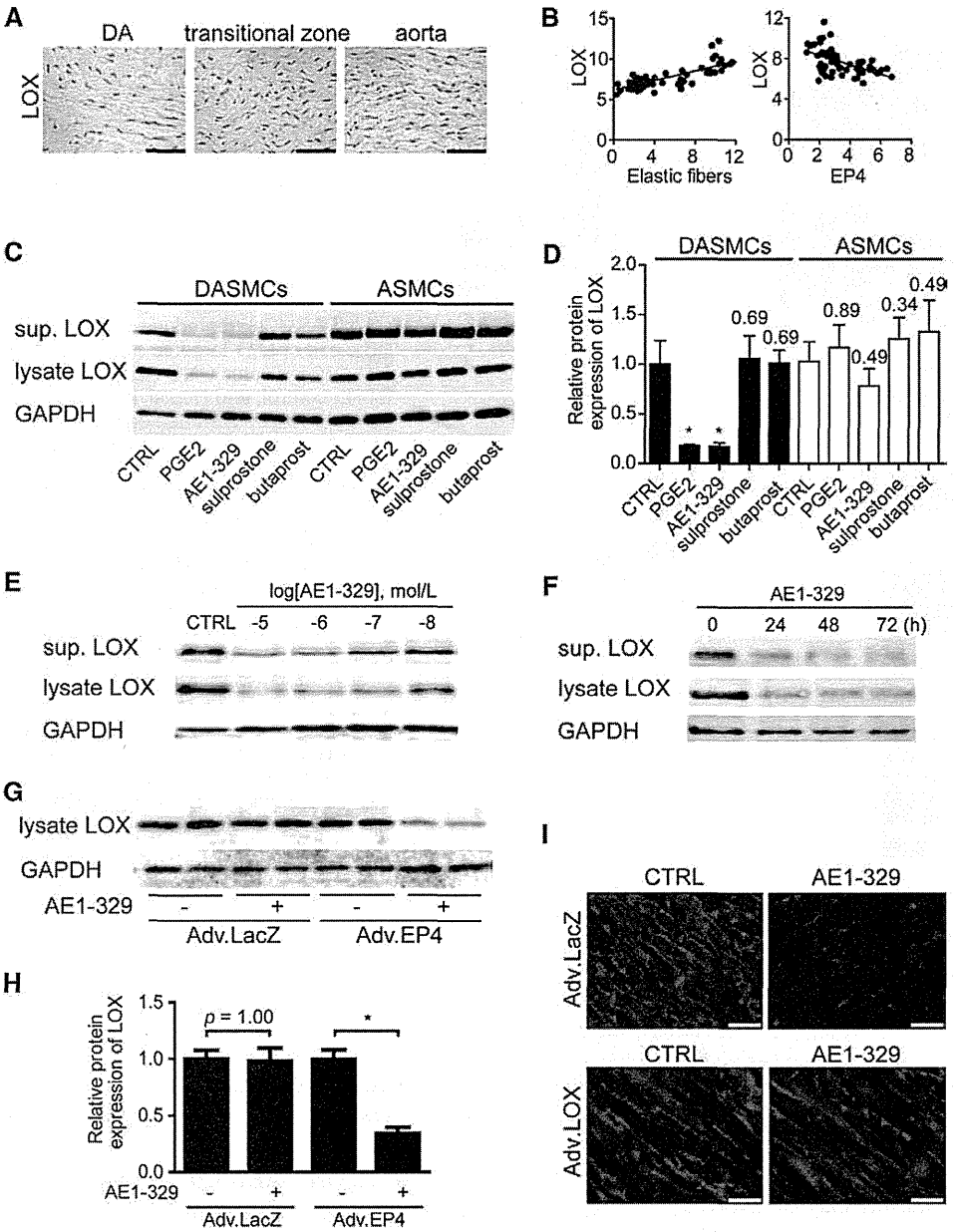


Figure 4. EP4 signaling attenuated elastic fiber formation via decreased lysyl oxidase (LOX) expression. **A**, Representative images of immunohistochemistry for LOX in human neonatal vessels. **B**, Representative results of quantification of elastic fiber formation, LOX, and EP4 protein expression using a color extraction method. Values are shown in arbitrary units. **C**, Western blotting for LOX in surpernatant (sup.) and lysate of smooth muscle cells (SMCs) treated for 72 h. **D**, Quantification of **C**. n=4, **P*<0.05 vs CTRL. Numbers on the bars indicate *P* values. Administration of prostaglandin E_2 (PGE_2) or the EP4 agonist AE1-329 decreased the mature LOX form in both supernatant and lysate of ductus arteriosus SMCs (DASMCs) but not in aortic SMCs (ASMCs). **E**, Dose-dependent effects of AE1-329 (24 h incubation) on LOX protein in DASMCs. **F**, Time-dependent reduction in LOX protein in DASMCs. **G**, Protein expression of LOX was decreased in EP4-overexpressing ASMCs (Adv. EP4) treated with AE-329 for 24 h. **H**, Quantification of **G**. n=4, **P*<0.05 vs CTRL. **I**, Immunostaining for elastin (red) and DNA (blue) of the LOX- or LacZ-overexpressing DASMCs treated with or without AE1-329. Each drug was used at 1 μmol/L. Scale bars, 50 μm (**A**); 20 μm (**I**).

Discussion

Although it is widely recognized in multiple species that the DA exhibits sparse elastic fibers in the middle layer and disassembly and fragmentation of the internal elastic lamina, the molecular mechanism for these has not yet been identified. The current study demonstrated a novel role of PGE_2 in spatially regulating elastogenesis by LOX protein degradation via the EP4-c-Src-PLCγ signal pathway in the DA, which contributes to the transition from fetal to neonatal circulation. Previous studies have demonstrated that abnormalities of elastic fibers are primarily responsible for PDA in some human cases.[10,11] According to the Gittenberger-de Groot group's[10] observation, there are several types of abnormal elastogenesis that can cause PDA. The following 2 types are of particular importance: (1) thickened subendothelial elastic lamina with sparse or slightly increased elastic fibers in the media, and (2) aortification of the ductal wall. PDA with aortification of the ductal wall in EP4-knockout mice resembles the latter phenotype. These abnormalities of elastic fibers are likely to prohibit intimal cushion formation and make it difficult to collapse the arterial wall. This suggests that the control of elastogenesis is clinically important. Pharmacological treatment for PDA, such as indomethacin after birth, may have an adverse effect on the inhibition of elastic fiber formation in the DA, especially in premature infants. This should be further investigated in a future study.

