――どこの大学でも、難しいと思いますよ。

森田　いざ実現に向けて動こうとしても、科学研究費を取るためには、過去5年間に外部資金をどれくらい獲得したか、など厳しい条件を突き付けられました。企業から移ったばかりの私はゼロですから、大きなハンディです。日本は、これから新しいことを行おうとする者に対して、どうしてこうも評価が下手かを思い知らされました。銀行も担保がないとお金を貸してくれません。素晴らしいアイデアを持っていても、アイデアだけではどうにもならない。それらの問題を解決したいと思ったのが、今回、特定非営利活動法人ライフイノベーション総合支援機構（略称：KSLION）を設立した動機です。

――NPO設立後は、いかがですか。

森田　設立後、まだ3カ月しか経っていませんが、正直、苦戦しています。ベンチャーキャピタルは、NPOには融資しません。国や自治体は、実績をあげているNPOにしか助成金を出しません。立ち上げ時には、まったく資金の手当てができないのです。仕方がないので、自己資金を出し、企業をリタイヤした年金生活の人たちに声をかけ、いわば霞を食べながら運営をしています（笑）。

――建物の建設資金などは、出す企業はありますね。

森田　それも、○○ホール、○○会館などという名前を付けてですね（笑）。目に見えないアイデアや計画には、なかなか出してくれませんね。

●KSLIONの重要な役割

――中小企業が、開発した製品を申請するためにPMDA（医薬品医療機器総合機構）に持っていくと、類似製品が米国にある場合はその調査のために渡航するPMDAの職員の航空運賃まで、申請者が負担するそうですね。

森田　中小企業が素晴らしいデバイスを開発しても、治験でつまずき申請すらしにくいことになりかねません。そこで、Pre-PMDA機能を持ち、開発の全期間におけるあらゆる相談に応じて、課題を解決するための支援を行うことにしたのです。

――中小企業の医療機器開発にとっては、助かりますね。

森田　医療機器開発では、デューク大学が最先端を走っています。HBD（Harmonization By Doing）というのがあり、日米共同でパイロット試験を実施し、炙り出た課題を解決して、さらに前へ進むというコンセプトで、日米間の医療機器規制の調和を図ろうとするアライアンスです。FDAなどもかかわっていますが、デューク大学がリーダーシップを発揮しています。ですから、デューク大学との連携も強めていきたいと思っています。

――KSLIONの将来展望をお聞かせください。

森田　CTTI（Clinical Trials Transformation Initiative）という、FDAとデューク大学で作った組織がありますが、日本版CTTIを作りたいと考えています。PMDA、経済産業省、厚生労働省、文部科学省、製薬協、医機連などにも入ってもらいたい組織です。

それから、日本で治験ができる環境を作ることを目指します。日本が世界初の治験を独立して行うには未だハードルが高いので、ハーバードやデュークなど米国と共同で行うのがよいでしょう。また、医療機器については、欧州と組むのがよいかもしれません。

