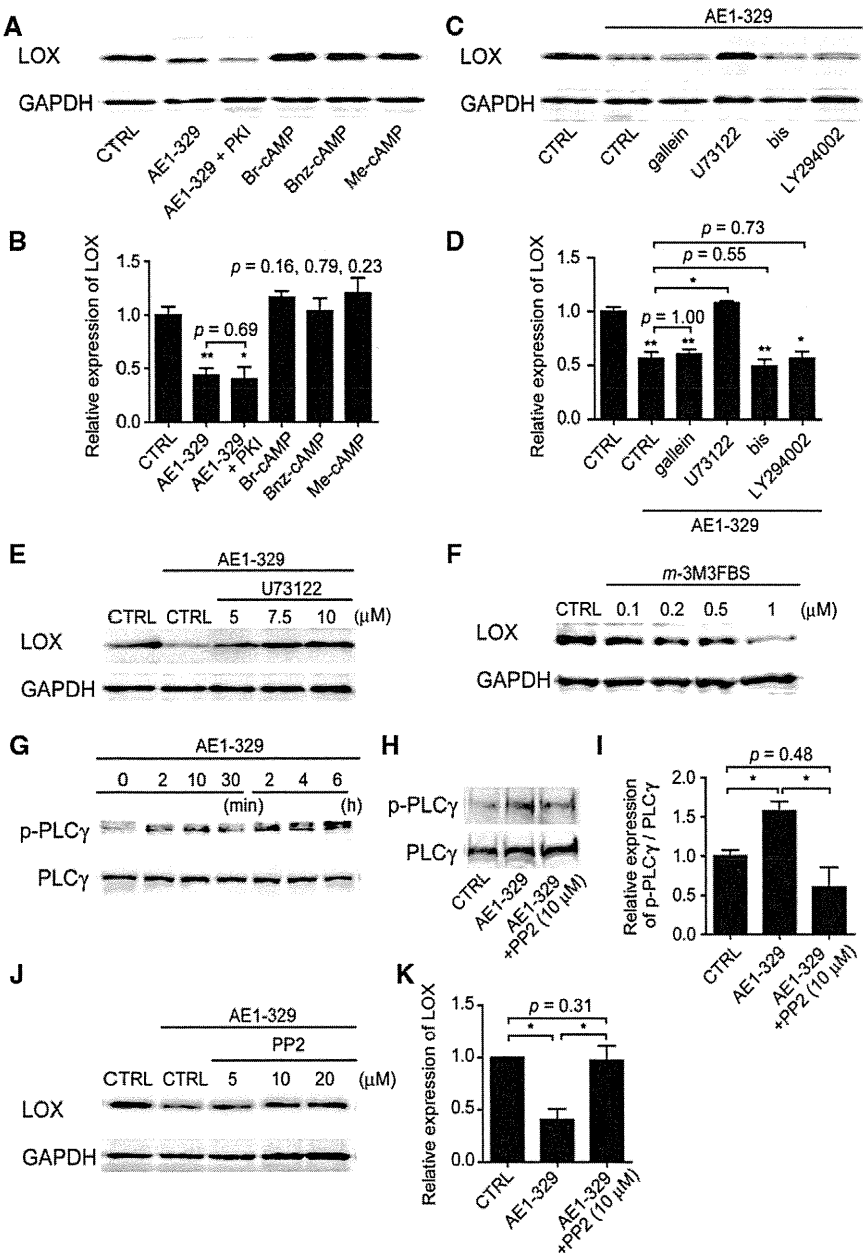

Figure 5. EP4 signaling decreased lysyl oxidase (LOX) protein via the c-Src-PLCγ pathway in ductus arteriosus smooth muscle cells (DASMCs). **A**, LOX protein expression in DASMCs treated with nonselective (Br-cAMP, 50 μmol/L), protein kinase A (PKA)-selective (Bnz-cAMP, 50 μmol/L) or Epac-selective (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

