, Schneider D, Sun X, Verheugt FW, Gibson CM. Rivaroxaban in patients with a recent acute coronary syndrome. *N Engl J Med.* 2012;366:9-19.
21. Eikelboom JW, Connolly SJ, Bosch J, Dagenais GR, Hart RG, Shestakovska O, Diaz R, Alings M, Lonn EM, Anand SS, Widimsky P, Hori M, Avezum A, Piegas LS, Branch KRH, Probstfield J, Bhatt DL, Zhu J, Liang Y, Maggioni AP, Lopez-Jaramillo P, O'Donnell M, Kakkar AK, Fox KAA, Parkhomenko AN, Ertl G, Stork S, Keltai M, Ryden L, Pogossova N, Dans AL, Lanas F, Commerford PJ, Torp-Pedersen C, Guzik TJ, Verhamme PB, Vinereanu D, Kim JH, Tonkin AM, Lewis BS, Felix C, Yusoff K, Steg PG, Metsarinne KP, Cook Bruns N, Misselwitz F, Chen E, Leong D, Yusuf S. Rivaroxaban with or without Aspirin in Stable Cardiovascular Disease. *N Engl J Med.* 2017;377:1319-1330.
22. Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med.* 2002;8:403-409.
23. Nishimoto S, Fukuda D, Higashikuni Y, Tanaka K, Hirata Y, Murata C, Kim-Kaneyama JR,

- Sato F, Bando M, Yagi S, Soeki T, Hayashi T, Imoto I, Sakaue H, Shimabukuro M, Sata M. Obesity-induced DNA released from adipocytes stimulates chronic adipose tissue inflammation and insulin resistance. *Sci Adv.* 2016;2:e1501332.
24. Gensini GG. A more meaningful scoring system for determining the severity of coronary heart disease. *Am J Cardiol.* 1983;51:606.
25. Yamaguchi K, Wakatsuki T, Soeki T, Niki T, Taketani Y, Oeduka H, Kusunose K, Ise T, Iwase T, Yamada H, Sata M. Effects of telmisartan on inflammatory cytokines and coronary plaque component as assessed on integrated backscatter intravascular ultrasound in hypertensive patients. *Circ J.* 2014;78:240-247.
26. Damiano BP, Cheung WM, Santulli RJ, Fung-Leung WP, Ngo K, Ye RD, Darrow AL, Derian CK, de Garavilla L, Andrade-Gordon P. Cardiovascular responses mediated by protease-activated receptor-2 (PAR-2) and thrombin receptor (PAR-1) are distinguished in mice deficient in PAR-2 or PAR-1. *J Pharmacol Exp Ther.* 1999;288:671-678.
27. Saifeddine M, al-Ani B, Cheng CH, Wang L, Hollenberg MD. Rat proteinase-activated receptor-2 (PAR-2): cDNA sequence and activity of receptor-derived peptides in gastric and vascular tissue. *Br J Pharmacol.* 1996;118:521-530.
28. Hezi-Yamit A, Wong PW, Bien-Ly N, Komuves LG, Prasad KS, Phillips DR, Sinha U. Synergistic induction of tissue factor by coagulation factor Xa and TNF: evidence for involvement of negative regulatory signaling cascades. *Proc Natl Acad Sci U S A.* 2005;102:12077-12082.
29. Nystedt S, Ramakrishnan V, Sundelin J. The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells. Comparison with the thrombin receptor.

- J Biol Chem.* 1996;271:14910-14915.
30. Sparkenbaugh EM, Chantrathammachart P, Mickelson J, van Ryn J, Hebbel RP, Monroe DM, Mackman N, Key NS, Pawlinski R. Differential contribution of FXa and thrombin to vascular inflammation in a mouse model of sickle cell disease. *Blood.* 2014;123:1747-1756.
  31. Svensson KJ, Kucharzewska P, Christianson HC, Skold S, Lofstedt T, Johansson MC, Morgelin M, Bengzon J, Ruf W, Belting M. Hypoxia triggers a proangiogenic pathway involving cancer cell microvesicles and PAR-2-mediated heparin-binding EGF signaling in endothelial cells. *Proc Natl Acad Sci U S A.* 2011;108:13147-13152.
  32. Borensztajn K, Peppelenbosch MP, Spek CA. Factor Xa: at the crossroads between coagulation and signaling in physiology and disease. *Trends Mol Med.* 2008;14:429-440.
  33. Krupiczkoj MA, Scotton CJ, Chambers RC. Coagulation signalling following tissue injury: focus on the role of factor Xa. *Int J Biochem Cell Biol.* 2008;40:1228-1237.
  34. Ringwala SM, Dibattiste PM, Schneider DJ. Effects on platelet function of a direct acting antagonist of coagulation factor Xa. *J Thromb Thrombolysis.* 2012;34:291-296.
  35. Borissoff JI, Heeneman S, Kilinc E, Kassak P, Van Oerle R, Winckers K, Govers-Riemslog JW, Hamulyak K, Hackeng TM, Daemen MJ, ten Cate H, Spronk HM. Early atherosclerosis exhibits an enhanced procoagulant state. *Circulation.* 2010;122:821-830.
  36. Antoniak S, Cardenas JC, Buczek LJ, Church FC, Mackman N, Pawlinski R. Protease-Activated Receptor 1 Contributes to Angiotensin II-Induced Cardiovascular Remodeling and Inflammation. *Cardiology.* 2017;136:258-268.
  37. Grimsey NJ, Trejo J. Integration of endothelial protease-activated receptor-1 inflammatory signaling by ubiquitin. *Curr Opin Hematol.* 2016;23:274-279.