――そのためには何が必要ですか。

森田　治験コストが、日本は高すぎます。コストを下げる必要があります。さらに効率を上げるためには、ICTを最大限に活用することが欠かせません。

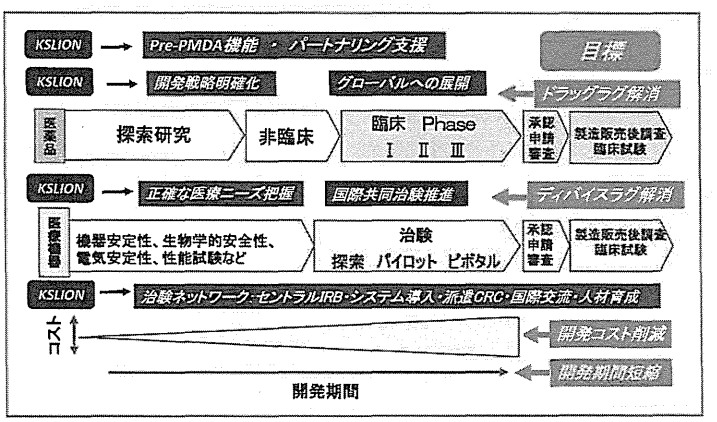

図1 ●●●●●●●●●●

イザイトップインタビュー
Top Interview

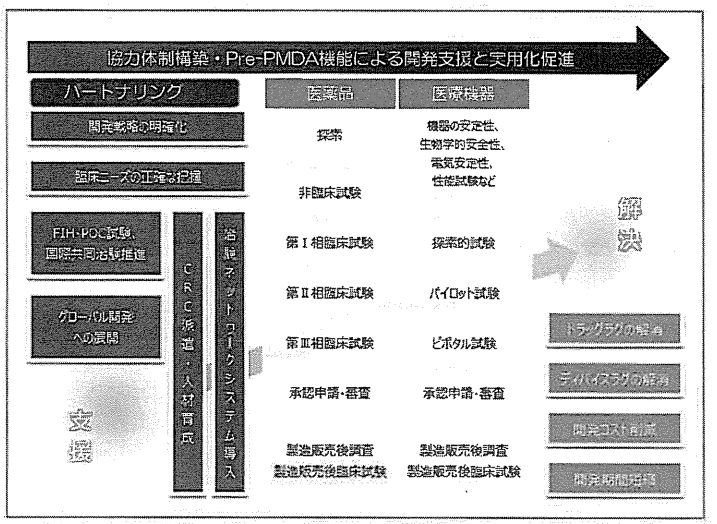

図2 ●●●●●●●●●●

今、具体的な話がインドから来ています。インドで治験サンプル数を確保し、コスト4割削減、期間3分の1短縮して治験を実施するという話です。この治験をPMDAが認めるかどうかが問題でしょう。治験の質が良いかどうかにかかっています。まずは、バイオシミラーがよいと考えています。未承認薬とは異なり、後発品なので大体の予想がつきます。ジェネリックメーカーも、バイオシミラーに手を出したいのですが、開発コストや開発期間がネックでした。インドで症例数を稼ぐことができれば、コスト削減と期間短縮は可能です。中国関係でも、まだオープンにはできませんが、次々に案件が舞い込みつつあります。今後、サクセスストーリーが、2つ、3つできれば、私たちも忙しくて、寝る暇もなくなると思っています。

●治験関連の仕事はさらに増える

――アイデアが尽きないようですね。

森田 SMO（Site Management Organization：治験施設支援機関）ビジネスも将来的には、リサーチナースにした方がよいと考えています。または、派遣型CRCにして、週に2日はA病院に、後の3日はB病院に行くなどというシステムも考えられます。さらに、神経疾患やがん領域など、高い専門性を持った人材を派遣できればよいでしょうね。あるいは、バイリンガルの人材は、国際共同治験にも耐えられるCRCとして、活躍の場は広がります。そうすれば、メーカーも医療機関側も、双方が助かるわけです。

――治験は、数を増やすために、開業医にも依頼したいそうですね。

森田 はい、その通りです。今言ったような仕組みを作れば、治験は進むと思います。いろいろなアイデアがありますが、低コストでアイデアを生かすには、NPOしか考えられません。NPOの強みは、大学ともPMDAともFDAとも、対等に渡り合えることです。講演でも、私は自由に発言しています。企業にいた時は、日本の臨床のトップリーダーに会うと、うつ向き気味に、遠慮しながら話さないと、生意気だといわれました（笑）。

――Pre-PMDAについて、もう少し、説明いただけますか。

森田 相談案件を持って、私も一緒にPMDAについて行ったことがありますが、「質問には答える」という姿勢でした。質問しないことには決して答えない。申請者と一緒になって、開発しよう、申請者を支援しようという立場ではありません。

――米国のFDAの場合はいかがですか。

森田 先ほどお話しした抗がん剤の申請時、FDAに行く前に、動物実験を含めプレクリニカルデータをたくさん送っておきました。最初に言われたことは「よくもこんなたくさんデータを集めましたねえ。もっと前に、治験に入れましたよ。このような良い薬であれば、大勢の患者さんが待っています」でした。さらに、第Ⅰ相試験では安全な用量から徐々に投与量を増やしていくのですが、「なんでこんな低いところから始めるのですか」といわれました。「がん患者さんは、いろいろな薬を試して効かなかったから、新薬の治験に挑んでいるのです。少々副作用が出ようとも、何より効果を期待しているのです。効果が出るような用量から、なぜ始めないのですか」と叱られました。

そして、FDAが推奨してくれた用量から始めたところ、けっこうな抗が

ん作用が認められ、しかも軽い副作用で収まるという、理想的な初回投与量から治験を開始できました。FDAはたいしたものだ、さすがFDAだと思いましたね。

●成功体験を積み上げ、次世代に引き継ぎたい

――日本の申請・承認の仕組みを変える必要がありそうですね。

森田　CTTIが典型ですが、政府から、民間から、大学から、さらに投資家までが入っています。そして、法律家、患者団体まで参加している。まさに、オール国家体制なのです。

