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Defective autophagy in vascular smooth muscle cells enhances plaque instability

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Abbreviated title: *defective autophagy in smooth muscle cells*

Abstract

Autophagy is considered as an evolutionarily preserved cellular catabolic process. Defective autophagy has been implicated in various human diseases, including cardiovascular diseases. Recently, we and others demonstrated that defective autophagy in vascular smooth muscle cells (SMCs) promotes the progression of atherosclerosis. In this study, we investigated the role of autophagy in SMCs on plaque instability *in vivo*. We generated mice with a defect in atg7 in which is an essential gene for autophagy, in SMCs by crossing *Atg7*$^{f/f}$ mice with *Tagln* (transgelin)*Cre*$^{+/0}$ mice (*Atg7cKO*). Then, *Atg7cKO* and apolipoprotein E (apoe)-deficient (*apoeKO*) mice were crossed to generate *Atg7cKO:apoeKO* mice. To generate a mice model of plaque instability, we conducted to form a tandem stenosis in the carotid artery of *Atg7cKO:apoeKO* mice and their controls (*apoeKO* mice) at the age of 10 weeks. At 5 weeks after surgery, the percentage of cross-sectional stenosis area in the operated common carotid artery of *Atg7cKO:apoeKO* mice was significantly higher than that in *apoeKO* mice. In addition, thrombus, which was not observed in *apoeKO* mice, was frequently found in *Atg7cKO:apoeKO* mice. Furthermore, the number of Berlin blue staining positive areas which indicated intraplaque haemorrhage, was significantly higher in *Atg7cKO:apoeKO* mice than in control *apoeKO* mice. Taken together, our data suggest that defective autophagy in SMCs enhances plaque instability and the risk of plaque rapture.

Keywords: Autophagy, Smooth muscle cells, plaque instability, atheroma

Abbreviations

*apoe*KO, apolipoprotein E-deficient

SMA, smooth muscle actin

SMCs, smooth muscle cells

SQSTMQ, Sequestosome 1

TS, tandem stenosis

Introduction

Macroautophagy (hereafter referred to as autophagy) is a highly evolutionarily conserved basal cellular process responsible for cellular renovation and homeostasis [1]. Autophagy acts to degrade proteins and organelles under basal conditions to eliminate defective or damaged organelles as well as to promote cell survival by ensuring an adequate supply of metabolic components, via the degradation of cytoplasmic components. Thus, defective autophagy has been implicated in various human diseases, including neuro-degenerative diseases [2], cancers [3], diabetes mellitus [4], glomerular diseases [5], and atherosclerotic diseases [6,7].

Atherosclerosis is a chronic progressive inflammatory disease that is associated with the incidence of cardiovascular diseases, such as myocardial infarction and stroke, which are major causes of morbidity and mortality [8]. The direct trigger of myocardial infarction and some cases of stroke in humans is the rupture of atherosclerotic plaques, which are known to be associated with thin fibrous caps, and are accumulations of infiltrated inflammatory cells, intraplaque newly formed blood vessels, intraplaque blood cells, intravascular thromboses, etc. [9]. Accordingly, an animal model of plaque instability reflecting the characteristics of human atherosclerosis would be useful to gain insight into the underlying pathophysiology of plaque rupture, toward the development of novel preventative therapies of plaque rupture. Large animals, such as pigs, monkeys, and rabbits are known to be useful models of plaque instability and rupture; however, ethical issues, high costs of breeding, and restricted availability limit the use of such animals. Instead, small animals, such as mice, have the potential to over- come these problems. Indeed, these

models, including Apoe (apolipoprotein E)-deficient mice (apoeKO) have been frequently used to study the pathogenesis and therapy of atherosclerosis [10]. However, in mouse models of atherosclerosis, such as apoeKO mice under normal conditions, plaque instability and rupture are not commonly observed [10]. In this regard, Chen et al. recently developed a novel plaque rupture model by generating a tandem stenosis (TS) in the carotid artery of apoeKO mice [11]. This model elicits low wall shear stress and high tensile stress in the carotid artery of apoeKO mice. Atherosclerotic lesions formed in this model show similarities to human plaque instability, in terms of a thin and disrupted fibrous cap, the existence of necrotic cores, accumulation lipids, accumulation of monocytes/macrophages, intraplaque hemorrhage, and intravascular hemorrhage [11]. In addition, pla- que rupture is frequently observed in this model [11]. These fea- tures of atherosclerotic lesions were not readily observed in previously published murine models of plaque instability [12-15].

In advanced atherosclerotic lesions, smooth muscle cells (SMCs) play a substantial role in preventing the rupture of fibrous caps [16]. Autophagy appears to play protective roles in the homeostasis of SMCs, similarly to in many other cell types. Indeed, a recent study demonstrated that the number of autophagosomes was increased in SMCs of human atherosclerotic lesions evaluated by trans- mission electron microscopy [17]. In addition, we and others independently demonstrated that genetically induced autophagic failure specifically in SMCs enhanced the progression of athero- sclerosis in apoeKO mice fed a Western diet [7,18]. However, in these models, the pathological features of plaque instability and rupture were rarely observed. Therefore, the role of SMC autophagy in plaque rupture has remained largely unknown. Based on this information, in this study, we

applied modified TS surgery to further investigate the role of SMC autophagy in the susceptibility of plaque rupture in vivo.