The EP4 receptor is highly expressed in the DA compared to the adjacent arteries[16]; it is coupled to Gαs and increases intracellular cAMP formation. The roles of EP4-cAMP signaling have been well studied in the DA. We and others have demonstrated that EP4 signaling induces vasodilation and hyaluronan-mediated vascular remodeling of the DA through cAMP-dependent protein kinase A[16,21,23] and that it promotes the migration of DASMCs and subsequent intimal thickening

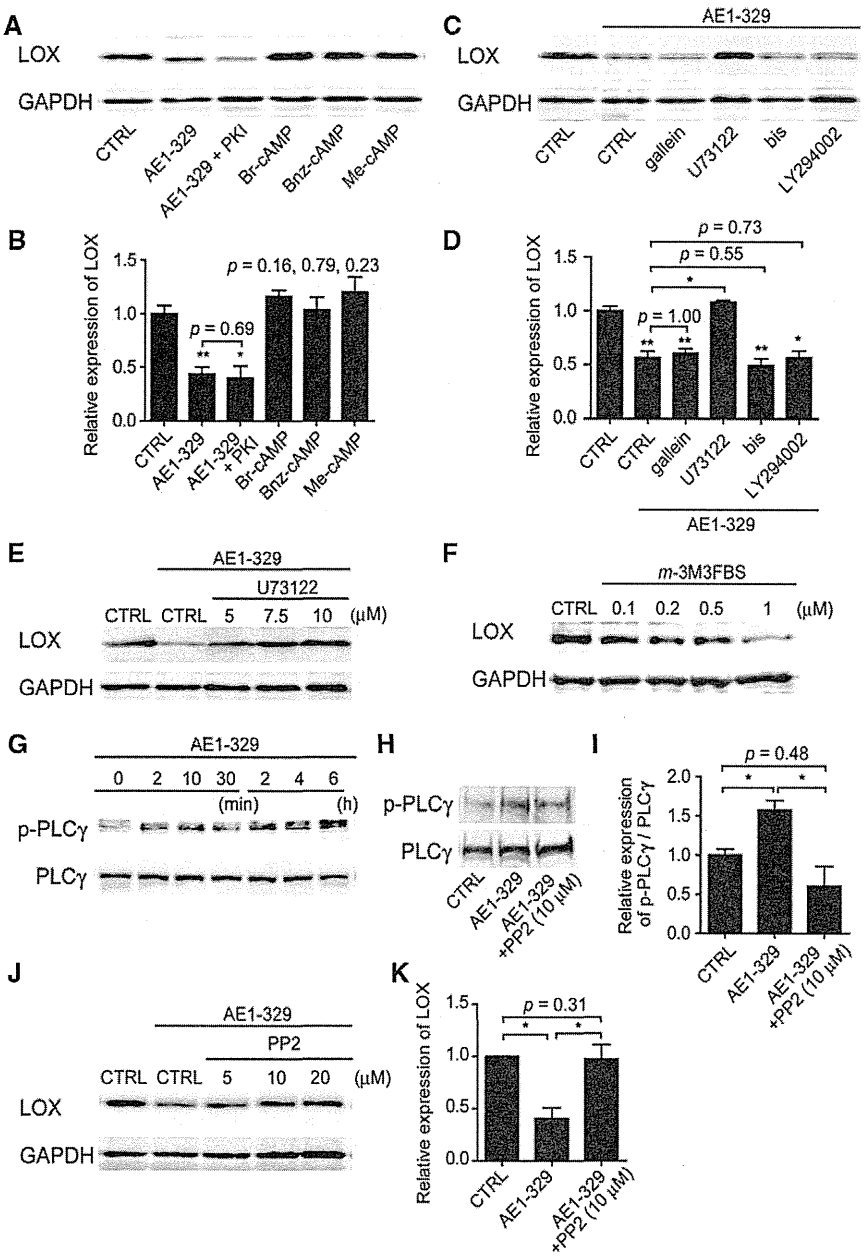


Figure 5. EP4 signaling decreased lysyl oxidase (LOX) protein via the c-Src-PLCγ pathway in ductus arteriosus smooth muscle cells (DASMCs). **A**, LOX protein expression in DASMCs treated with nonselective (Br-cAMP, 50 μmol/L), protein kinase A (PKA)-selective (Bnz-cAMP, 50 μmol/L) or Epac-selective (Me-cAMP, 50 μmol/L) cAMP analogs for 24 h. The AE1-329 was administered in the presence or absence of the PKA inhibitor PKI (10 μmol/L). **B**, Quantification of **A**. n=4–6, *P<0.05, **P<0.01 vs CTRL. cAMP signaling did not affect the EP4-mediated reduction of LOX protein. **C**, LOX protein expression in DASMCs treated with gallein (Gβγ inhibitor, 10 μmol/L), U73122 (PLC inhibitor, 10 μmol/L), bis (bisindolylmaleimide: PKC inhibitor, 10 μmol/L), or LY294002 (PI3K inhibitor, 1 μmol/L) in the presence of AE1-329. **D**, Quantification of **C**. n=4–6, *P<0.05, **P<0.01 vs CTRL. Inhibition of PLC eliminated the EP4-mediated reduction of LOX protein. **E**, Dose-dependent effect of U73122 in DASMCs treated with AE1-329. **F**, Dose-dependent inhibitory effect of *m*-3M3FBS on LOX protein expression. **G** and **H**, Phosphorylation of PLCγ in DASMCs treated with PP2 (Src inhibitor) or AE1-329. **I**, Quantification of **H**. n=4–6, *P<0.05. Inhibition of Src attenuated the EP4-mediated phosphorylation of PLCγ. **J**, PP2 attenuated the EP4-mediated reduction of LOX protein. **K**, Quantification of **J**. n=4–6. *P<0.05. Whole cell lysate was used for LOX detection. AE1-329 was used at 1 μmol/L.

through exchange protein activated by the cAMP.[22] Additional downstream signaling pathways of EP4 have been demonstrated in other cell types. EP4 uses Gαi and phosphoinositide 3-kinase, which are generally activated by Gβγ.[36] In addition to these well-known signaling pathways of EP4, our findings revealed c-Src-PLCγ signaling as a novel EP4 downstream pathway, as well as the inhibitory role played by EP4 signaling in elastogenesis. This EP4-signaling pathway was found to be independent of cAMP signaling, including protein kinase A and exchange protein activated by the cAMP, and Gβγ and phosphoinositide 3-kinase. Studies using colorectal and lung cancer cells have suggested that β-arrestin1 bound to EP4 activates c-Src.[31,32] In the DA, however, we did not identify an association between EP4–c-Src signaling and β-arrestin1 using β-arrestin1–targeted siRNA (data not shown). Ma et al[37] clearly demonstrated that Gαs and Gαi proteins directly stimulate the kinase activity of c-Src. Because the EP4 receptor is coupled to Gαs and Gαi, direct association between these G proteins and c-Src may activate its downstream signaling in EP4-mediated degradation of LOX protein. This possibility should be validated in a future study.