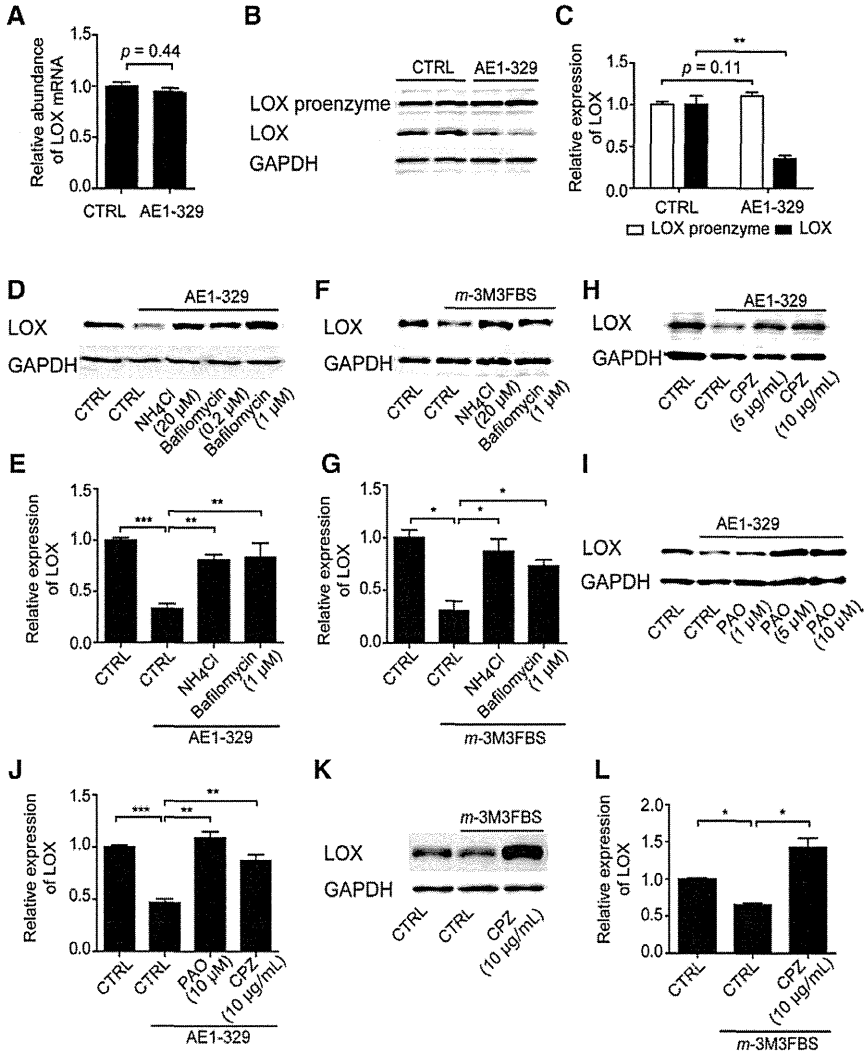

Figure 6. EP4 signaling decreased lysyl oxidase (LOX) protein through lysosomal degradation. **A** and **B**, Expression of LOX mRNA, pro-LOX, and LOX protein in ductus arteriosus smooth muscle cells (DASMCs) treated with AE1-329. mRNA of LOX and pro-LOX protein were not decreased by EP4 agonist in DASMCs. **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.

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Disclosures

None.

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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.