Materials and Methods

2.1. Animals.

apoeKO mice and transgelin (Tagln) Cre $^{+/0}$ mice purchased from the Jackson Laboratory were housed in specific pathogen-free barrier facilities. Atg7$^{f/f}$ mice [19] were bred with Tagln Cre$^{+/0}$ mice to generate mice homologous for the floxed allele and hemizygious for the Cre transgene (Atg7cKO), as smooth muscle- specific Atg7-deficient mice [18]. These mice were backcrossed at least 10 times on a C57BL/6 background. Littermate controls were homogeneous for the floxed allele atg7 gene, but did not carry the Cre transgene (Atg7$^{f/f}$) [20]. Then, Atg7cKO and Atg7$^{f/f}$ were bred with apoeKO mice (C57BL/6 background) to generate Atg7cKO:apoeKO mice and Atg7$^{f/f}$apoeKO mice (control apoeKO mice) that were used for this study (Charles River Japan, Ibaraki, Japan). Mice were maintained under a 12-h light/dark cycle, and fed a standard rodent diet (22.6% protein, 53.8% carbohydrate, 5.6% fat, 6.6% mineral and vitamin mixture, and 3.3% fiber; total: 356 kcal/ 100 g, CRF-1, Charles River Japan) with water ad libitum. The study protocol was reviewed and approved by the Animal Care and Use Committee of Juntendo University.

2.2. TS surgery.

TS surgery was conducted using a modified version of a previously described protocol [11]. Briefly, at the age of 10 weeks, an incision was made in the neck after anaesthesia and the connective and adipose tissues were removed from the right common carotid artery. A needle with a diameter of 150 mm was inserted along the right common carotid artery. The right common carotid artery at the carotid artery bifurcation (the proximal point) and the point 3 μm distal from the proximal point (the distal point) were tied with 6-0 nylon suture threads, together with the needle. The needle was subsequently removed. Then, the skin incision was closed with a wound clip. After surgery, mice were fed a Western diet (1.25% cholesterol, 15% fat, and 0.5% sodium cholate) (D12108C, EPS EKISHIN Co., Ltd., Tokyo, Japan). The sham group underwent all aforementioned procedures except for suturing of the right com- mon carotid artery.

2.3. Quantification of atherosclerotic lesions.

At 5 weeks after TS surgery, mice were sacrificed under anaes- thesia induced by intraperitoneal injection of sodium pentobarbital, to quantify the number of atherosclerotic lesions. The aorta was flushed with normal saline followed by 10% buffered formalin, as described previously [18]. The operated common carotid arteries were collected. Then, the connective and adipose tissues were removed from the artery. Images were captured with a digital single-lens reflex camera. For quantitative analysis of atheroscle- rotic lesions, the carotid artery was embedded in optimal cutting temperature compound, and 4-μm thick cross-sections at 100 μm intervals from the distal point that was tied with a 6-0 nylon suture thread were prepared using a cryostat. Three consecutive sections were taken sequentially,

dried at room temperature for 30 min, and stained with hematoxylin and eosin to calculate atherosclerotic areas. Then, immunohistochemistry was performed using a guinea pig anti-human sequestosome 1 (SQSTM1)/p62 polyclonal anti- body (GP62-C, Progen Biotechnik, GmbH), or mouse anti-human ACTA2: actin, alpha 2, smooth muscle, aorta (ACTA2)/a-smooth muscle actinstaining antibody (M0851, Dako). Collagen-positive area was analyzed by staining with Azan stain (Muto Pure Chemicals, Tokyo, Japan). Berlin blue staining (Muto Pure Chemicals) was used for the detection of iron. Berlin blue staining-positive areas were counted in three consecutive sections per mice. The average number of Berlin blue staining-positive area per mouse was eval- uated. The percentage of cross-sectional stenosis area was calcu- lated by dividing the nonoccluded area by the area of the region inside the outer layer of the tunica media. Images were captured and analyzed using ImagePro Plus software.

2.4. Laboratory data.

Blood samples were collected from the mice at the time of sacrifice. Concentrations of cholesterol, chylomicron, very-low- density lipoprotein, low-density-lipoprotein cholesterol, and high-density-lipoprotein cholesterol were measured by Skylight Biotech, Inc. (Tokyo, Japan).

2.5. Statistical analysis.

Differences between two groups were analyzed for statistical significance using the Mann-Whitney t-test for continuous variables or the Fisher exact test for categorical

variables. P-values less than 0.05 were considered to indicate a statistically significant difference between two groups. Results are expressed as the mean ± SEM. Data were analyzed using the Statistical Package for Social Science computer software program, version 18 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Autophagy deficiency in SMCs is associated with macroscopic atherosclerotic plaque hemorrhage

To investigate the roles of SMC autophagy in plaque rupture, TS was surgically introduced in the right common carotid artery of control apoeKO mice and Atg7cKO:apoeKO mice at the age of 10 weeks. After the procedure, the mice were fed a Western diet. At 5 weeks after surgery, there was no significant difference in body weights and lipid parameters between control apoeKO mice and Atg7cKO:apoeKO mice (Supplementary Table 1). Chen et al. showed that TS surgery in high- fat fed apoeKO mice induces macroscopic atherosclerotic plaque hemorrhage in the common carotid artery [11]. Consistent with this finding, we found macroscopic atherosclerotic plaque hemorrhage in some mice; however, the frequency of this change was different between groups. Macroscopic atherosclerotic plaque hemorrhage was observed in 7 out of 14 control apoeKO mice, whereas it was observed in 13 out of 14 Atg7cKO:apoeKO mice (Fig. 1).

3.2. Autophagy deficiency in SMCs enhances progression of atherosclerosis.

We analyzed the pathological changes of sham-operated common carotid arteries of control apoeKO mice and Atg7cKO:apoeKO mice based on the analysis of serial cross sections. As shown in Fig. 2A and B, obvious atheromas were not found in sham-operated common carotid arteries of both control apoeKO mice and Atg7cK- O:apoeKO mice at 5 weeks after undergoing surgery. Using these sections, we analyzed the expression of SQSTM1, an adaptor protein that is degraded by autophagosomes, and its cellular accumulation, which has been widely used as a diagnostic maker for autophagic dysfunction [21]. As predicted, whereas a very small number of SQSTM1-positive cells were observed in cells located in the media of the sham-operated common carotid artery of control apoeKO mice, most cells at this location in Atg7cKO:apoeKO mice were positive for SQSTM1 positive (Fig. 2A and B).