## Figure legends

### Figure 1. Effect of genetic deletion of RAR-2 on atherosclerotic lesion formation in *ApoE*<sup>-/-</sup> mice.

En face Sudan IV staining of the aortic arch showed that *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice had reduced atherosclerotic lesions compared with *ApoE*<sup>-/-</sup> mice (n=9-13). Scale bar: 1 mm. \**P*<0.05. All values are mean ± SEM.

### Figure 2. Effect of genetic deletion of PAR-2 on characteristics of atherosclerotic plaques in *ApoE*<sup>-/-</sup> mice.

**(A)** Oil red O staining of atherosclerotic plaques in the aortic root. Lipid deposition in plaques was decreased in *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice compared with that in *ApoE*<sup>-/-</sup> mice. Scale bar: 100 μm. **(B)** Picrosirius red staining observed under polarization microscopy. Collagen content in plaques was increased in *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice compared with that in *ApoE*<sup>-/-</sup> mice. Scale bar: 100 μm. **(C-E)** Immunostaining against Mac-3 (C), MCP-1 (D), and MMP-9 (E). *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice showed reduced accumulation of macrophages and less expression of MCP-1 and MMP-9 in atherosclerotic plaques compared with *ApoE*<sup>-/-</sup> mice. Scale bar: 50 μm. (n=10 in A and B, n = 5 in C through E, per group). Mac-3 indicates macrophage antigen-3; MCP-1, monocyte chemotactic protein-1; and MMP-9, matrix metalloproteinase-9. \**P*<0.05 and \*\**P*<0.01. All values are mean ± SEM.

### Figure 3. Effect of genetic deletion of PAR-2 on vascular inflammation.

**(A)** qPCR analyses using abdominal aorta revealed that genetic deletion of PAR-2 decreased the

expression of F4/80, a macrophage marker. PAR-2 deletion also reduced the expression of inflammatory molecules, such as MCP-1, MIP-1 $\alpha$ , IL-6 and MMP-9, in the atherosclerotic aorta (n=10-12). **(B)** The expression of I $\kappa$ B $\alpha$  was examined by western blotting. The degradation of I $\kappa$ B $\alpha$  was reduced in PAR-2 deficient animals, suggesting suppression of NF- $\kappa$ B activity (n=5). **(C)** Expression of PARs in the aorta. qPCR analyses using abdominal aorta revealed that genetic deletion of PAR-2 did not change the expression of PAR-1, PAR-3, and PAR-4. (n=10). *ApoE*<sup>-/-</sup> indicates apolipoprotein E-deficient; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP-1  $\alpha$ , macrophage inflammatory protein  $\alpha$ ; MMP-9, matrix metalloproteinase-9; N.D., not detectable; and TNF-  $\alpha$ , tumor necrosis factor- $\alpha$ . \**P*<0.05. All values are mean  $\pm$  SEM.

**Figure 4. Role of PAR-2 in hematopoietic cells in atherosclerotic lesion formation.**

**(A)** En face Sudan IV staining of the aortic arch demonstrated that hematopoietic deletion of PAR-2 in *ApoE*<sup>-/-</sup> mice reduced atherosclerotic lesion progression in the aortic arch compared with the control BM chimeric mice (n=9-10). **(B)** En face Sudan IV staining of the aortic arch demonstrated that hematopoietic restoration of PAR-2 in *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice accelerated atherosclerotic lesion progression in the aortic arch compared with the control BM chimeric mice (n=5-7). Scale bar: 1 mm. BM indicates bone marrow. \**P*<0.05 and \*\**P*<0.01. All values are mean  $\pm$  SEM.