――日本は何をやっているのか、という気持ちが強くなりますね。

森田　今、様々な公的団体や機関、さらにはIT企業などと組んで、仕組みを作ろうとしています。

――日本だけではなく、海外との関係も強められているようですね。

森田　中国には301病院というのがあります。中国解放軍関連のトップの病院です。治外法権になっており、中国で未承認の医薬品でも医療機器でも、良いものは何でも輸入して使うのだそうです。医薬品は、米国から入ってきますが、医療機器は、日本に期待しています。ただし、301病院に送る医療機器については根回しが必要です。これが実現すれば、軍の病院から中国全土の病院に、日本の医療機器、機材などを広げていくことも可能です。

――利益を優先するのではなく、NPOとして活動を広げていただきたいと思います。

森田　もちろんです。今後、若い人たちと一緒に成功体験を積み、次の世代に引き継いでいきたい。より多くの人にKSLIONを知っていただき、ご支援をいただければありがたいですね。

――本日は、ご多忙のところ、ありがとうございました。

（文責・インタビュー：井澤　泰）

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Prostaglandin E_2 Inhibits Elastogenesis in the Ductus Arteriosus via EP4 Signaling

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Background—Elastic fiber formation begins in mid-gestation and increases dramatically during the last trimester in the great arteries, providing elasticity and thus preventing vascular wall structure collapse. However, the ductus arteriosus (DA), a fetal bypass artery between the aorta and pulmonary artery, exhibits lower levels of elastic fiber formation, which promotes vascular collapse and subsequent closure of the DA after birth. The molecular mechanisms for this inhibited elastogenesis in the DA, which is necessary for the establishment of adult circulation, remain largely unknown.

Methods and Results—Stimulation of the prostaglandin E_2 (PGE_2) receptor EP4 significantly inhibited elastogenesis and decreased lysyl oxidase (LOX) protein, which catalyzes elastin cross-links in DA smooth muscle cells (SMCs), but not in aortic SMCs. Aortic SMCs expressed much less EP4 than DASMCs. Adenovirus-mediated overexpression of LOX restored the EP4-mediated inhibition of elastogenesis in DASMCs. In EP4-knockout mice, electron microscopic examination showed that the DA acquired an elastic phenotype that was similar to the neighboring aorta. More importantly, human DA and aorta tissues from 7 patients showed a negative correlation between elastic fiber formation and EP4 expression, as well as between EP4 and LOX expression. The PGE_2-EP4-c-Src-phospholipase C (PLC)γ–signaling pathway most likely promoted the lysosomal degradation of LOX.

Conclusions—Our data suggest that PGE_2 signaling inhibits elastogenesis in the DA, but not in the aorta, through degrading LOX protein. Elastogenesis is spatially regulated by PGE_2-EP4 signaling in the DA. ***(Circulation.* 2014;129:487-496.)**

Key Words: elasticity ■ muscle, smooth ■ pediatrics ■ prostaglandins ■ signal transduction

Elastic fibers are the largest structures in the extracellular matrix. Beginning with the onset of pulsatile blood flow in the developing aorta and pulmonary artery, smooth muscle cells (SMCs) in the vessel wall produce a complex extracellular matrix that ultimately defines the mechanical properties that are critical for proper function of the neonatal and adult vascular system.[1] As such, hemodynamics and mechanical stress are considered to be the main regulators in the formation of the vascular elastic fiber system during development.[2]

Clinical Perspective on p 496

The ductus arteriosus (DA) and its connecting elastic arteries (ie, the descending aorta and the main pulmonary trunk) are exposed to essentially the same mechanical forces and hemodynamics. However, since 1914, it has been widely recognized in multiple species that the DA exhibits sparse elastic fibers in the middle layer compared with adjacent elastic arteries, as well as disassembly and fragmentation of the internal elastic lamina.[3–8] In the human fetal aorta, newly synthesized uncrosslinked elastin appears at 23 weeks of gestational age to be unevenly distributed on the surface of microfibrils, where it forms continuous strips of variable width.[9] However, the DA exhibits fewer elastic fibers than the aorta.[4,6] This decreased elastogenesis is the hallmark of the vascular remodeling of the DA in humans and a variety of other species.[3–7] It has been suggested that this muscular phenotype of the DA allows it to collapse easily at birth when prostaglandin E_2 (PGE_2) is withdrawn and blood flow between the aorta and the pulmonary artery is reduced, thereby permitting immediate postnatal closure of the DA. Conversely, it is known that abnormalities of elastic fibers and elastic lamina are primarily responsible for the persistence of the DA in some human cases.[10,11] These abnormalities likely prevent intimal cushion formation and make it difficult to collapse the arterial wall. Therefore, it is