Next, we analyzed the pathological changes of the operated common carotid artery of control apoeKO mice and Atg7cK- O:apoeKO mice. As shown in Fig. 2C, large atheromas were readily found in the operated common carotid artery of Atg7cKO:apoeKO mice at 5 weeks after TS surgery. These lesions showed features of various stages of atherosclerotic lesions, such as accumulation of macrophage-derived foam cells, migration of SMCs into the subendothelial space of atheromas, and necrotic tissue containing cholesterol crystals (Fig. 2C). Furthermore, a few regions with thin fibrous caps were identified, although in general fibrous caps were thick (Fig. 2C). In control apoeKO mice, these atherosclerotic lesions were rarely found, and typically, only small atheromas were found (Fig. 2D). In addition, in control apoeKO mice, the number of SQSTM1-expressing cells in the media of the operated carotid artery was higher than in the sham-operated carotid artery (Fig. 2E).

Furthermore, in Atg7cKO:apoeKO mice, the number of SQSTM1- positve cells in the operated carotid arteries was much higher than in sham-operated carotid arteries (Fig. 2F). In particular, in the operated carotid arteries of Atg7cKO:apoeKO mice, SQSTM1- positive cells were observed not only in the media, but also in the atheroma. Consistent with this change, the percentage of crosssectional stenosis area calculated by dividing the nonoccluded area by the area of the region inside the outer layer of the tunica media in the operated common carotid artery of Atg7cKO:apoeKO mice, was significantly higher than in control apoeKO mice (Fig. 2G). Taken together, these data suggest that autophagy deficiency in SMCs enhances the progression of atherosclerosis.

3.3 Autophagy deficiency in SMCs enhances plaque rupture and formation of thrombosis.

Next, to investigate the effect of autophagy deficiency in SMCs on plaque instability, we stained SMCs with Berlin blue to detect the presence of intraplaque hemorrhage. Whereas Berlin blue- positive areas were rarely found in SMCs from apoeKO mice (Fig. 3A), Berlin blue staining-positive areas were frequently found in the operated common carotid artery of Atg7cKO:apoeKO mice (Fig. 3B). Indeed, the number of Berlin blue staining-positive areas was significantly higher in Atg7cKO:apoeKO mice than in apoeKO mice (Fig. 3C). In addition, hemosiderin identified with Berlin blue stain was found in foam cells, suggesting that its formation may be associated with phagocytosis of red blood cells and hemoglobin when mice lived (Fig. 3D).

Regarding plaque instability-associated lesions, 5 out of 14 Atg7cKO:apoeKO mice showed formed thrombus, whereas none of the control apoeKO mice showed such lesions. Among the 5 lesions, there were 2 luminal thrombi that were composed of new capillary vessels derived from the vascular wall (Fig. 4A), myofibroblasts (Fig. 4B), and collagen (Fig. 4C). These features suggest that these thrombi are organized thrombi. The other 3 thrombi were atherothrombi characterized by the presence of neutrophil infiltration and neovessels (Fig. 4D). Taken together, these data suggest that Atg7cKO:apoeKO mice after TS surgery are susceptible to plaque rupture and thrombosis formation.

4. Discussion

In this study, we demonstrated that autophagic failure in SMCs enhanced plaque instability and the risk of plaque rupture in apoeKO mice undergoing TS surgery. This data clearly suggests the essential role of SMC autophagy in the protection against plaque instability. These results confirmed previous findings that autophagy deficiency in SMCs promotes the progression of atherosclerosis [7,18].

In the original study of a mouse model of plaque rupture, Chen et al. performed surgery to induce a TS in the right common carotid artery of 12-week old apoeKO mice fed a high-fat diet containing 0.15% cholesterol for 6 weeks [11], and subsequently analyzed plaque progression. At 2 weeks after surgery, they found only small stable atherosclerotic plaques. At 4 weeks, atherosclerotic plaques with thick fibrous caps and large areas of necrotic tissue containing cholesterol crystals were observed. On the other hand, some shoulder lesions showed susceptibility to plaque rupture, and were characterized by thinner cellular

caps. At 7 weeks after surgery, evidence of ruptured fibrous caps, intraplaque hemorrhage, and luminal thrombi were found. These lesions further progressed to 11 weeks after surgery. In our study, we also found intraplaque hemorrhage in some control apoeKO mice at 5 weeks after TS surgery. Compared with the above previous study, the progression of atherosclerosis in our study appeared to be mild, which may be because we used a slightly modified version of the protocol used in the original study. Specifically, we applied TS to the carotid artery of Atg7cKO:apoeKO mice and control apoeKO mice at the age of 10 weeks, rather than 12 weeks. Furthermore, we replaced the Western diet containing 1.25% cholesterol to a normal chow diet. However, we finally confirmed more advanced atherosclerotic le- sions characterized by luminal thrombus and an almost fully occluded carotid artery not only in Atg7cKO:apoeKO mice but also in control apoeKO mice at 10 weeks after surgery (data not shown). Thus, our slightly modified protocol might also be helpful to investigate the mechanism of plaque instability, similarly to the original protocol.

Previously, we demonstrated that autophagy deficiency in SMCs promotes the progression of atherosclerosis [18]. In that study, we demonstrated that SMC death by autophagy deficiency was associated with plaque growth. In this present study, we further confirmed the role of autophagy in SMCs in more advanced atherosclerotic stages, including those showing intraplaque hemorrhage and luminal thrombosis. The mechanisms by which autophagy deficiency in SMCs enhances plaque instability remain unclear at present. However, we found that the apoptosis of SMCs caused by autophagy deficiency in SMCs enhanced the progression of atherosclerosis, including some characteristics of plaque instability, such as an enlarged necrotic core and a thinned fibrous cap (Fig. 2C). In

addition, previous studies demonstrated that apoptosis in SMCs induced characteristics of plaque instability in atheromas [22,23]. Thus, death of SMCs by autophagy deficiency appears to be involved in enhanced plaque instability.