**Figure 5. Effect of hematopoietic deletion of PAR-2 on characteristics of atherosclerotic plaques in aortic root.**

**(A)** Oil red O staining of atherosclerotic plaques in the aortic root. PAR-2 deletion in BM cells reduced lipid deposition compared with the control group. Scale bar: 100  $\mu$ m. **(B)** Picrosirius red

staining observed under polarization microscopy. Collagen content was increased in *ApoE*<sup>-/-</sup> mice lacking PAR-2 in BM cells compared with the control group. Scale bar: 100  $\mu$ m. **(C-E)** Immunostaining against Mac-3 (C), MCP-1 (D), and MMP-9 (E). *ApoE*<sup>-/-</sup> mice lacking PAR-2 in BM cells demonstrated reduced accumulation of macrophages and less expression of MCP-1 and MMP-9 in atherosclerotic plaques compared with the control chimeric mice. Scale bar: 50  $\mu$ m. (n=9-10 in A and B, n=5-10 in C through E, per group). BM indicates bone marrow; Mac-3, macrophage antigen-3; MCP-1, monocyte chemotactic protein-1; MIP-1  $\alpha$ , macrophage inflammatory protein  $\alpha$ ; and MMP-9, matrix metalloproteinase-9. \*\**P*<0.01. All values are mean  $\pm$  SEM.

**Figure 6. Role of PAR-2 in hematopoietic cells in expression of inflammatory molecules in abdominal aorta.**

**(A)** qPCR analyses using abdominal aorta revealed that hematopoietic deletion of PAR-2 in *ApoE*<sup>-/-</sup> mice decreased the expression of F4/80, a macrophage marker, and inflammatory molecules in the atherosclerotic aorta (n=7-8). **(B)** qPCR analyses using abdominal aorta revealed that hematopoietic restoration of PAR-2 in *PAR-2*<sup>-/-</sup>*ApoE*<sup>-/-</sup> mice increased inflammatory molecules in the atherosclerotic aorta (n=4-7). IL indicates interleukin; MCP-1, monocyte chemotactic protein-1; MIP-1  $\alpha$ , macrophage inflammatory protein  $\alpha$ ; MMP-9, matrix metalloproteinase-9; and TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . \**P*<0.05 and \*\**P*<0.01. All values are mean  $\pm$  SEM.

**Figure 7. Role of PAR-2 signaling in macrophage activation.**

**(A)** qPCR analysis demonstrated that FXa or AP-2 significantly increased the expression of

inflammatory molecules in BM-derived macrophages from wild-type mice compared with those from *PAR-2*<sup>-/-</sup> mice (n=4, per group). **(B)** The expression of I $\kappa$ B $\alpha$  in BM-derived macrophages was examined by western blotting. FXa enhanced the degradation of I $\kappa$ B $\alpha$  in wild-type macrophage but not in PAR-2-deficient macrophages (n=5-6). **(C)** Lipid uptake by BM-derived macrophages was examined by Oil red O staining. The activation of PAR-2 signaling by FXa or AP-2 significantly increased uptake of oxidized low-density lipoprotein (oxLDL) to BM-derived macrophages from wild-type mice compared with those from *PAR-2*<sup>-/-</sup> mice. The ratio of Oil red O positive area/cell area (%) was calculated in 100 cells under light microscopy and averaged. Scale bar: 10  $\mu$ m. (n=4 per group). AP-2 indicates specific agonist peptide of PAR-2; BM, bone marrow; FXa, activated factor X; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MW, molecular weight; NT, nontreatment; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ . and WT, wild-type. \**P*<0.05 and \*\**P*<0.01. All values are mean  $\pm$  SEM.

**Figure 8. Relationship between plasma FXa level and severity of coronary atherosclerosis in humans.**

**(A)** Plasma FXa level in patients with stable angina showed a positive correlation with Gensini score. **(B)** Plasma FXa level in patients with stable angina tended to correlate with coronary plaque volume as determined by IVUS study. FXa indicates activated factor X; and IVUS, intravascular ultrasound.



**Table 1. Effects of PAR-2 deletion on metabolic parameters.**

	<i>ApoE<sup>-/-</sup></i>	<i>PAR-2<sup>-/-</sup> ApoE<sup>-/-</sup></i>	<i>P</i> -value
Body weight, g	38.8±1.8	43.0±1.5	0.07
Heart rate /min	667±14	645±12	0.28
Systolic BP, mmHg	110.5±3.7	115.7±3.0	0.29
Diastolic BP, mmHg	80.1±3.8	84.2±3.1	0.41
FXa, ng/ml	10.8±1.1	8.7±0.9	0.29
Plasma glucose, mg/dl	133.7±6.2	131.5±5.0	0.78
Triglyceride, mg/dl	117.0±31.2	114.5±26.4	0.95
Total cholesterol, mg/dl	1084.3±179.0	1316.4±151.3	0.33
HDL-C, mg/dl	20.5±5.0	26.0±4.3	0.41
LDL-C, mg/dl	1040.3±173.0	1298.2±151.7	0.27

All values are mean±SEM. ApoE<sup>-/-</sup> indicates apolipoprotein E-deficient; BP, blood pressure, FXa, activated factor X; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and PAR-2, protease-activated receptor.