LOX is a copper-dependent amine oxidase that catalyzes the cross-linking of elastin and collagen and ensures the stability of the extracellular matrix.[38] Because LOX is the isoform responsible for 80% of the LOX activity in aortic SMCs,[39] it is essential to the maintenance of the tensile and elastic features of the vascular system.[38] LOX is synthesized as a pre-protein. After signal peptide hydrolysis, enzyme glycosylation, cooper incorporation, and lysine tyrosylquinone generation, the enzyme is released into the extracellular space. Then, BMP-1 processes LOX, yielding the mature LOX form and its propeptide.[27] The mechanisms of the transcriptional regulation of

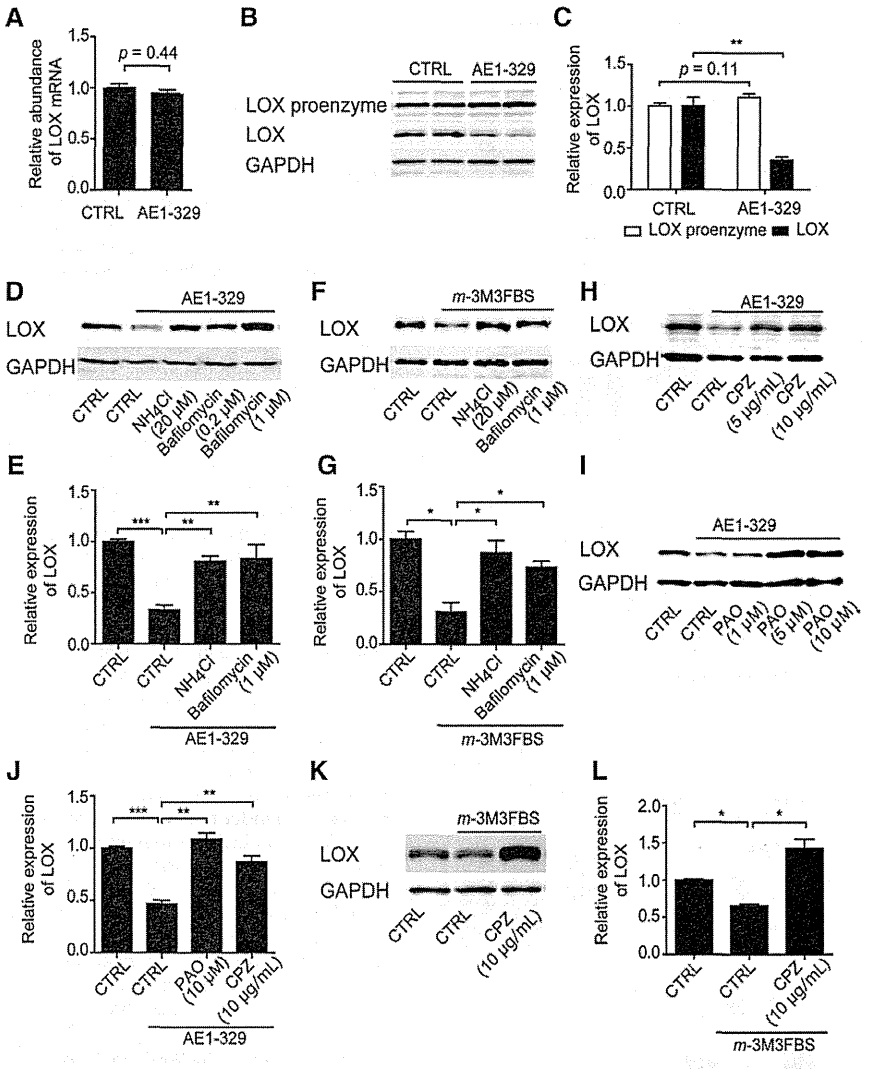


Figure 6. EP4 signaling decreased lysyl oxidase (LOX) protein through lysosomal degradation. **A** and **B**, Expression of LOX mRNA, pro-LOX, and LOX protein in ductus arteriosus smooth muscle cells (DASMCs) treated with AE1-329. mRNA of LOX and pro-LOX protein were not decreased by EP4 agonist in DASMCs. **C**, Quantification of **B**. n=4. **D** and **F**, Administration of lysosomal inhibitors (NH_4Cl or bafilomycin) for 24 h restored the AE1-329- or *m*-3M3FBS (0.5 μmol/L)-induced reduction of LOX protein. **E** and **G**, Quantification of **D** and **F**, respectively. n=6. **H** and **I**, Administration of clathrin-mediated endocytosis inhibitors (chlorpromazine or phenylarsine oxide) for 24 h restored the AE1-329-induced reduction of LOX protein. **J**, Quantification of **H** and **I**. n=4–6. **K**, Administration of chlorpromazine for 24 h restored the *m*-3M3FBS (0.5 μmol/L) -induced reduction of LOX protein. **L**, Quantification of **K**. **P*<0.05, ***P*<0.01. ****P*<0.001. Whole cell lysate was used for LOX detection. AE1-329 was used at 1 μmol/L.

LOX have been extensively studied. Interferon-γ, transforming growth factor-β, platelet-derived growth factor, connective tissue growth factor, and angiotensin II induce LOX gene expression via the interferon regulatory factor 1 transcriptional factor in multiple tissues, including blood vessels.[40,41] On the other hand, atherogenic concentrations of low-density lipoprotein and tumor necrosis factor α reduce LOX mRNA.[42,43] Song et al[44] have also shown that interferon-γ inhibits LOX gene expression through binding to the antagonistic transcriptional factor, interferon regulatory factor 2, in vascular SMCs.

In contrast to our understanding of these transcriptional regulations of LOX, little is known regarding LOX protein metabolism. In the present study, we demonstrated for the first time that the PGE_2-EP4 signal promoted lysosomal degradation of LOX protein. Recently, 1 study that used lysosomal inhibitors and Vps18-deficient mice demonstrated that LOX protein was degraded through lysosomes in Purkinje cells.[45] However, the detailed molecular mechanisms triggering the degradation of LOX protein have not been reported and should be examined in future studies. Once LOX is cleaved from the proenzyme, it acts as a highly reactive enzyme. The mature LOX form catalyzes an oxidative deamination of lysine and hydroxylysine residues to peptidyl α-aminoadipic-δ-semialdehydes. These highly reactive semialdehydes can spontaneously condense to form intra- and intermolecular covalent cross-linkages.[27] Elastic fiber formation must be highly regulated to ensure the integrity of vascular and other tissues. Therefore, in addition to transcriptional regulation, the existence of protein regulation of LOX that we demonstrated in this study is physiologically reasonable.