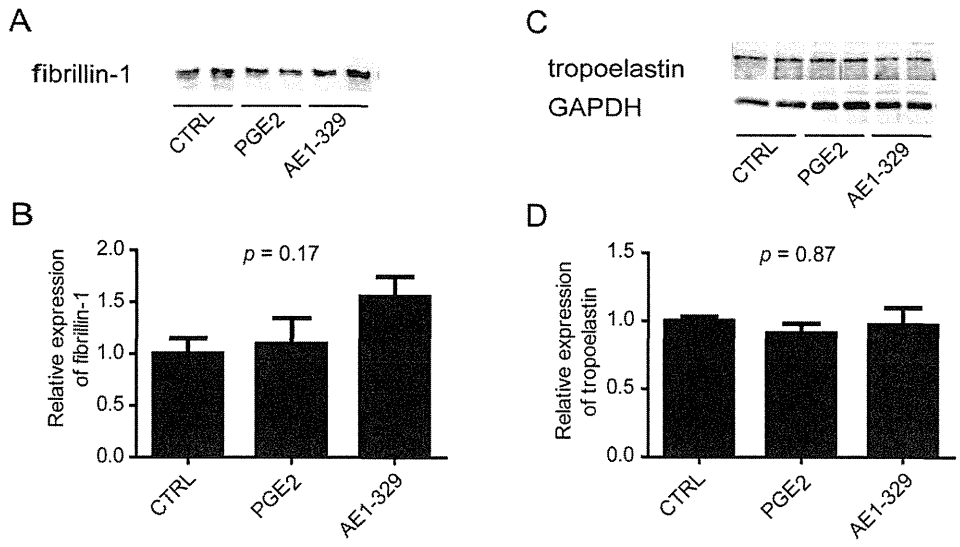
Supplemental table 2.

Correlation between elastic fiber formation and expression of EP4 and LOX

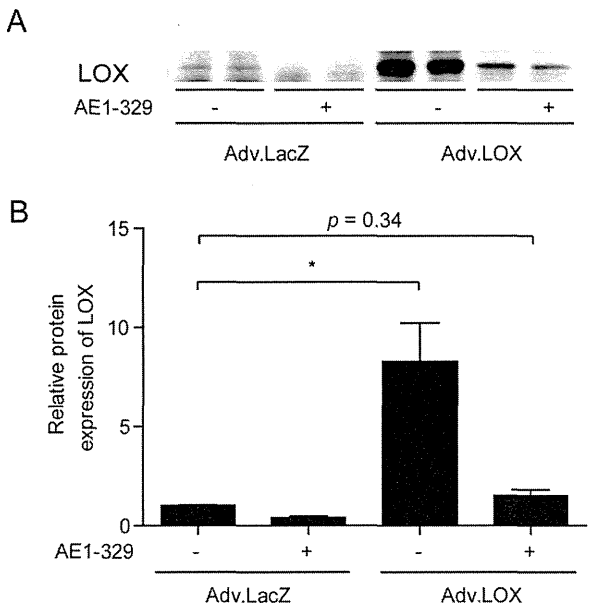
	Elastic fiber formation - EP4			Elastic fiber formation - LOX			EP4 - LOX		
case	r	n	*p* value	r	n	*p* value	r	n	*p* value
1	-0.7164	68	< 0.0001***	0.8095	68	< 0.0001***	-0.6723	68	< 0.0001***
2	-0.8277	22	< 0.0001***	0.6043	22	0.0029**	-0.6101	22	0.0026**
3	-0.8869	44	< 0.0001***	0.6431	44	< 0.0001***	-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