In the present study, intraplaque hemorrhage, which is regarded as a characteristic of plaque instability and rupture [15], was more frequently observed in Atg7cKO:apoeKO mice than control apoeKO mice. There are at least 2 possible causes of intraplaque hemorrhage. One possible cause is rupture of intra- plaque neovascularization, and the other is plaque rupture leading to erythrocyte invasion from the lumen into the plaque. Within plaque lesions, the accumulation of erythrocytes derived from intraplaque hemorrhage causes increased local cholesterol levels. These higher cholesterol levels contribute to enlargement of the necrotic core and triggers monocyte/macrophage infiltration into atherosclerotic lesions, leading to plaque rupture [14,24]. Indeed, Atg7cKO:apoeKO mice had larger necrotic core lesions than control apoeKO mice. These plaque lesions with larger necrotic cores contained factors derived from macro- phages as well as oxidated lipids, resulting in thrombus-prone plaques.

Given that plaque rupture and subsequent thrombus are direct triggers of acute coronary syndrome and cardiovascular death, luminal thrombus and atherothromobosis are regarded as prominent characteristics found in Atg7cKO:apoeKO mice [25]. In a mouse model of plaque destabilization, blood-plaque in- teractions through plaque disruption and a concomitant increase in thrombogenicity were shown to be triggering factors of thrombus [26]. Taken together, we found that intraplaque hem- orrhage, luminal thrombus, and

atherothrombosis are the typical features of plaque instability and rupture in Atg7cKO:apoeKO mice. These data suggest the clinical importance of SMC autophagy.

In conclusion, we demonstrated that autophagy deficiency in SMCs enhances plaque instability and plaque rupture. Based on the possible implication of SMC autophagy, the induction of autophagy may help to prevent the progression of atherosclerosis in patients.

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Disclosure of potential conflict of interest

None

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References

[1] D.J. Klionsky, Autophagy: from phenomenology to molecular understanding in less than a decade, Nat. Rev. Mol. Cell Biol. 8 (2007) 931e937.

[2] M. Komatsu, S. Waguri, T. Chiba, et al., Loss of autophagy in the central ner- vous system causes neurodegeneration in mice, Nature 441 (2006) 880e884.

[3] L. Lin, E.H. Baehrecke, Autophagy, cell death, and cancer, Mol Cell Oncol 2 (2015) e985913.

[4] C. Ebato, T. Uchida, M. Arakawa, et al., Autophagy is important in islet ho-meostasis and compensatory increase of beta cell mass in response to high-fat diet, Cell Metabol. 8 (2008) 325e332.

[5] J. Matsuda, T. Namba, Y. Takabatake, et al., Antioxidant role of autophagy in maintaining the integrity of glomerular capillaries, Autophagy 14 (2018) 53e65.

[6] X. Liao, J.C. Sluimer, Y. Wang, et al., Macrophage autophagy plays a protective role in advanced atherosclerosis, Cell Metabol. 15 (2012) 545e553.

[7] M.O. Grootaert, P.A. da Costa Martins, N. Bitsch, I. Pintelon, et al., Defective autophagy in vascular smooth muscle cells accelerates senescence and pro- motes neointima formation and atherogenesis, Autophagy 11 (2015) 2014e2032.

[8] R. Ross, Atherosclerosis–an inflammatory disease, N. Engl. J. Med. 340 (1999) 115e126.

[9] A.V. Finn, M. Nakano, J. Narula, et al., Concept of vulnerable/unstable plaque, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 1282e1292.

[10] Y. Nakashima, A.S. Plump, E.W. Raines, et al., ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree, Arte- rioscler. Thromb. 14 (1994) 133e140.

[11] Y.C. Chen, A.V. Bui, J. Diesch, et al., A novel mouse model of atherosclerotic plaque instability for drug testing and mechanistic/therapeutic discoveries using gene and microRNA expression profiling, Circ. Res. 113 (2013) 252e265.

[12] M.E. Rosenfeld, M.M. Averill, B.J. Bennett, et al., Progression and disruption of advanced atherosclerotic plaques in murine models, Curr. Drug Targets 9 (2008) 210e216.

[13] C. Cheng, D. Tempel, R. van Haperen, et al., Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress, Circulation 113 (2006) 2744e2753.

[14] J.H. von der Thusen, B.J. van Vlijmen, R.C. Hoeben, et al., Induction of atherosclerotic plaque rupture in apolipoprotein E-/- mice after adenovirus- mediated transfer of p53, Circulation 105 (2002) 2064e2070.

[15] T. Sasaki, M. Kuzuya, K. Nakamura, et al., A simple method of plaque rupture induction in apolipoprotein E-deficient mice, Arterioscler. Thromb. Vasc. Biol. 26 (2006) 1304e1309.

[16] M.R. Bennett, S. Sinha, G.K. Owens, Vascular smooth muscle cells in athero- sclerosis, Circ. Res. 118 (2016) 692e702.

[17] W. Martinet, G.R. De Meyer, Autophagy in atherosclerosis: a cell survival and death phenomenon with therapeutic potential, Circ. Res. 104 (2009) 304e317.

[18] Y. Osonoi, T. Mita, K. Azuma, et al., Defective autophagy in vascular smooth muscle cells enhances cell death and atherosclerosis, Autophagy (2018) 1991e2006.

[19] M. Komatsu, S. Waguri, T. Ueno, et al., Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice, J. Cell Biol. 169 (2005) 425e434.

[20] V. Subramanian, J. Golledge, T. Ijaz, , et al.A. Daugherty, Pioglitazone-induced reductions in atherosclerosis occur via smooth muscle cell-specific interaction with PPAR{gamma}, Circ. Res. 107 (2010) 953e958.

[21] D.J. Klionsky, K. Abdelmohsen, A. Abe, et al., Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition), Autophagy 12 (2016) 1e222.