The Rabinovitch group has extensively studied the molecular mechanisms of the sparse elastic fiber formation in the medial layer of the DA. Their studies have demonstrated that LOX activity does not differ between the lamb DA, aorta, and pulmonary artery.[46] Our study demonstrated that LOX protein was dramatically decreased by EP4 signaling in rodents and humans, suggesting that LOX activity is decreased in these DAs. Currently, we do not have a clear explanation for the apparent inconsistency in terms of LOX expression and activity. Further research is required to determine the species difference in LOX protein metabolism and activity. The Rabinovitch group also demonstrated that there is decreased insolubilization of elastin in the DA that is associated with the truncated 52-kDa tropoelastin that lacks the C terminus,[12] which is unrelated to heightened elastolytic activity.[46] Similarly, our results showed that matrix metalloproteinase 2

activity does not differ between the DA and the aorta, suggesting that impaired elastogenesis rather than enhanced elastolytic activity provides a muscular arterial property to the DA.

The present study demonstrated that LOX expression is important during development. However, LOX expression is known to be markedly responsive to a variety of pathological states, including wound repair, aging, and tumorigenesis.[41] In particular, strong evidence exists regarding the involvement of a reduction in LOX activity in the pathogenesis of vascular diseases characterized by destructive remodeling of the arterial wall. Previous reports demonstrated that aortic aneurysm and coronary dissections were related to a disturbance in LOX expression in animal models and humans.[47,48] Therefore, the regulation of LOX expression is considered an attractive therapeutic target. In this study, it should be noted that there seems to be a threshold value for EP4 expression to induce a decrease in elastic fibers and LOX (Figures 2C and 4B). In our previous report, analyses of human aortic aneurysmal tissues demonstrated that EP4 expression is greater in aneurysmal legions than that in nondiseased areas.[49] Further studies are required to investigate whether EP4-mediated LOX regulation plays a role in pathological conditions.

Taken together, these findings suggest that PGE$_2$-EP4 signaling inhibits elastogenesis in the DA by degrading LOX protein. The PGE$_2$-EP4-mediated LOX protein regulation via a previously unrecognized signaling pathway may also provide the basis for therapeutic strategies that target vascular elastogeneis.

Acknowledgments

We thank Professor S. Narumiya (Department of Pharmacology, Kyoto University Faculty of Medicine, Kyoto, Japan) for kindly providing EP4$^{-/-}$ mice. We thank Professor S. Morita and Dr M. Taguri (Department of Biostatistics and Epidemiology, Yokohama City University, Yokohama, Japan) for assistance with statistical analysis. The adenoviruses of EP4 and LOX were kindly provided from Dr Y. Kobayashi (Matsumoto Dental University, Matsumoto, Japan) and Dr K. Yoshimura (Yamaguchi University, Yamaguchi, Japan), respectively. We also thank Yuka Sawada for histological analyses.

Sources of Funding

This work was supported by grants from the Ministry of Health, Labor, and Welfare of Japan (Y.I.), the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Y.I., U.Y., S.S., M.M., S.M.), a Grant-in-Aid for Scientific Research on Innovative Areas (23116514 and 25116719 to U.Y., 22136009 to Y.I.), the Kitsuen Kagaku Research Foundation (Y.I.), the Foundation for Growth Science (S.M.), the Yokohama Foundation for Advanced Medical Science (U.Y., S.M.), the "High-Tech Research Center" Project for Private Universities: MEXT (S.M.), MEXT-Supported Program for the Strategic Research Foundation at Private Universities (S.M.), the Vehicle Racing Commemorative Foundation (U.Y., S.M.), Miyata Cardiology Research Promotion Funds (U.Y., S.M.), the Takeda Science Foundation (Y.I., U.Y., S.M.), the Japan Heart Foundation Research Grant (U.Y.), the Kowa Life Science Foundation (U.Y.), the Sumitomo Foundation (U.Y), and the Shimabara Science Promotion Foundation (S.M.).

Disclosures

None.

References

1. Wagenseil JE, Mecham RP. Vascular extracellular matrix and arterial mechanics. *Physiol Rev*. 2009;89:957–989.
2. Wagenseil JE, Ciliberto CH, Knutsen RH, Levy MA, Kovacs A, Mecham RP. The importance of elastin to aortic development in mice. *Am J Physiol Heart Circ Physiol*. 2010;299:H257–H264.
3. Jager BV, Wollenman OJ. An Anatomical Study of the Closure of the Ductus Arteriosus. *Am J Pathol*. 1942;18:595–613.
4. de Reeder EG, van Munsteren CJ, Poelmann RE, Patterson DF, Gittenberger-de Groot AC. Changes in distribution of elastin and elastin receptor during intimal cushion formation in the ductus arteriosus. *Anat Embryol (Berl)*. 1990;182:473–480.
5. Ho SY, Anderson RH. Anatomical closure of the ductus arteriosus: a study in 35 specimens. *J Anat*. 1979;128(pt 4):829–836.
6. Toda T, Tsuda N, Takagi T, Nishimori I, Leszczynski D, Kummerow F. Ultrastructure of developing human ductus arteriosus. *J Anat*. 1980;131(pt 1):25–37.
7. Tada T, Kishimoto H. Ultrastructural and histological studies on closure of the mouse ductus arteriosus. *Acta Anat (Basel)*. 1990;139:326–334.
8. Schaeffer JP. The behavior of elastic tissue in the postfetal occlusion and obliteration of the ductus arteriosus (BOTALLI) in SUS SCROFA. *J Exp Med*. 1914;19:129–142.
9. Jaques A, Serafini-Fracassini A. Morphogenesis of the elastic fiber: an immunoelectronmicroscopy investigation. *J Ultrastruct Res*. 1985; 92:201–210.
10. Gittenberger-de Groot AC. Persistent ductus arteriosus: most probably a primary congenital malformation. *Br Heart J*. 1977;39:610–618.
11. Gittenberger-de Groot AC, Moulaert AJ, Hitchcock JF. Histology of the persistent ductus arteriosus in cases of congenital rubella. *Circulation*. 1980;62:183–186.
12. Hinek A, Rabinovitch M. The ductus arteriosus migratory smooth muscle cell phenotype processes tropoelastin to a 52-kDa product associated with impaired assembly of elastic laminae. *J Biol Chem*. 1993;268:1405–1413.
13. Hinek A, Mecham RP, Keeley F, Rabinovitch M. Impaired elastin fiber assembly related to reduced 67-kD elastin-binding protein in fetal lamb ductus arteriosus and in cultured aortic smooth muscle cells treated with chondroitin sulfate. *J Clin Invest*. 1991;88:2083–2094.
14. Mitchell MD, Lucas A, Etches PC, Brunt JD, Turnbull AC. Plasma prostaglandin levels during early neonatal life following term and pre-term delivery. *Prostaglandins*. 1978;16:319–326.
15. Woodward DF, Jones RL, Narumiya S. International Union of Basic and Clinical Pharmacology. LXXXIII: classification of prostanoid receptors, updating 15 years of progress. *Pharmacol Rev*. 2011;63:471–538.
16. Yokoyama U, Minamisawa S, Quan H, Ghatak S, Akaike T, Segi-Nishida E, Iwasaki S, Iwamoto M, Misra S, Tamura K, Hori H, Yokota S, Toole BP, Sugimoto Y, Ishikawa Y. Chronic activation of the prostaglandin receptor EP4 promotes hyaluronan-mediated neointimal formation in the ductus arteriosus. *J Clin Invest*. 2006;116:3026–3034.
17. Segi E, Sugimoto Y, Yamasaki A, Aze Y, Oida H, Nishimura T, Murata T, Matsuoka T, Ushikubi F, Hirose M, Tanaka T, Yoshida N, Narumiya S, Ichikawa A. Patent ductus arteriosus and neonatal death in prostaglandin receptor EP4-deficient mice. *Biochem Biophys Res Commun*. 1998;246:7–12.
18. Smith GC, Wu WX, Nijland MJ, Koenen SV, Nathanielsz PW. Effect of gestational age, corticosteroids, and birth on expression of prostanoid EP receptor genes in lamb and baboon ductus arteriosus. *J Cardiovasc Pharmacol*. 2001;37:697–704.
19. Leonhardt A, Glaser A, Wegmann M, Schranz D, Seyberth H, Nüsing R. Expression of prostanoid receptors in human ductus arteriosus. *Br J Pharmacol*. 2003;138:655–659.
20. Rabinovitch M. Cell-extracellular matrix interactions in the ductus arteriosus and perinatal pulmonary circulation. *Semin Perinatol*. 1996;20:531–541.
21. Yokoyama U, Minamisawa S, Ishikawa Y. Regulation of vascular tone and remodeling of the ductus arteriosus. *J Smooth Muscle Res*. 2010;46:77–87.
22. Yokoyama U, Minamisawa S, Quan H, Akaike T, Suzuki S, Jin M, Jiao Q, Watanabe M, Otsu K, Iwasaki S, Nishimaki S, Sato M, Ishikawa Y. Prostaglandin E2-activated Epac promotes neointimal formation of the rat ductus arteriosus by a process distinct from that of cAMP-dependent protein kinase A. *J Biol Chem*. 2008;283:28702–28709.
23. Yokoyama U, Minamisawa S, Katayama A, Tang T, Suzuki S, Iwatsubo K, Iwasaki S, Kurotani R, Okumura S, Sato M, Yokota S, Hammond HK, Ishikawa Y. Differential regulation of vascular tone and remodeling via stimulation of type 2 and type 6 adenylyl cyclases in the ductus arteriosus. *Circ Res*. 2010;106:1882–1892.
24. Hirai M, Ohbayashi T, Horiguchi M, Okawa K, Hagiwara A, Chien KR, Kita T, Nakamura T. Fibulin-5/DANCE has an elastogenic organizer activity that is abrogated by proteolytic cleavage in vivo. *J Cell Biol*. 2007;176:1061–1071.