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important to understand the molecular mechanisms of how elastogenesis is regulated in the DA. Although Hinek et al[12,13] have demonstrated that truncated 52-kDa tropoelastin and the reduction of elastin binding protein negatively regulates elastic fiber formation in the DA, the mechanisms for impaired elastogenesis in the DA wall are not yet fully understood, despite nearly a century of research.[8]

During mid- to late gestation, fetuses are exposed to abundant PGE_2 that is released from the placenta[14] in accordance with the time course of impaired elastic fiber formation in the DA. The biological effects of PGE_2 depend on the prostanoid EP receptor subtypes EP1 through EP4[15]. Among the EP subtypes, EP4 is highly expressed in the DA of multiple species, including mice, rats, and humans, and regulates the DA muscular tone.[16–19] In addition to the DA muscular contraction, remodeling of the extracellular matrix during the fetal and neonatal period is necessary to complete the anatomical closure of the DA.[16,20,21] Our previous studies have demonstrated the role of PGE_2-EP4 signaling in DA remodeling, in which EP4 stimulation promotes intimal thickening, which is characteristic of the remodeling of the DA, in a hyaluronan-dependent and -independent manner.[16,21–23]

In this context, we hypothesized that PGE_2 inhibits elastogenesis in the DA through PGE_2-EP4 signaling. In the present study, we examined the molecular mechanisms of the inhibitory regulation of elastogenesis in human DA tissues and rodent DASMCs. We demonstrated that activation of EP4 promoted degradation of the mature lysyl oxidase (LOX) protein, a cross-linking enzyme for elastic fibers, only in the DA (and not in the aorta), leading to poor elastogenesis.

Methods

Expanded methods are described in the online-only Data Supplement.

Animals and Tissues

We used Wistar rat fetuses from timed-pregnant mothers (SLC Inc., Hamamatsu, Japan). Pooled tissues of the DA, aorta, and pulmonary arteries were obtained from rats on day 21 of gestation (n>60). Generation and phenotypes of EP4-knockout mice have been described previously.[17] All mice were C57BL/6 background littermates from heterozygote crosses. All animal studies were approved by the institutional animal care and use committees of Yokohama City University and Waseda University.

Human Tissues of the DA

Human DA tissues were obtained from Yokohama City University Hospital and Kanagawa Children's Medical Center at the time of corrective operations. Detailed patient information is summarized in Table I in the online-only Data Supplement. The study was approved by the human subject committees at both Yokohama City University and Kanagawa Children's Medical Center. All samples were obtained after receiving written informed parental consent.

Tissue Staining and Immunohistochemistry

Elastic fiber formation was evaluated by Elastica van Gieson staining. Immunohistochemical analysis was performed as previously described.[16] A color extraction method using BIOREVO bz-9000 and associated software (KEYENCE, Osaka, Japan) was performed to quantify elastic fiber formation and expression of EP4 and LOX. Three serial paraffin-embedded sections per each patient were subjected to elastica staining and immunohistochemistry. More than 19 fields in the smooth muscle layer of the DA and aorta were examined in each slide. The area stained dark purple indicated elastic fibers and diaminobenzidine (DAB)-stained colors, EP4- or LOX-positive areas, were extracted from matched area and counted using the software. Correlations of elastic fiber formation and EP4 and LOX expression were examined using >19 independent fields within 1 patient. We examined sections from a total of 7 patients, and the correlation coefficient and *P* value of each patient are shown in Table II in the online-only Data Supplement.

Immunocytochemistry

Vascular SMCs were plated on glass coverslips in 10% FBS in DMEM. The culture medium was then changed to 10% FBS in DMEM/F-12 alone, PGE_2, AE1-329, sulprostone, butaprost, or β-aminopropionitrile fumarate (day 1). Each drug was added on day 4. To examine the effect of silencing EP4 on elastic fiber formation, reverse transfection of DASMCs with EP4-targeted siRNA was performed according to the manufacturer's instructions on days 1 and 4, and treated with AE1-329 on days 2 and 5. To examine the effect of overexpression of LOX or EP4, the cells were infected with adenoviruses at 10 multiplicities of infection on days 1 and 4. AE1-329 was added to the cells on days 2 and 5. All cells were fixed in 10% buffered formalin on day 7. The fixed cells were stained with anti-elastin antibody as previously described.[22] All images were taken using a Nikon TE2000 (Nikon Instruments Inc, Tokyo, Japan) and processed under the same settings.