**Table 2. Effects of PAR-2 deletion in BM on metabolic parameters.**

<b>Donor Recipient</b>	<b><i>ApoE</i><sup>-/-</sup> <i>ApoE</i><sup>-/-</sup></b>	<b><i>PAR-2</i><sup>-/-</sup><i>ApoE</i><sup>-/-</sup> <i>ApoE</i><sup>-/-</sup></b>	<b><i>P</i>-value</b>
Body weight, g	28.8±1.7	27.6±1.5	0.30
Heart rate /min	667±14	667±14	0.49
Systolic BP, mmHg	102.3±7.5	106.5±6.5	0.35
Diastolic BP, mmHg	62.7±7.2	69.8±6.3	0.91
Plasma glucose, mg/dl	131.3±6.3	122.8±5.5	0.25
Triglyceride, mg/dl	186.3±23.9	223.8±29.7	0.34
Total cholesterol, mg/dl	983.1±42.2	1147.0±79.5	0.09
HDL-C, mg/dl	26.4±2.9	21.1±2.0	0.14
LDL-C, mg/dl	919.4±44.3.4	1081.0±80.7	0.10

All values are mean±SEM. ApoE<sup>-/-</sup> indicates apolipoprotein E-deficient; BM, bone marrow; BP, blood pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and PAR-2, protease-activated receptor.

**Table 3. Patients' characteristics.**

	(N = 32)
Age, yrs	69.6±1.1
Male/female	20 (63) / 12 (37)
Smoking	19 (59)
Hypertension	30 (94)
HbA1c, %	6.1±0.2
Triglyceride, mg/dl	125±11
Total cholesterol, mg/dl	176±6
HDL-C, mg/dl	105±5
LDL-C, mg/dl	52±2

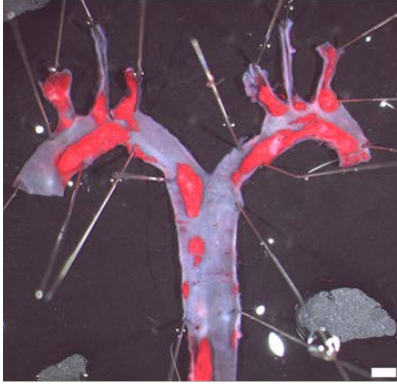
Data presented are mean±SEM or number (percentage). HDL-C indicates high-density lipoprotein cholesterol; and LDL-C, low-density lipoprotein cholesterol.

**Table 4. Multivariate analysis for prediction of the severity of coronary atherosclerosis.**

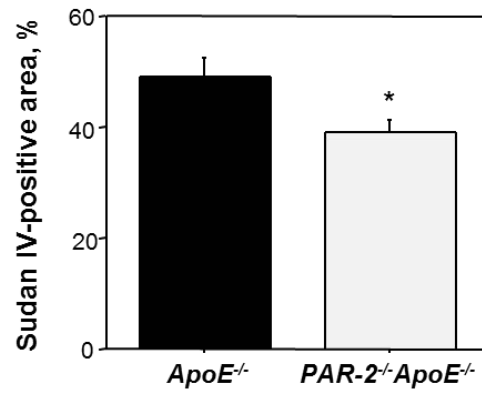
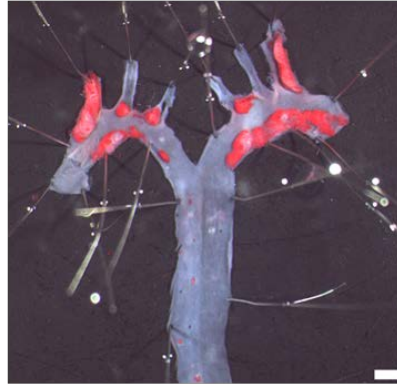
	Gensini score (log)		Coronary plaque volume	
Corrected R <sup>2</sup>	0.29		0.44	
P-value	0.04		0.006	
	Standard regression coefficient	P-value	Standard regression coefficient	P-value
Plasma FXa level, ng/ml	0.520	0.003	0.568	0.0005
Age, y	0.153	0.36	-0.104	0.48
Male sex	0.097	0.62	0.399	0.03
Smoking	0.265	0.16	-0.233	0.16
Hypertension	-0.128	0.44	0.272	0.07
HbA1c, %	0.003	0.99	0.511	0.002
HDL-C, mg/dL	-0.287	0.21	-0.180	0.37
Triglyceride, mg/dL	0.006	0.98	-0.182	0.35
Total cholesterol, mg/dL	0.421	0.05	0.124	0.51