25. Nguyen M, Camenisch T, Snouwaert JN, Hicks E, Coffman TM, Anderson PA, Malouf NN, Koller BH. The prostaglandin receptor EP4 triggers remodelling of the cardiovascular system at birth. *Nature*. 1997;390:78–81.
26. Nakamura T, Lozano PR, Ikeda Y, Iwanaga Y, Hinek A, Minamisawa S, Cheng CF, Kobuke K, Dalton N, Takada Y, Tashiro K, Ross J Jr, Honjo T, Chien KR. Fibulin-5/DANCE is essential for elastogenesis in vivo. *Nature*. 2002;415:171–175.
27. Rodríguez C, Martínez-González J, Raposo B, Alcudia JF, Guadall A, Badimon L. Regulation of lysyl oxidase in vascular cells: lysyl oxidase as a new player in cardiovascular diseases. *Cardiovasc Res*. 2008;79:7–13.
28. Mäki JM, Räsänen J, Tikkanen H, Sormunen R, Mäkikallio K, Kivirikko KI, Soininen R. Inactivation of the lysyl oxidase gene Lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice. *Circulation*. 2002;106:2503–2509.
29. Elzenga NJ, Gittenberger-de Groot AC. Localised coarctation of the aorta. An age dependent spectrum. *Br Heart J*. 1983;49:317–323.
30. Jimenez M, Daret D, Choussat A, Bonnet J. Immunohistological and ultrastructural analysis of the intimal thickening in coarctation of human aorta. *Cardiovasc Res*. 1999;41:737–745.
31. Kim JI, Lakshmikanthan V, Frilot N, Daaka Y. Prostaglandin E2 promotes lung cancer cell migration via EP4-betaArrestin1-c-Src signalsome. *Mol Cancer Res*. 2010;8:569–577.
32. Buchanan FG, Gorden DL, Matta P, Shi Q, Matrisian LM, DuBois RN. Role of beta-arrestin 1 in the metastatic progression of colorectal cancer. *Proc Natl Acad Sci USA*. 2006;103:1492–1497.
33. Wang J, Yin G, Menon P, Pang J, Smolock EM, Yan C, Berk BC. Phosphorylation of G protein-coupled receptor kinase 2-interacting protein 1 tyrosine 392 is required for phospholipase C-gamma activation and podosome formation in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2010;30:1976–1982.
34. Mazharian A, Thomas SG, Dhanjal TS, Buckley CD, Watson SP. Critical role of Src-Syk-PLC{gamma}2 signaling in megakaryocyte migration and thrombopoiesis. *Blood*. 2010;116:793–800.
35. Min C, Kirsch KH, Zhao Y, Jeay S, Palamakumbura AH, Trackman PC, Sonenshein GE. The tumor suppressor activity of the lysyl oxidase propeptide reverses the invasive phenotype of Her-2/neu-driven breast cancer. *Cancer Res*. 2007;67:1105–1112.
36. Regan JW. EP2 and EP4 prostanoid receptor signaling. *Life Sci*. 2003;74:143–153.
37. Ma YC, Huang J, Ali S, Lowry W, Huang XY. Src tyrosine kinase is a novel direct effector of G proteins. *Cell*. 2000;102:635–646.
38. Kagan HM, Li W. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. *J Cell Biochem*. 2003;88:660–672.
39. Mäki JM, Sormunen R, Lippo S, Kaarteenaho-Wiik R, Soininen R, Myllyharju J. Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. *Am J Pathol*. 2005;167:927–936.
40. Yoshimura K, Aoki H, Ikeda Y, Fujii K, Akiyama N, Furutani A, Hoshii Y, Tanaka N, Ricci R, Ishihara T, Esato K, Hamano K, Matsuzaki M. Regression of abdominal aortic aneurysm by inhibition of c-Jun N-terminal kinase. *Nat Med*. 2005;11:1330–1338.
41. Smith-Mungo LI, Kagan HM. Lysyl oxidase: properties, regulation and multiple functions in biology. *Matrix Biol*. 1998;16:387–398.
42. Rodríguez C, Alcudia JF, Martínez-González J, Raposo B, Navarro MA, Badimon L. Lysyl oxidase (LOX) down-regulation by TNFalpha: a new mechanism underlying TNFalpha-induced endothelial dysfunction. *Atherosclerosis*. 2008;196:558–564.
43. Rodríguez C, Raposo B, Martínez-González J, Casaní L, Badimon L. Low density lipoproteins downregulate lysyl oxidase in vascular endothelial cells and the arterial wall. *Arterioscler Thromb Vasc Biol*. 2002;22:1409–1414.
44. Song YL, Ford JW, Gordon D, Shanley CJ. Regulation of lysyl oxidase by interferon-gamma in rat aortic smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2000;20:982–988.
45. Peng C, Yan S, Ye J, Shen L, Xu T, Tao W. Vps18 deficiency inhibits dendritogenesis in Purkinje cells by blocking the lysosomal degradation of Lysyl Oxidase. *Biochem Biophys Res Commun*. 2012;423:715–720.
46. Zhu L, Dagher E, Johnson DJ, Bedell-Hogan D, Keeley FW, Kagan HM, Rabinovitch M. A developmentally regulated program restricting insolubilization of elastin and formation of laminae in the fetal lamb ductus arteriosus. *Lab Invest*. 1993;68:321–331.
47. Nakashima Y, Sueishi K. Alteration of elastic architecture in the lathyritic rat aorta implies the pathogenesis of aortic dissecting aneurysm. *Am J Pathol*. 1992;140:959–969.
48. Sibon I, Sommer P, Lamaziere JM, Bonnet J. Lysyl oxidase deficiency: a new cause of human arterial dissection. *Heart*. 2005;91:e33.
49. 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, Ishikawa Y. Inhibition of EP4 signaling attenuates aortic aneurysm formation. *PLoS ONE*. 2012;7:e36724.