[22] M.C. Clarke, N. Figg, J.J. Maguire, et al., Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis, Nat. Med. 12 (2006) 1075e1080.

[23] M.C. Clarke, T.D. Littlewood, N. Figg, J.J. Maguire, et al., Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration, Circ. Res. 102 (2008) 1529e1538.

[24] J.B. Michel, R. Virmani, E. Arbustini, et al., Intraplaque haemorrhages as the trigger of plaque vulnerability, Eur. Heart J. 32 (2011), 1977-1985, 1985a, 1985b, 1985c.

[25] R. Virmani, F.D. Kolodgie, A.P. Burke, et al., Lessons from sudden coronary death: a comprehensive morphological classification scheme for atheroscle- rotic lesions, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1262e1275.

[26] X. Liu, M. Ni, L. Ma, et al., Targeting blood thrombogenicity precipitates atherothrombotic events in a mouse model of plaque destabilization, Sci. Rep. 5 (2015) 10225.

Figure legends

Figure 1. Macroscopic changes of common carotid artery after tandem stenosis surgery.

Representative common carotid artery from a control apoeKO mouse (A) and an Atg7cKO:apoeKO mouse (B) harvested at 5 weeks after tandem stenosis surgery are shown. Scale bars: 5 mm.

Figure 2. Autophagy deficiency in SMCs enhances progression of atherosclerosis.

(A, B) Representative histological sections of a sham-operated right common carotid artery of a control apoeKO mouse and Atg7cKO:apoeKO mouse stained with a SQSTM1 antibody and hematoxylin. (C) Representative histological section of the right common carotid artery of a Atg7cKO:apoeKO mouse stained with hematoxylin and eosin. Atheroma found in Atg7cKO:apoeKO mice showed features of the various stages of atherosclerotic lesions. The upper boxed area shows migration of SMCs into the subendothelial space in an atheroma. The lower boxed area indicates the accumulation of macrophage-derived foam cells. (D) Representative histological section of the right common carotid artery of a control apoeKO mouse stained with hematoxylin and eosin. Small atheromas were found in apoeKO mice. The boxed area indicates a fatty streak. (E, F) Representative histological sections of a TS- operated right common carotid artery from a control apoeKO mouse and an Atg7cKO:apoeKO mouse stained with a SQSTM1 antibody and hematoxylin. (G) Percentage luminal stenosis of control apoeKO mice (n=14) and Atg7cKO:apoeKO mice

(n=14). Data are shown as the mean ± SEM of 14 samples. *P < 0.05 vs Control. Scale bars: 200 mm.

Figure 3. Autophagy deficiency in SMCs enhances plaque instability.

(A, B) Representative histological sections of an operated common carotid artery from a control apoeKO mouse (A) and an Atg7cKO:apoeKO mouse (B) stained with Berlin blue. Scale bars: 200 μm. Arrows indicate Berlin blue staining-positive areas. (C) The number of Berlin-blue positive areas were analyzed. Data are the mean ± SEM of 14 apoeKO mice and 14 Atg7cKO:apoeKO mice. *P<0.05 vs Control. (D) Berlin blue staining-positive areas were found in foam cells. Arrows indicate Berlin blue staining- positive areas.

Figure 4. Thrombosis observed in *Atg7cKO:apoeKO* mice.

Sections of the operated common carotid artery from Atg7cKO:apoeKO mice at 5 weeks after tandem stenosis surgery. (A) Representative histological section of the operated common carotid artery stained with hematoxylin and eosin. The luminal thrombosis was contained new capillary vessels, indicated by arrows. (B) Representative histo- logical section of an operated common carotid artery stained with ACTA2/a-smooth muscle actin. The luminal thrombosis contained myofibroblasts, indicated by the arrows. (C) Representative histological section of an operated common carotid artery stained with azan dye. The luminal thrombosis contained collagen. (D) Representative histological section of an operated common carotid artery with atherothrombosis stained with hematoxylin and eosin. The upper boxed area indicates foam cells. The lower boxed area indicates an

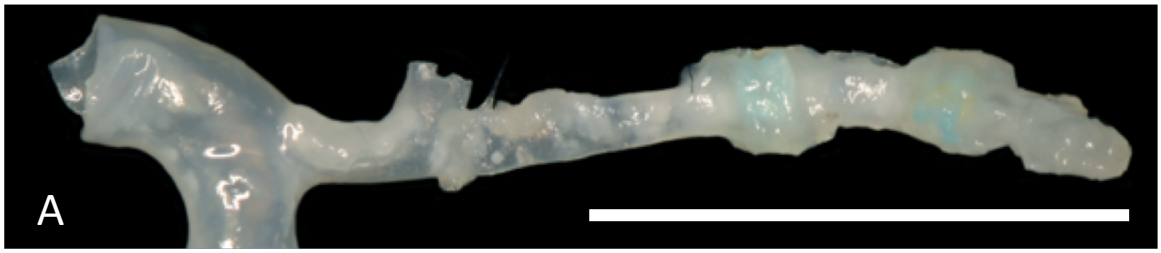
atheroma. The arrows indicate neutrophil cells and the arrowheads indicate neovessels. Scale bar: 200 μm.

Supplementary Table 1. Anthropometric data in control *apoeKO* mice and *Atg7cKO:apoeKO* mice at 5 weeks after tandem stenosis surgery.