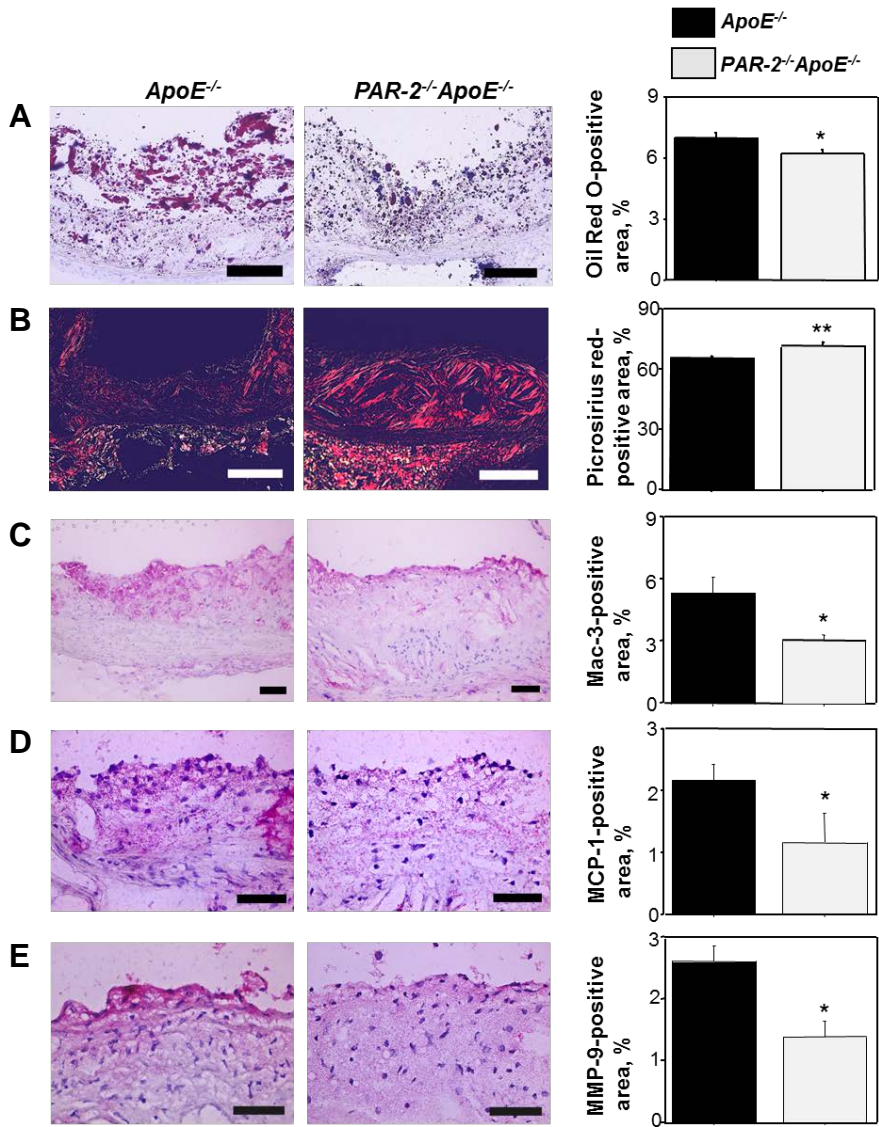
FXa indicates activated factor X; and HDL-C, high-density lipoprotein cholesterol.