CLINICAL PERSPECTIVE

The ductus arteriosus (DA) is a fetal bypass artery between the aorta and the pulmonary artery. Although the DA closes immediately after birth, it remains open in some infants, a condition known as patent DA. Patent DA remains a frequent problem among premature infants with significant morbidity and mortality. Both vascular contraction and remodeling (ie, intimal thickening) are required for complete anatomical closure of the DA. Decreased elastogengesis is known as a hallmark of DA remodeling and is thought to contribute to intimal thickening of the DA. However, the molecular mechanisms of decreased elastogenesis are not fully understood. Herein, we show that prostaglandin E_2 (PGE_2) receptor EP4 signaling promotes degradation of the mature lysyl oxidase protein, a cross-linking enzyme for elastic fibers, only in the DA, leading to decreased elastogenesis. The newly recognized PGE-EP4-c-Src-PLCγ-signaling pathway most likely contributes to the lysosomal degradation of lysyl oxidase. Based on these data, it appears that PGE-EP4 signaling is required for DA remodeling and that inhibition of this signaling by cyclooxygenase inhibitors may attenuate DA remodeling after birth, especially in premature infants in which the DA is not fully remodeled. Activation of the c-Src-PLCγ signaling pathway may be an additional strategy to promote anatomical closure of the immature DA.

SUPPLEMENTAL MATERIAL

Supplemental Methods

Reagents

8-p-Methoxyphenylthon-2-Omethyl-cAMP (pMe-cAMP) and N6-benzoyladenosine-cAMP (Bnz-cAMP) were purchased from BioLog Life Science Institute (Bremen, Germany) and Sigma (St. Louis, MO), respectively. PGE_2, sulprostone, butaplost, gallein, BAPN, bisindolylmaleimide (bis), U73122, U0126, LY294002, PAO, EIPA, and 8-Bromo-cAMP (Br-cAMP) were purchased from Sigma-Aldrich (St. Louis, MO). CPZ, MβCD, MG132, and NH_4Cl were obtained from Wako (Osaka, Japan). The PKA inhibitor (14–22), bafilomycin A1, PP2, and m-3M3FBS were obtained from Calbiochem (Darmstadt, Germany). ONO-AE1-329 was kindly provided by ONO Pharmaceutical Company (Osaka, Japan). Antibodies for LOX and pro-LOX for immunoblotting were obtained from Abcam (Cambridge, UK) and Novus Biological (Littleton, CO), respectively. Anti-LOX antibody for immunohistochemistry and anti-BMP-1 were obtained from US Biological (Swampscott, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-elastin and anti-EP4 antibodies were obtained from Elastin Products Company (Owensville, MO) and Caymanchemical (Ann Arbor, MI), respectively. Anti-PLCγ and anti-phosphorylated PLCγ antibodies were obtained from Cell Signaling (Beverly, MA). Anti-MMP-2 and anti-MMP-9 antibodies were from R&D Systems (Minneapolis, MN). Anti-fibrillin-1 antibody was kindly

provided from Dr. Nakamura (Kansai University, Japan).

Isolation and culture of rat smooth muscle cells (SMCs)

Vascular SMCs were obtained from the DA and aorta of Wistar rat fetuses on the 21st day of gestation (SLC Inc.) as previously described[1]. Using the same protocol, pulmonary SMCs were isolated from the branch extralobular pulmonary arteries from Wistar rats on the 21st day of gestation. SMCs were used at passages 4 to 6.

Immunoblot analysis

Proteins from whole cells were analyzed by immunoblotting as previously described [1].

Adenovirus construction

Adenovirus of EP4 was kindly provided from Dr. Y. Kobayashi (Matsumoto Dental University, Japan)[2]. A control adenovirus vector with LacZ was used at the same multiplicity of infection.

RNA interference (siRNA)

Double-stranded siRNAs to the selected regions of EP4 (stealth RNAi RSS331316) and the negative siRNA purchased from Invitrogen (San Diego, CA). According to the manufacturer's instructions, cells were transfected with siRNA (300 pmol), using Lipofectamin RNAiMAX (Invitrogen).

Quantitative and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Isolation of total RNA and generation of cDNA were performed and RT-PCR analysis was done

as previously described [1]. The primers were designed based on the rat nucleotide sequences of EP4 (5'-CTC GTG GTG CGA GTG TTC AT-3' and 5'-AAG CAA TTC TGA TGG CCT GC-3') and BMP-1 (5'-CAT CTC CAT CGG CAA GAA C-3' and 5'-CTC GAC TTC CTG AAC TTC CAT C-3'). Each primer set was designed between multiple exons. The abundance of each gene was determined relative to the 18S transcript.

Electron microscopy

Electron microscopic analysis for elastic fiber formation was performed as previously described [3].

Gelatin zymography

MMP activity was examined by gelatin zymography as previously described [4].