Quantitative Measurement of Insoluble Elastin

Newly synthesized insoluble elastin was measured as previously described.[24] Briefly, DASMCs were subconfluently plated on 60-mm dishes. Three days after plating, 20 μCi [^{3}H]valine was added to each dish (day 0). AE1-329 (1 μmol/L) or phosphate-buffered saline was added on days 0 and 4. The cells were harvested in 0.1 mol/L acetic acid on ice on day 7. The cells were boiled in 0.1N NaOH for 1 h. The insoluble pellets were boiled with 5.7N HCl for 1 h. The radioactivity was measured with a scintillation counter.

Statistical Analysis

Data are shown as the mean±SEM of independent experiments. The Mann–Whitney *U* test, Kruskal-Walis test, and Pearson correlation coefficient were used to determine the statistical significance of the data. A value of $P<0.05$ was considered significant.

Results

EP4 Signaling Inhibits Elastogenesis in the DA In Vivo

In the late gestation period, the DA exhibits disassembly and fragmentation of the internal elastic lamina and sparse elastic fibers in the middle layer compared to its two connecting arteries, the aorta and the pulmonary artery (Figure 1A), despite the fact that they are exposed to essentially the same hemodynamics. The expression of rat EP4 is greater in the DA than in the aorta and the pulmonary artery on the 21st day of gestation (day 21; Figure 1B).[16] We examined the association between the expression of EP4 mRNA and elastogenesis in developing mouse fetuses (Figure 1C). In day 12.5 mice, organized elastic fibers were not observed in either the DA or the aorta, whereas in situ hybridization analysis revealed that the expression of EP4 mRNA was clearly higher in the DA than in the aorta or the pulmonary artery. In day 16.5 and day 18.5 mice, the formation of elastic fibers was observed more clearly in the aorta than in the DA. In these developing stages, obvious abundant expression of EP4 mRNA was observed in the DA, but not in the aorta. To examine the effect of EP4