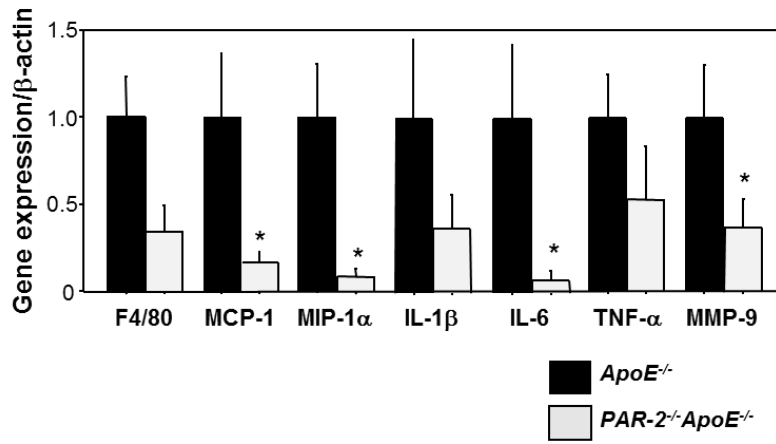
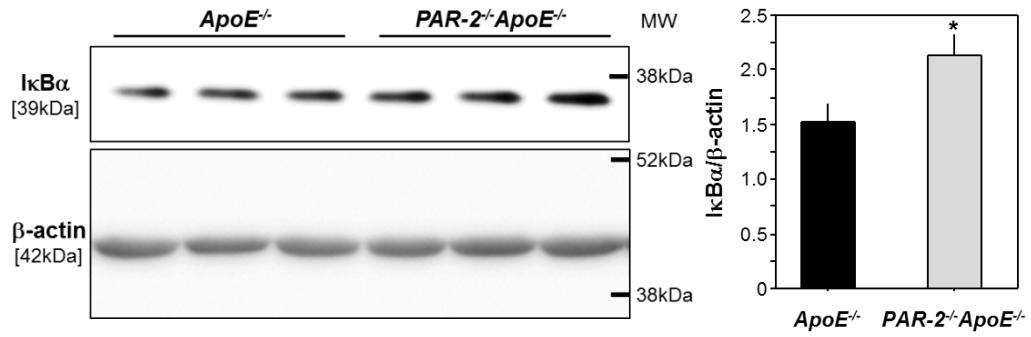
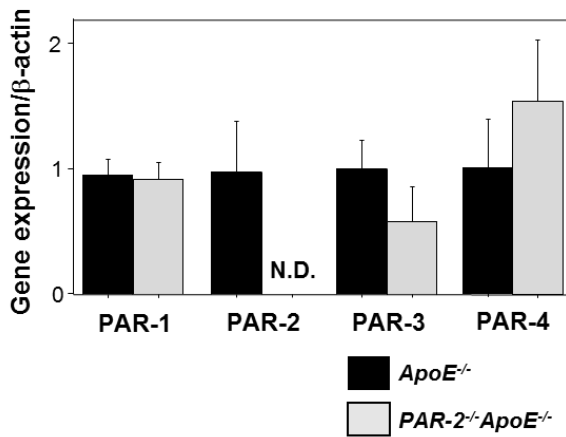
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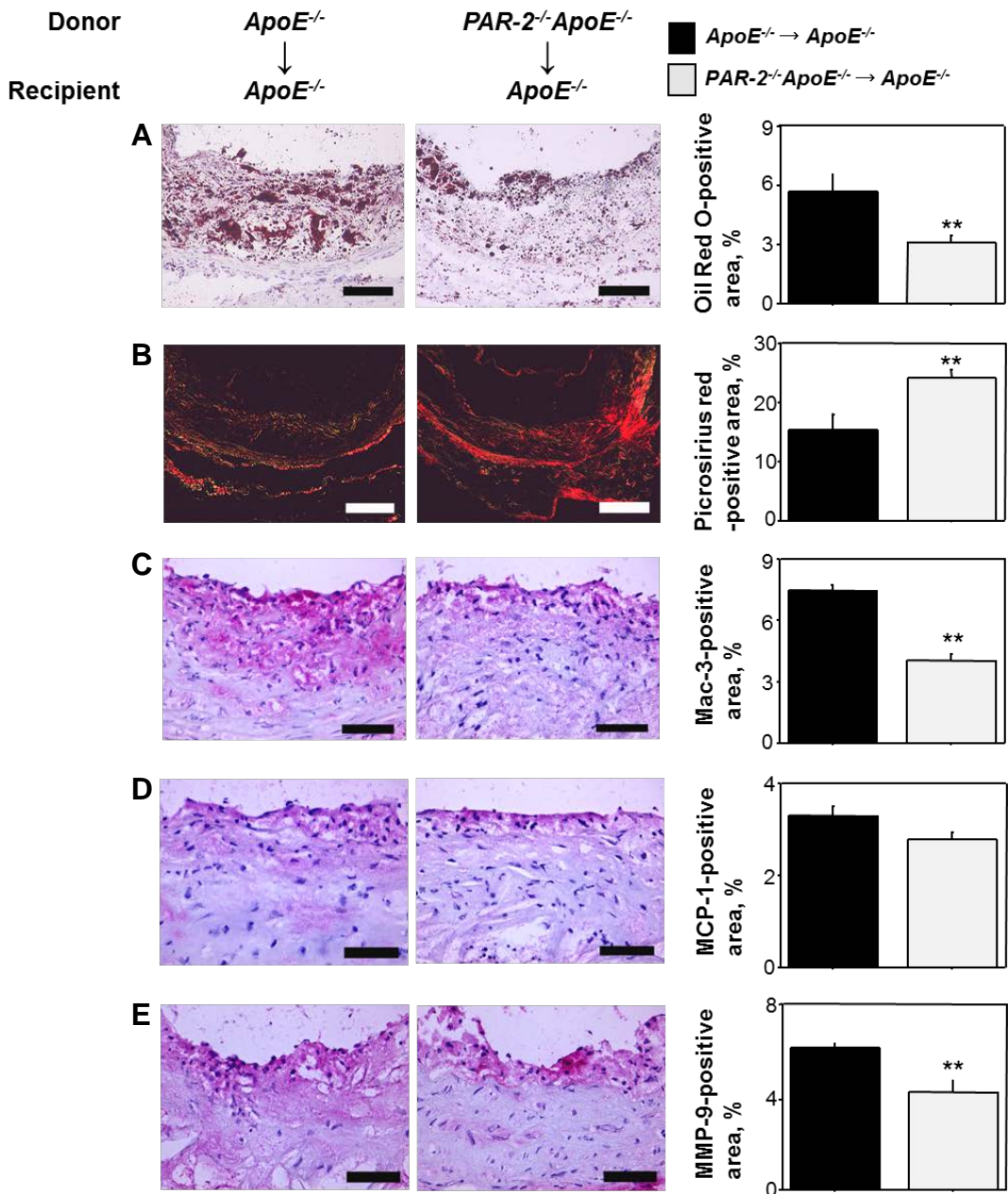