In situ hybridization

Expression of EP4 mRNA in mice fetuses on day 12.5, 16.5, and 18.5 of gestation was evaluated by *in situ* hybridization. A 543 bp DNA fragment corresponding to nucleotide positions 1373 to 1915 of mouse EP4 cDNA (Gen-Bank NM_008965) was cloned into pGEMT-Easy vector (Promega, Madison, WI) and used for the generation of sense and antisense RNA probes. Digoxigenin-labeled RNA probes were prepared with DIG RNA Labeling Mix (Roche, Basel, Switzerland). Hybridization was performed with probes at concentrations of 300 ng/ml in the Probe Diluent-1 (Genostaff, Tokyo, Japan) at 60°C for 16 h. After treatment with 0.5% blocking reagent (Roche) in TBST for 30 min, the sections were incubated with anti-DIG AP conjugate (Roche) diluted 1:1000 with TBST for 2 hr at room temperature (RT). Coloring reactions were

performed with NBT/BCIP solution overnight and then washed with PBS. The sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan), and mounted with CC/Mount (DBS).

Supplemental Table 1.

Summary of patient profile

Case	Age at Operation	Diagnosis
1	2 days	CoA, VSD
2	3 days	TGA, CoA
3	4 days	CoA, VSD
4	4 days	CoA, VSD
5	13 days	CoA, VSD
6	13 days	CoA, VSD
7	1 month	hypoLV, CoA, VSD

CoA: Coarctation of the Aorta, VSD: Ventricular Septum Defect,

TGA: Transposition of the Great Arteries, hypoLV: hypoplastic Left Ventricule.

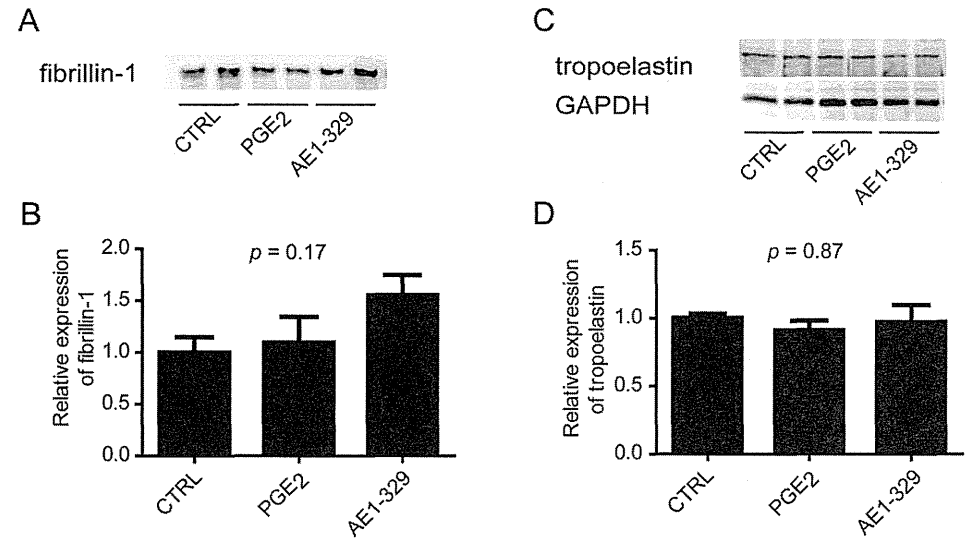
Supplemental table 2.

Correlation between elastic fiber formation and expression of EP4 and LOX

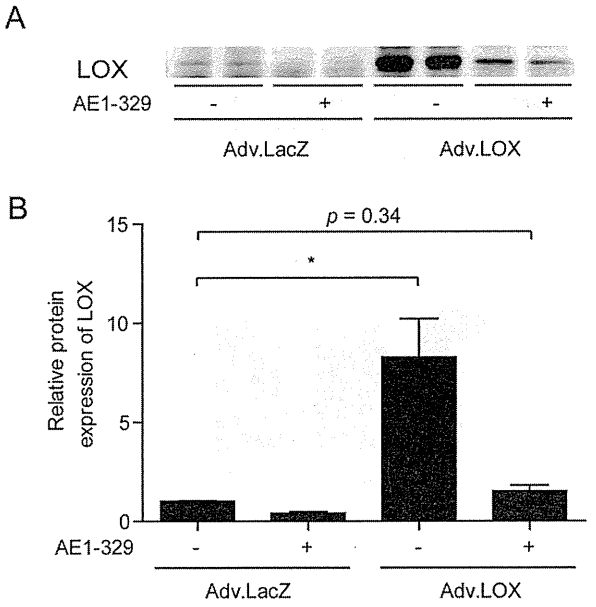
	Elastic fiber formation - EP4			Elastic fiber formation - LOX			EP4 - LOX		
case	r	n	*p* value	r	n	*p* value	r	n	*p* value
1	-0.7164	68	< 0.0001***	0.8095	68	< 0.0001***	-0.6723	68	< 0.0001***
2	-0.8277	22	< 0.0001***	0.6043	22	0.0029**	-0.6101	22	0.0026**
3	-0.8869	44	< 0.0001***	0.6431	44	< 0.0001***	-0.5626	44	< 0.0001***
4	-0.7116	62	< 0.0001***	0.765	62	< 0.0001***	-0.4875	62	< 0.0001***
5	-0.547	35	0.0007***	0.7561	35	< 0.0001***	-0.5335	35	0.001***
6	-0.523	28	0.0043**	0.6032	28	0.0007***	-0.6066	28	0.0006***
7	-0.7851	19	< 0.0001***	0.8649	19	< 0.0001***	-0.5765	19	0.0098**

r: correlation coefficient; n: number of sampling points. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Supplemental Figure 1