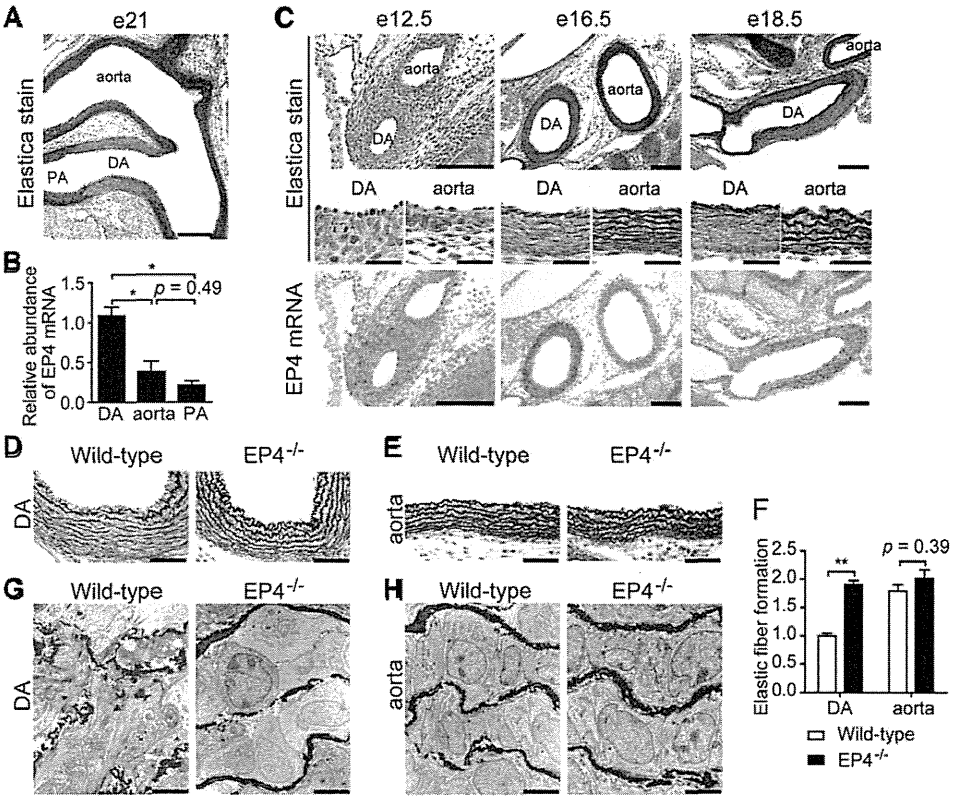

Figure 1. EP4 signaling attenuated elastic fiber formation in vivo. **A**, Elastica van Gieson stain (elastica stain) of rat fetus on day 21 of gestation (e21). **B**, Expression of EP4 mRNA of the rat ductus arteriosus (DA), aorta, and pulmonary artery (PA) on day 21 of gestation. n=6. **C**, Developmental changes in elastic fiber formation and EP4 mRNA by in situ hybridization in mouse fetus on days 12.5 (e12.5), 16.5 (e16.5), and 18.5 (e18.5) of gestation. Expression of EP4 mRNA was higher in the DA than in the aorta and pulmonary artery. Conversely, elastic fiber formation is sparser in the DA than in the other arteries. **D**, **E**, **G**, and **H**, Elastica stain and electron microscopic images of wild-type and EP4$^{-/-}$ mice on day 18.5 of gestation. Elastic fiber formation was restored in the DA of EP4$^{-/-}$ mice. **F**, Quantification of the elastic fiber formation of **D** and **E** using a color extraction method. n=8. *P<0.05, **P<0.01. Scale bars, 200 μm (**A**); 100 μm (**C**, **upper** and **lower**); 50 μm (**D**, **E**); 20 μm (**C**, **middle**); 5 μm (**G**, **H**).

on elastogenesis in vivo, we examined elastic fiber formation in the DA of EP4$^{-/-}$ mice. In EP4$^{-/-}$ mice, which die postnatally as a result of persistent patent DA (PDA),[17,25] we found that the DA acquired an elastic phenotype that was similar to that of the neighboring aorta, as determined by elastica staining (Figure 1D and 1E), a color extraction method of elastica staining (Figure 1F), and electron microscopic examination (Figure 1G and 1H).

Human Vascular Tissues Show a Negative Correlation Between Elastic Fibers and EP4 Expression

We also investigated the relationship between elastic fiber formation and EP4 expression in surgical samples from 7 patients with coarctation of the aorta who underwent surgical repair of aortic narrowing (Figure 2A, Table I in the online-only Data Supplement). In concurrence with the findings in rodents, there was less elastic fiber formation in the DA than in the normal aorta, and the cells stained with anti-EP4 antibody were far more abundant in the DA (Figure 2B). Indeed, statistical analysis revealed that the correlation was significant between the amount of EP4 expression and the degree of inhibited elastic fiber formation (Figure 2C, Table II in the online-only Data Supplement). Thus, elastogenesis is inhibited when EP4 is abundant. Taken together, these in vivo data suggest that EP4 plays a primary role in the inhibition of elastogenesis of the DA in humans and rodents.

EP4 Signaling Inhibits Elastogenesis in DASMCs

To clarify the role of EP4 in elastogenesis in detail, we evaluated the elastic fiber assemblies in rat DASMCs using an in vitro system, as reported previously.[24] In the control group, DASMCs developed an abundant meshwork of elastic fibers (Figure 3A). In the presence of PGE$_2$ or the EP4 agonist ONO-AE1-329, however, DASMCs developed a poor meshwork of elastic fibers. Neither the EP1/3 agonist sulprostone nor the EP2 agonist butaprost had any effect on elastic fiber development. LOX is a cross-linking enzyme that forms insoluble mature elastic fibers. Its specific small molecule inhibitor β-aminopropionitrile fumarate impaired elastic fiber formation (Figure 3A). To quantify the amount of mature (ie, cross-linked) elastic fibers inhibited by EP4 stimulation, we metabolically labeled newly synthesized elastin with [^{3}H] valine, and measured the incorporation of [^{3}H]valine in the NaOH-insoluble fraction of these cells, which reflects the amount of newly synthesized mature elastic fibers.[24] As shown in Figure 3B, in DASMCs, we detected a significant decrease in the incorporation of [^{3}H]-valine into the insoluble fraction when ONO-AE1-329 was added to the medium (Figure 3B). When the expression of EP4 mRNA was decreased by 89%