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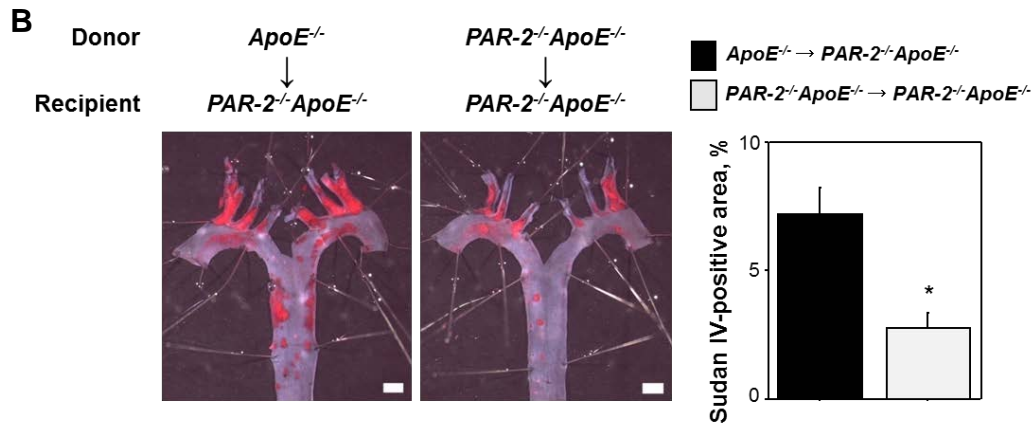
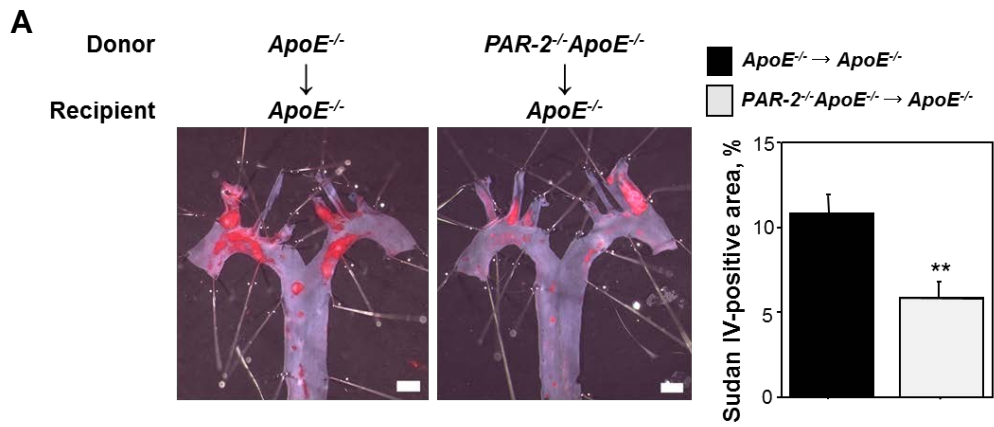


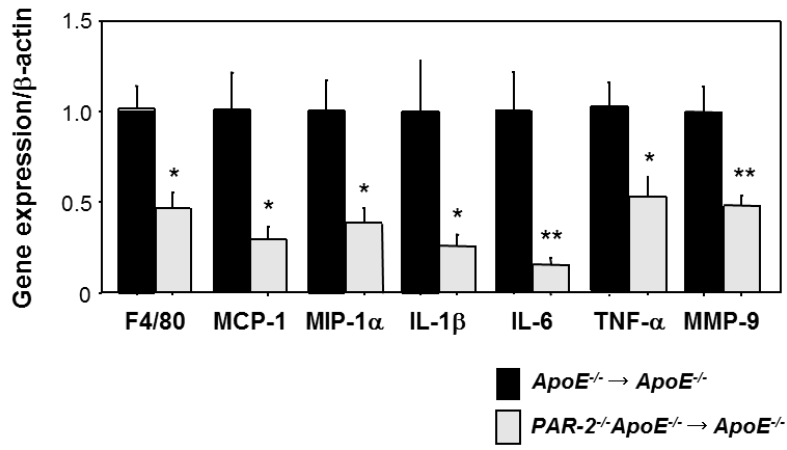
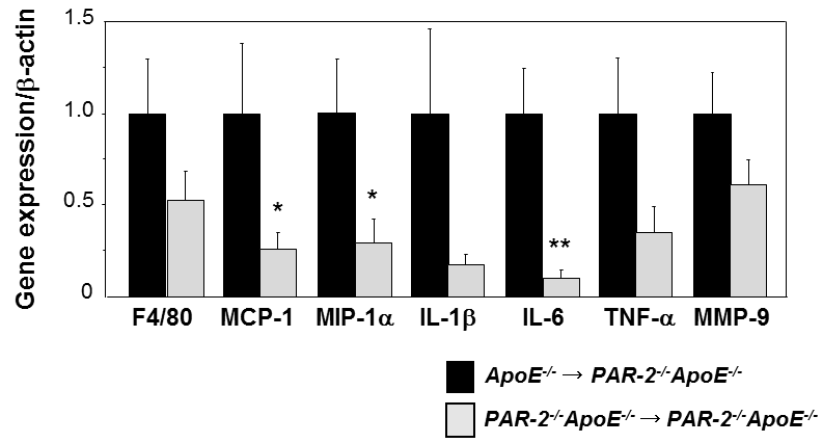