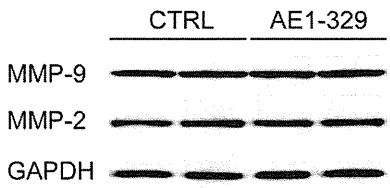


Supplemental Figure 2

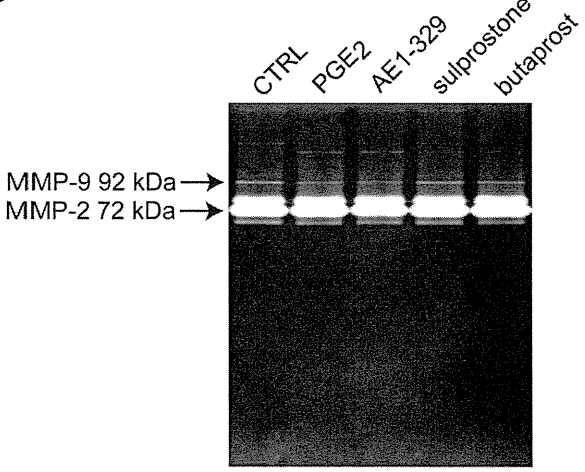


Supplemental Figure 3

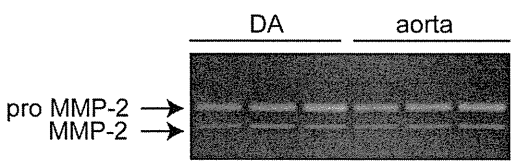
A



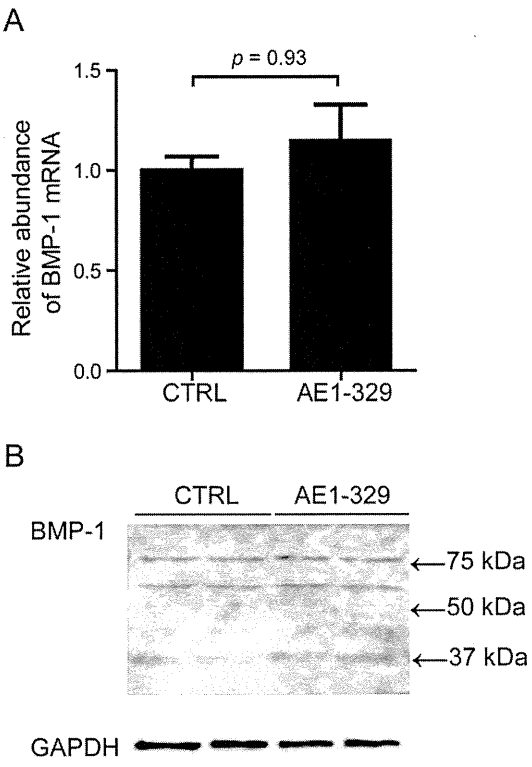
B



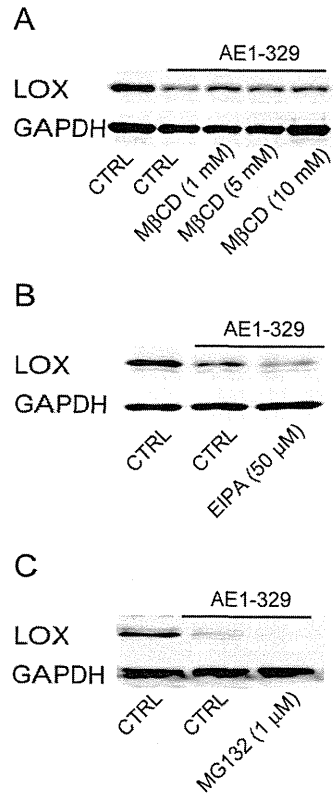
C



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).

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Three-dimensional multilayers of smooth muscle cells as a new experimental model for vascular elastic fiber formation studies

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ABSTRACT

Objective: Elastic fiber formation is disrupted with age and by health conditions including aneurysms and atherosclerosis. Despite considerable progress in the understanding of elastogenesis using the planar culture system and genetically modified animals, it remains difficult to restore elastic fibers in diseased vessels. To further study the molecular mechanisms, in vitro three-dimensional vascular constructs need to be established. We previously fabricated vascular smooth muscle cells (SMCs) into three-dimensional cellular multilayers (3DCMs) using a hierarchical cell manipulation technique, in which cells were coated with fibronectin-gelatin nanofilms to provide adhesive nano-scaffolds. Since fibronectin is known to assemble and activate elastic fiber-related molecules, we further optimized culture conditions.
Methods and results: Elastica stain, immunofluorescence, and electron microscopic analysis demonstrated that 3DCMs, which consisted of seven layers of neonatal rat aortic SMCs cultured in 1% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium, exhibited layered elastic fibers within seven days of being in a static culture condition. In contrast, the application of adult SMCs, 10% FBS, ε-poly(-lysine) as an alternative adhesive for fibronectin, or four-layered SMCs, failed to generate layered elastic fiber formation. Radioimmunoassay using [^{3}H]valine further confirmed the greater amount of cross-linked elastic fibers in 3DCMs than in monolayered SMCs. Layered elastic fiber formation in 3DCMs was inhibited by the lysyl oxidase inhibitor β-aminopropionitrile, or prostaglandin E_2. Furthermore, infiltration of THP-1-derived macrophages decreased the surrounding elastic fiber formation in 3DCMs.
Conclusion: 3DCMs may offer a new experimental vascular model to explore pharmacological therapeutic strategies for disordered elastic fiber homeostasis.