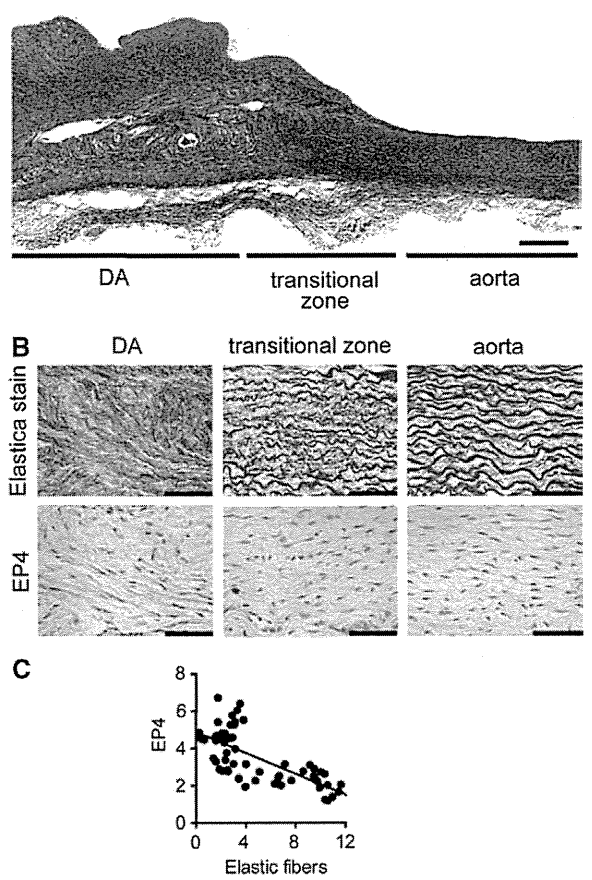

Figure 2. Human vascular tissues show a negative correlation between elastic fibers and EP4 expression. **A**, A representative image of the elastica stain of a human neonatal vessel. **B**, Elastica stain and immunohistochemistry for EP4 in human neonatal vessels. **C**, Representative results of quantification of elastic fiber formation and EP4 protein expression using a color extraction method. Values are shown in arbitrary units. Scale bars, 200 μm (**A**); 50 μm (**B**).

by RNA interference, DASMCs developed elastic fiber formation even in the presence of ONO-AE1-329 (Figure 3C). To further confirm the existence of EP4-mediated impaired elastic fiber formation, we used rat aortic SMCs, which express much less EP4 than DASMCs (Figure 3D). When EP4 was forcibly expressed in aortic SMCs by EP4 gene transfer, elastogenesis was markedly impaired by ONO-AE1-329, whereas ONO-AE1-329 did not attenuate elastic fiber formation in the LacZ control (Figure 3E). These in vitro results indicate that PGE_2-EP4 stimulation is responsible for the impaired elastogenesis of the DA.

EP4 Signaling Inhibits Elastic Fiber Formation by Decreasing LOX Protein

In the process of elastic fiber assembly, soluble elastin precursors (tropoelastin) are deposited on microfibrils.[1,26] They are then cross-linked by LOX, which confers elastic properties to elastic fibers.[27] Inactivation of the *LOX* gene is known to cause structural alterations in the arterial walls, leading to cardiovascular abnormalities.[28] In this context, we investigated the expression of LOX protein in human surgical samples. In contrast to EP4, there were significantly fewer cells stained with anti-LOX antibody in the DA (Figure 4A). When elastic fiber formation and the expression of EP4 and LOX were quantified, LOX expression was positively correlated with elastic fiber formation, whereas it was negatively correlated with EP4 expression (Figure 4B, Table II in the online-only Data Supplement). Interestingly, elastic fiber formation and EP4- or LOX-positive cells in the transitional zone appeared intermediate between the DA and aorta. We think that this finding supports previous reports that suggested that the coarctative ridge, a narrowed pathological segment in the aorta, is formed by mixed tissues from the native aorta and migrated tissues of DA origin.[29,30]

Next, we examined the effect of EP4 stimulation on LOX protein expression. We found that the amount of mature LOX form was significantly decreased in the culture media of DASMCs and DASMC lysates in the presence of PGE_2 and ONO-AE1-329 (Figure 4C and 4D). The effects of ONO-AE1-329 were dose- and time-dependent (Figure 4E and 4F). Other EP isoform-specific agonists had little effect. Interestingly, stimulation of EP4 did not change the expression levels of tropoelastin and fibrillin-1 proteins, which are the main components of elastic fibers (Figure IA–ID in the online-only Data Supplement). In the next LOX detection, we used whole cell lysate containing both intracellular and extracellular LOX protein. Although these EP4-mediated effects were not detected in aortic SMCs (ASMCs), the EP4 agonist significantly decreased the expression of LOX protein in ASMCs when EP4 expression was induced using the adenovirus (Figure 4G and 4H). When LOX expression was induced using the adenovirus (Figure IIA and IIB in the online-only Data Supplement), elastogenesis was largely restored in the ONO-AE1-329–treated DASMCs (Figure 4I). Thus, the reduction in LOX played a primary role in the EP4-mediated impairment of elastogenesis. The expression levels of matrix metalloproteinases and their activity were not altered in the DASMCs by EP4 stimulation (Figure IIIA and IIIB in the online-only Data Supplement). Nor was there any difference in matrix metalloproteinase 2 activity between rat tissues of the DA and aorta (Figure IIIC in the online-only Data Supplement), suggesting that EP4 signaling plays a role in inhibiting elastogenesis, but not in promoting elastolysis in the DA.