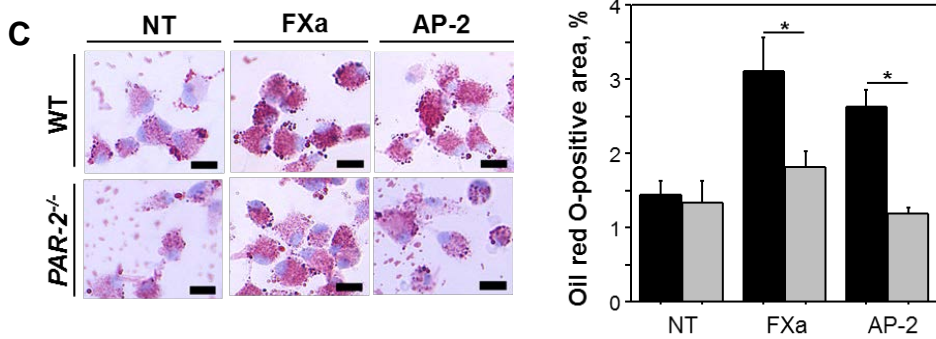
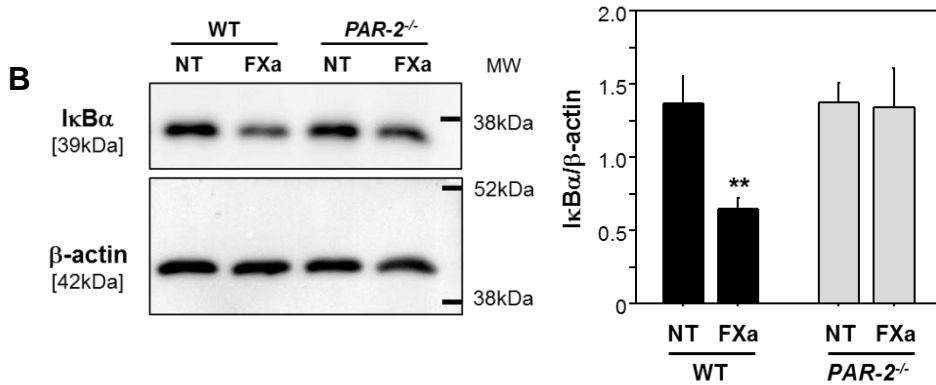
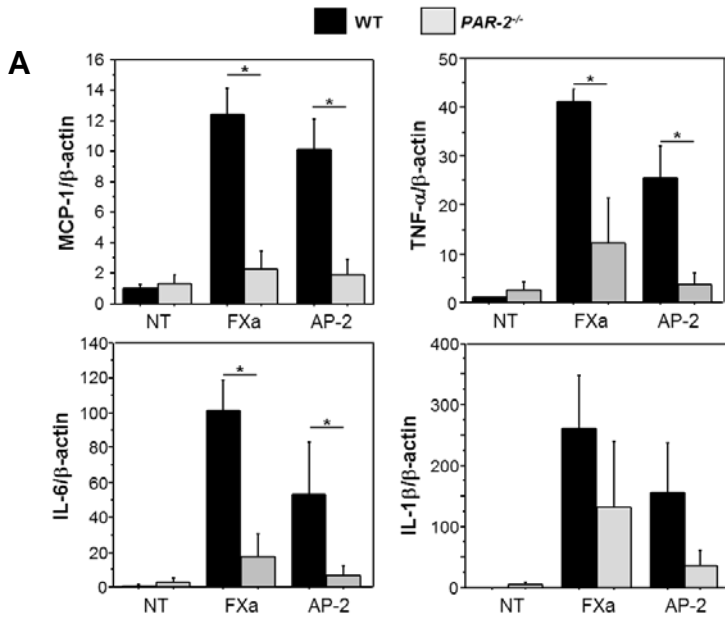
**A****B****C**

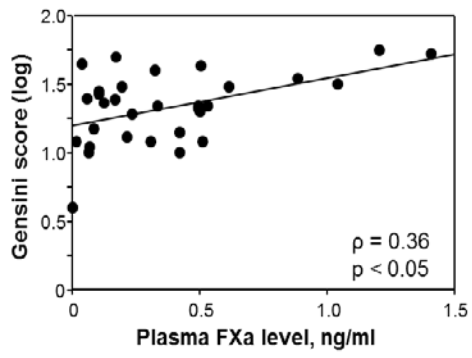






**A****B**



**A****B**