1. Introduction

Arterial walls have a highly organized layer structure that consists of various cells and extracellular matrix (ECM) components. In particular, the vascular media is composed of a dense population of concentrically organized smooth muscle cells (SMCs) and elastic fibers, and plays a pivotal role in maintaining sufficient blood pressure, even during variations in hemodynamic stress. In physiological conditions, SMCs synthesize elastin and other specific molecules, which are incorporated into elastic fibers and arranged into concentric rings of elastic lamellae around the arterial media [1]. In contrast, arterial compliance and distensibility are impaired in the presence of cardiovascular disease and risk factors such as aortic aneurysm, atherosclerosis, ischemic heart disease, aging, hypertension, cigarette smoking, and diabetes [2]. Hence, impaired elastic properties are associated with arterial dysfunction and pathophysiology [2,3].

Changes in arterial elastic properties are the result of alterations in the intrinsic structural properties of the artery, including the fracturing and thinning of elastic fibers. Current approaches to

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examining the elastogenesis and degradation of elastic fibers rely heavily on the use of the planar culture of vascular SMCs and genetically modified animals. These approaches have been instrumental in numerous discoveries and have been modified to create very elegant experimental designs [1,4–7]. Currently, however, no pharmacological strategy to promote elastogenesis and prevent the degradation of elastic fiber formation is available, and the molecular mechanisms of the regulation of elastic fiber formation remain to be studied. The two-dimensional (2D) monolayer culture system is a useful method for isolating specific factors and their effects on specific cell types [5–7], but it lacks the native-like layered structure of elastic fibers. Therefore, changes in the spatial arrangement of elastic fibers induced by various stimuli and the infiltration of immune cells cannot be observed. In-vivo analysis, on the other hand, often fails to discriminate among the various and complex factors. In this context, in vitro reconstruction vessel models overcoming these limitations are considered potential platforms of vascular biology that can provide further insights into the spatio-temporal molecular mechanisms of elastic fiber formation.

We previously developed a novel three-dimensional (3D) cell construction method [8] and created 3D-layered blood vessel constructs consisting of human umbilical arterial SMCs and human umbilical vascular endothelial cells [9]. To develop the 3D-cellular multilayers (3DCMs), we fabricated nanometer-sized cell adhesives like ECM scaffolds onto the surface of a cell membrane, which enables another cellular layer to adhere to the coated cell surface. We employed a layer-by-layer (LbL) technique to fabricate fibronectin–gelatin nanofilms onto living cell membranes, because the LbL technique produces nanometer-sized polymer films with a controllable nanometer thickness through the alternate immersion into interactive polymer solutions. We found that approximately 6 nm of fibronectin-based nanofilms were suitable for developing stable adhesive scaffolds and for creating allogeneic or xenogeneic multiple cell layers.

In addition to the cell adhesion effect, fibronectin has been known to orchestrate the assembly of the ECM [10–15]. In particular, recent reports suggest that fibronectin fiber assembles pericellularly into fibrillin microfibrils that have a complex structural organization and are widespread in elastic tissues [10,16]. Furthermore, fibronectin binds to lysyl oxidase (LOX), a cross-linking enzyme for elastic fibers, and acts as a scaffold for enzymatically active 30 kDa LOX [14]. Using scanning electron microscopy and transmission electron microscopy, we observed that fibronectin formed extracellular fibrils in the abovementioned 3DCMs within 24 h cultures [9], suggesting that fibronectin-coated SMCs have the potential to produce elastic fiber assemblies. In this context, we aimed to create the first scaffold-free 3D cellular multilayers (3DCMs) that are specifically designed for investigating the spatial regulation of vascular elastic fibers by employing this LbL assembly technique. The present study demonstrates that the optimized culture conditions provided layered elastic fiber formation in the 3DCMs consisting of neonatal rat SMCs within seven days of static culture conditions. In the 3DCMs, it was observed in the vertical view that macrophage infiltration or prostaglandin E_2 (PGE_2) changed the spatial arrangement of elastic fibers.