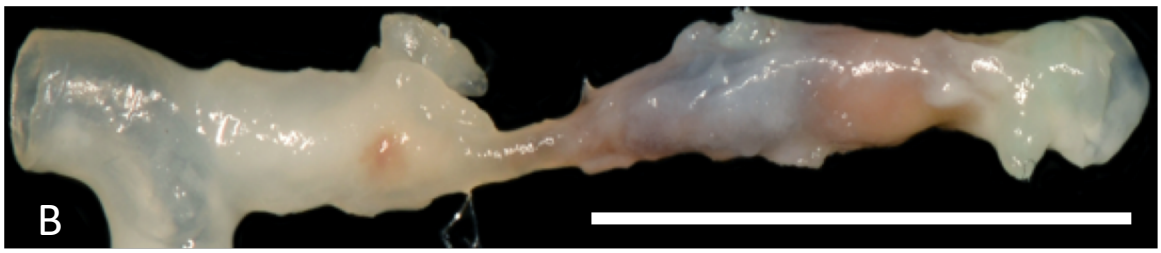
	Control apoeKO mice	*Atg7cKO:apoeKO* mice	P value
Body Weight (g)	29.7 ± 0.54 (n=14)	29.82± 0.88 (n=15)	0.90
Total-cholesterol (mg/dl)	1161.5 ± 80.1 (n=14)	1132.4 ± 105.8 (n=14)	0.83
Chylomicron (mg/dl)	186.4 ± 12.3 (n=14)	164.3± 9.5 (n=14)	0.17
Very Low Density Lipoprotein cholesterol (mg/dL)	786.8 ± 52.9 (n=14)	705.0 ± 76.3 (n=14)	0.39
Low Density Lipoprotein cholesterol (mg/dL)	215.1 ± 16.1 (n=14)	214.5 ± 26.1 (n=14)	0.98
High Density Lipoprotein cholesterol (mg/dL)	18.6 ± 0.6 (n=14)	18.9 ± 1.3 (n=14)	0.83

Each blood sample was obtained in the fasting state. Data are represented as mean ± SEM.