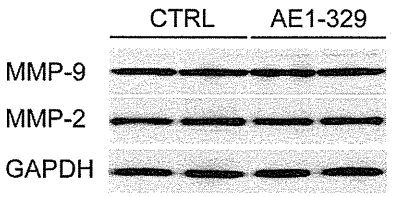


Supplemental Figure 2

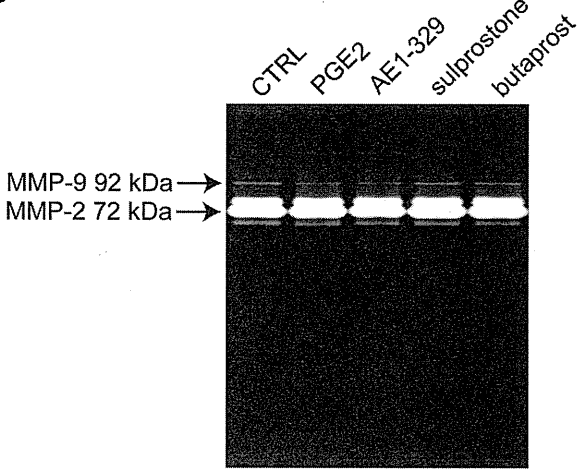


Supplemental Figure 3

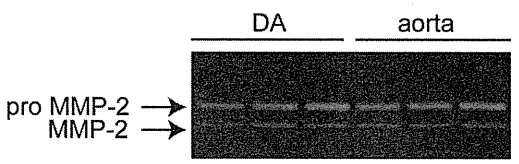
A



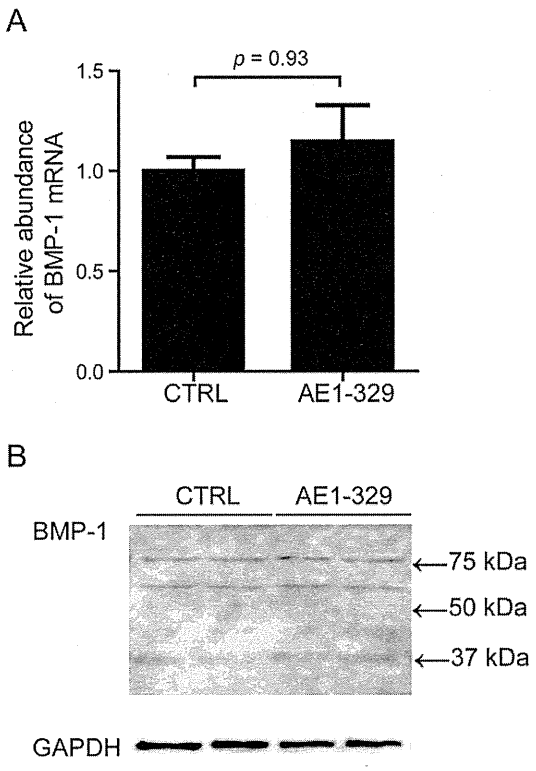
B



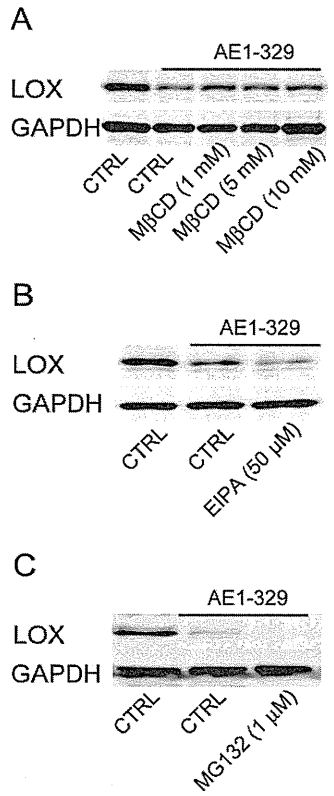
C



Supplemental Figure 4



Supplemental Figure 5



Supplemental Figure Legends

Supplemental Figure 1

EP4 signaling did not affect protein expression of tropoelastin and fibrillin-1.

(A) Protein expression of fibrillin-1 in culture medium of DASMCs treated with either PBS, PGE_2 (1 μM), or AE-329 (1 μM) for 72 h. (B) Quantification of (A), n = 4. (C) Protein expression of tropoelastin in whole cell lysate of DASMCs treated with either PBS, PGE_2 (1 μM), or AE-329 (1 μM) for 72 h. (D) Quantification of (C), n = 4.

Supplemental Figure 2

Overexpression of LOX protein in DASMCs transfected with Adv.LOX.

(A) Protein expression of LOX in culture medium of DASMCs transfected Adv.LacZ or Adv.LOX in the presence or absence of AE-329 (1 μM). The time-course of transfection and drug administration was same as Figure 4I. (B) Quantification of (A), n = 4, $*p < 0.05$.

Supplemental Figure 3

EP4 signaling did not affect expression or activation of MMP-2 or -9 in DASMCs.

(A) Protein expression of MMP-2 and-9 in DASMCs treated with or without AE1-329 (1 μM) for 72 h. (B) Gelatin zymography of DASMCs treated with 1 μM of PGE2 or each EP agonist. (C) Gelatin zymography of the rat DA and aorta on the 21st day of gestation.

Supplemental Figure 4

EP4 signaling did not change BMP-1 expression in DASMCs.

(A) Expression of BMP-1 mRNA in DASMCs treated with or without AE1-329 (1 μM) for 24 h. n = 4. (B) Representative image of protein expression of BMP-1 in DASMCs treated with or without AE1-329 (1 μM) for 72 h.

Supplemental Figure 5

LOX degradation was associated with caveolar endocytosis, macropinocytosis, and proteosome.

(A) Representative figures of protein expression of LOX in whole cell lysate of DASMCs treated with MbCD, EIPA, or MG132 in the presence of AE1-329 (1 μM).

References

1. Yokoyama U, Minamisawa S, Adachi-Akahane S, Akaike T, Naguro I, Funakoshi K, Iwamoto M, Nakagome M, Uemura N, Hori H, Yokota S, Ishikawa Y. Multiple transcripts of ca2+ channel alpha1-subunits and a novel spliced variant of the alpha1c-subunit in rat ductus arteriosus. *Am J Physiol Heart Circ Physiol*. 2006;290:H1660-1670

2. Kobayashi Y, Take I, Yamashita T, Mizoguchi T, Ninomiya T, Hattori T, Kurihara S, Ozawa H, Udagawa N, Takahashi N. Prostaglandin e2 receptors ep2 and ep4 are down-regulated during differentiation of mouse osteoclasts from their precursors. *J Biol Chem*. 2005;280:24035-24042

3. Nakamura T, Lozano PR, Ikeda Y, Iwanaga Y, Hinek A, Minamisawa S, Cheng CF, Kobuke K, Dalton N, Takada Y, Tashiro K, Ross Jr J, Honjo T, Chien KR. Fibulin-5/dance is essential for elastogenesis in vivo. *Nature*. 2002;415:171-175

4. Yoshimura K, Aoki H, Ikeda Y, Fujii K, Akiyama N, Furutani A, Hoshii Y, Tanaka N, Ricci R, Ishihara T, Esato K, Hamano K, Matsuzaki M. Regression of abdominal aortic aneurysm by inhibition of c-jun n-terminal kinase. *Nat Med*. 2005;11:1330-1338

Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association®

Prostaglandin E_2 Inhibits Elastogenesis in the Ductus Arteriosus via EP4 Signaling

Utako Yokoyama, Susumu Minamisawa, Aki Shioda, Ryo Ishiwata, Mei-Hua Jin, Munetaka Masuda, Toshihide Asou, Yukihiko Sugimoto, Hiroki Aoki, Tomoyuki Nakamura and Yoshihiro Ishikawa

Circulation. 2014;129:487-496; originally published online October 21, 2013;
doi: 10.1161/CIRCULATIONAHA.113.004726
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7322. Online ISSN: 1524-4539