2. Materials and methods

Expanded materials and methods are described in Supplemental data.

2.1. Animals

Neonate (day 1) and adult Wistar rats (4–5months old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). All animal studies were approved by the institutional animal care and use committees of Yokohama City University.

2.2. Cell culture

Vascular SMCs in primary culture were obtained from the aorta of rat neonates (day 1) as previously described [17–19]. Briefly, the minced tissues were digested with a collagenase-dispase enzyme mixture at 37 °C for 20 min. The cell suspensions were then centrifuged, and the medium was changed to a collagenase II enzyme mixture. After 12 min of incubation at 37 °C, cell suspensions were plated on 35 mm poly-L-lysine-coated dishes The growth medium contained Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA). Human adult aortic SMCs were obtained from Lonza (Walkersville, MD, USA). The confluent SMCs were used at passages 5–7. THP-1 cells were obtained from the Health Science Research Resources Bank (Osaka, Japan) and were maintained in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% FBS. All cells were cultured in a moist tissue culture incubator at 37 °C in 5% CO_2-95% ambient mixed air.

2.3. Construction of 3DCMs

Construction of 3DCMs was performed as previously described [8]. Briefly, a cell disk LF (Sumitomo Bakelite, Tochigi, Japan) was rinsed with 50 mM Tris–HCl buffer solution (pH 7.4) for 15 min and coated with fibronectin (0.2 mg/ml) for 30 min at 37 °C. SMCs were cultured on the cell disk (11×10^4 cells/cm^2) and incubated for 12 h in 10% FBS/DMEM. The monolayered SMCs were then immersed alternatively in a solution of fibronectin (0.2 mg/ml) and gelatin (0.2 mg/ml). After nine steps of LbL assembly with fibronectin and gelatin, a second cell layer was seeded on the first cell layer (11×10^4 cells/cm^2) and incubated for 6–12 h at 37 °C. The cycles of LbL nanofilm assembly and subsequent cell seeding were repeated six times in four days to construct seven-layered 3DCMs. During the first four days, the medium was refreshed daily. Twelve hours after the seeding of the last layer, the culture media was changed to DMEM or DMEM/F-12 (Gibco, Carlsbad, CA) containing 1% FBS or 10% FBS. The 3DCMs were incubated for an additional 48 h and either fixed in buffered 10% formalin or harvested in TRIzol (Invitrogen, Carlsbad, CA). The time-course of the 3DCM experiments is shown in Supplemental Fig. 1A. Stimulation by β-aminoproprionitrile (BAPN) or PGE_2 (1 μM) was performed simultaneously with the medium change on day 5. For macrophage infiltration assay, seven-layered 3DCMs at day 5 were put on a 24-well plate and applied with 500 μl of RPMI 1640 medium containing THP-1 cells (2.0×10^5 cells) with or without phorbol 12-myristate 13-acetate (PMA, 0.1 μM). The 3DCMs were incubated for 1 h at 37 °C. Next, unattached THP-1 cells were washed out with PBS and 3DCMs were incubated in 1% FBS/DMEM for 72 h until fixation. Control monolayered neonatal rat aortic SMCs (2D-SMCs) were plated on day 4 in the same density as a single layer in 3DCMs (11×10^4 cells/cm^2), and were incubated for 48 h. Four-layered 3DCMs were constructed from day 3 following the same time-course. The proportional increase in the thickness of 3DCMs with the number of seeding events was shown in Supplemental Fig. 1B. To confirm the effect of fibronectin nanofilms, ε-poly(lysine) (0.2 mg/ml) was used as an alternative adhesive polymer.

2.4. Quantitative measurement of insoluble elastin

Newly synthesized insoluble elastin was measured as previously described [20,21]. Briefly, after the seven-layered 3DCMs and 2D-