Control *apoeKO*



Atg7cKO:apoeKO



Sham

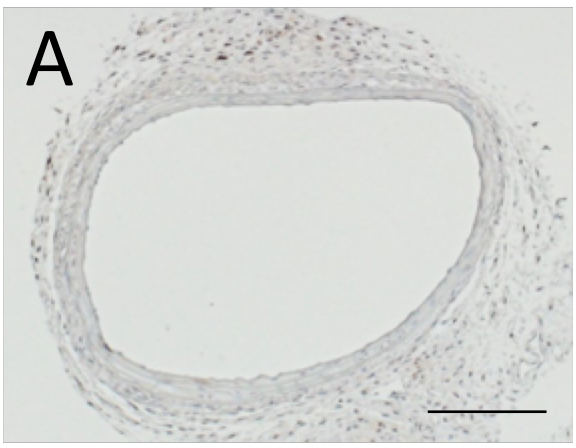
TS operation

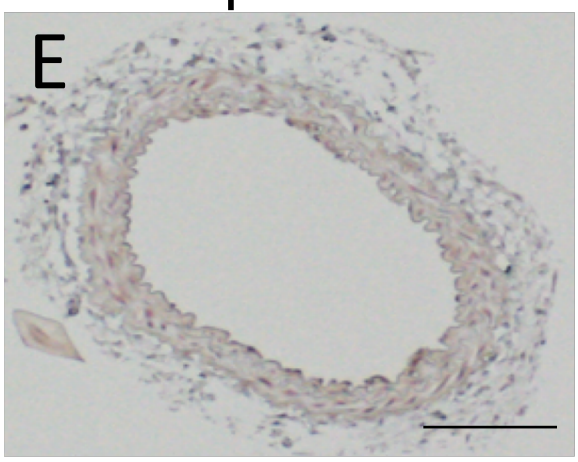
Control apoeKO

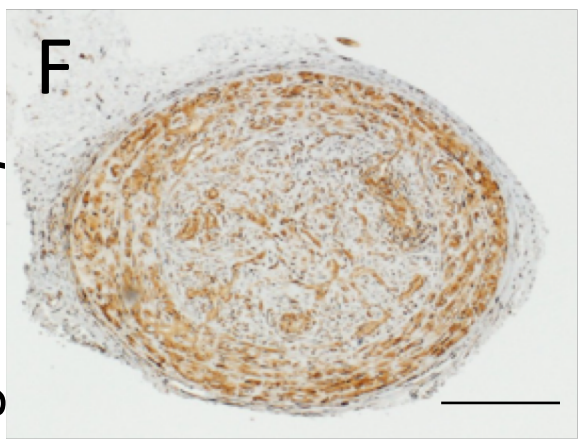
Atg7cKO:apoeKO

Control apoeKO

Atg7cKO:apoeKO

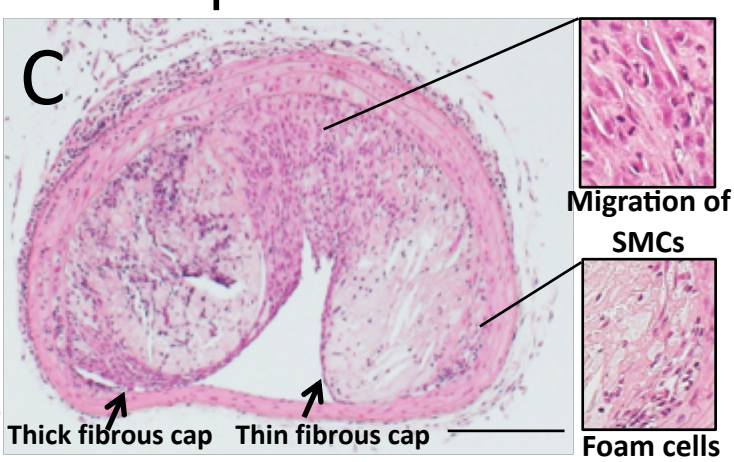




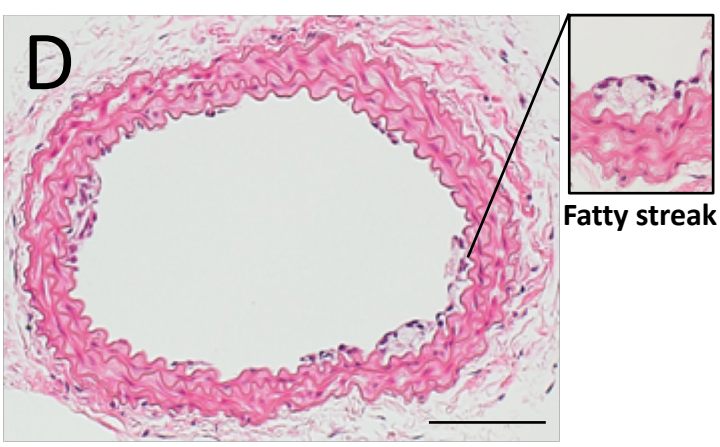


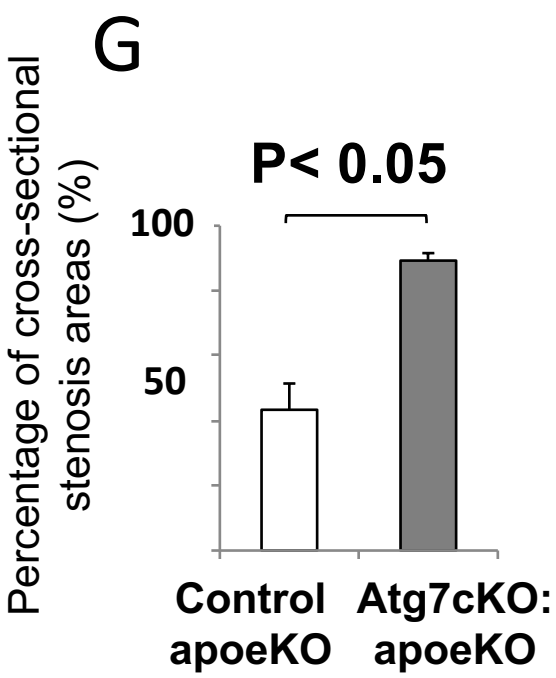
TS operation

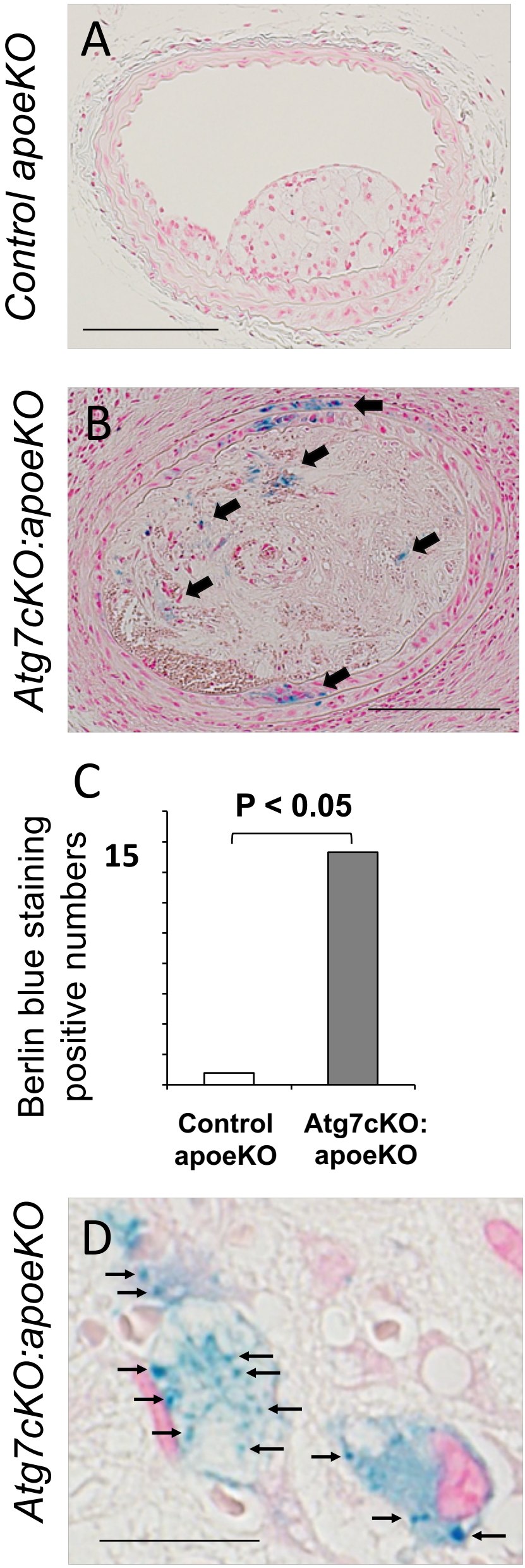
Atg7cKO:apoeKO

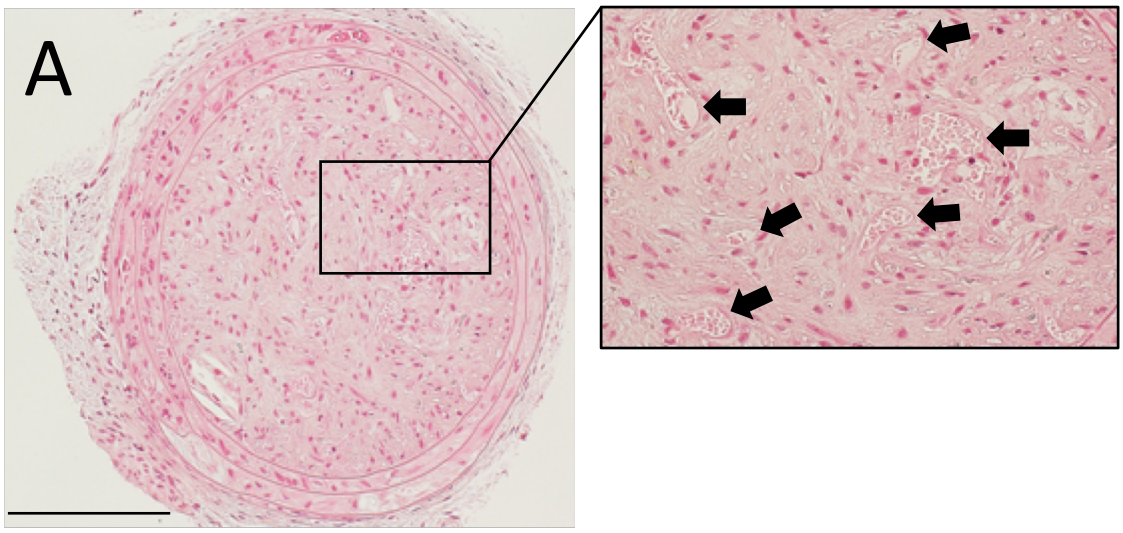


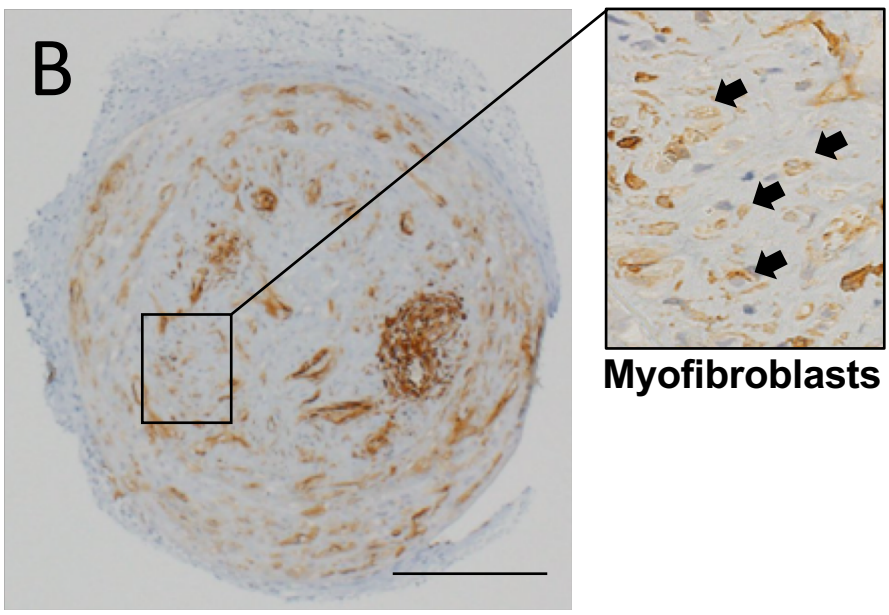
Control apoeKO



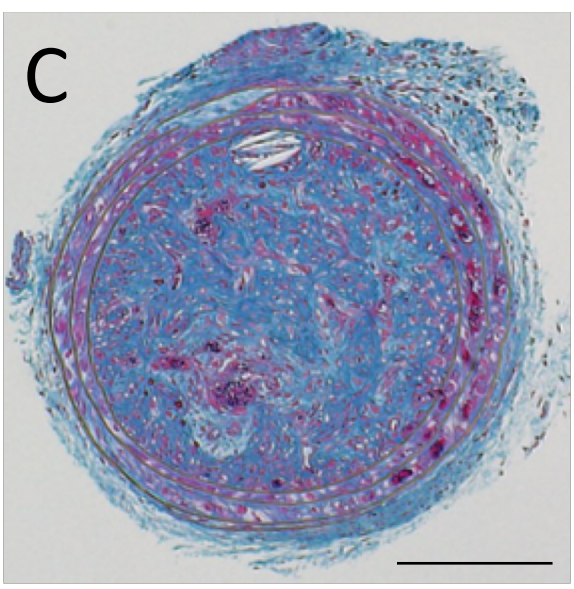


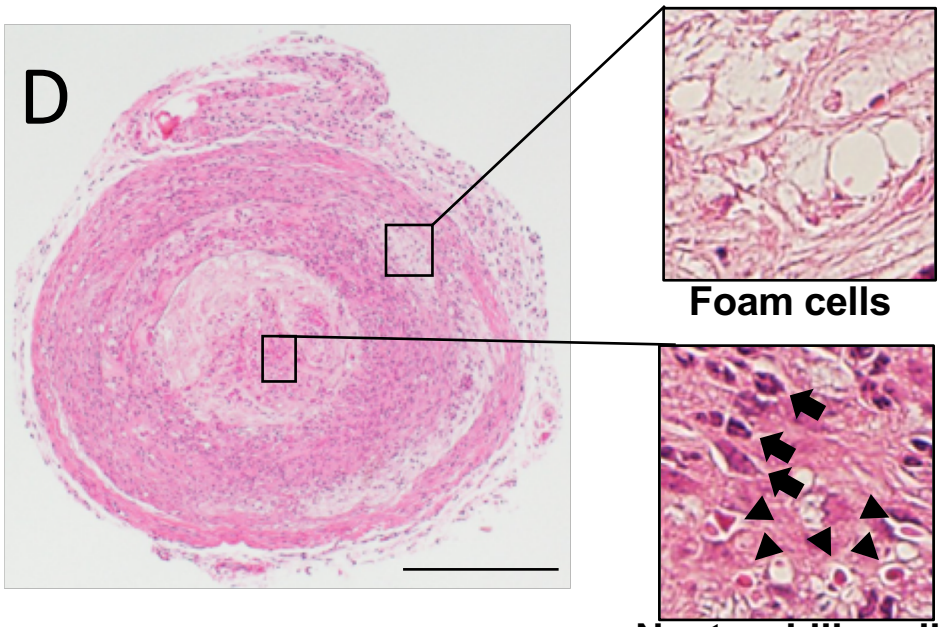

A
Control apoeKO
B
Atg7cKO:apoeKO
C
P < 0.05
15
Berlin blue staining positive numbers
Control apoeKO
Atg7cKO: apoeKO
D
Atg7cKO:apoeKO





Myofibroblasts





Foam cells

Neutrophilic cells and neovessels