The c-Src-PLC Signal Pathway Plays a Role in the PGE_2-EP4–Induced Reduction in LOX Protein

Next, we examined the downstream signal pathway responsible for the EP4-mediated reduction in LOX protein expression levels. Although our previous studies have demonstrated that cAMP and its downstream pathways play a primary role in EP4-mediated DA remodeling,[16,22,23] the cAMP–protein kinase A or cAMP–exchange protein activated by the cAMP pathway did not play a role in the EP4-mediated reduction in LOX protein (Figure 5A and 5B). Instead, we found that the EP4-induced reduction in LOX protein was restored by the PLC inhibitor U73122 (Figure 5C–5E), but not by Gβγ, protein kinase C, or phosphoinositide 3-kinase inhibitors (gallein, bisindolylmaleimide I, LY294002; Figure 5C and 5D). Furthermore, the PLC activator *m*-3M3FBS significantly decreased the expression levels of LOX protein in DASMCs

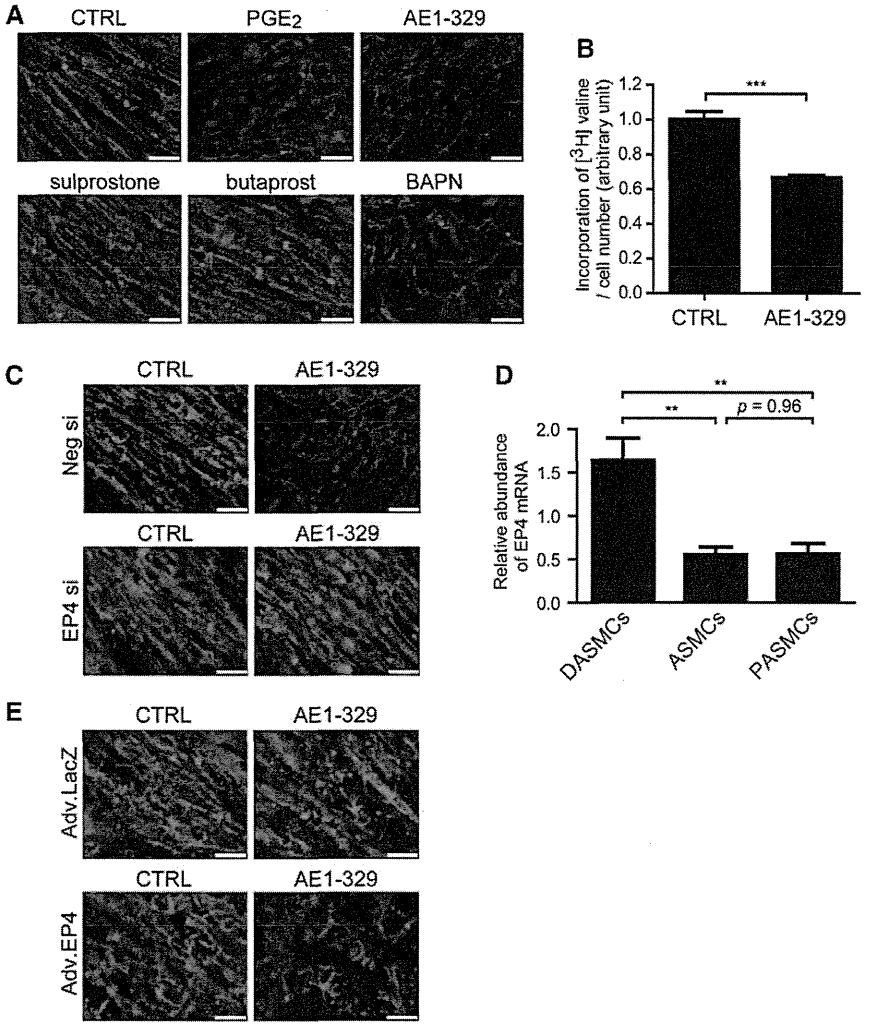

Figure 3. EP4 signaling attenuated elastic fiber formation in vitro. **A** and **C**, Immunostaining for elastin (red) and DNA (blue) of ductus arteriosus smooth muscle cells (DASMCs) treated with each drug indicated or EP4-targeted siRNA (EP4 si). AE1-329 indicates ONO-AE1-329; CTRL, control; and Neg si, negative control siRNA. Pharmacological activation of EP4 by ONO-AE1-329 attenuated elastic fiber formation. **B**, Incorporation of [^{3}H]valine was quantified in DASMCs treated with or without AE1-329. n=8, ***P<0.001. **D**, Expression of EP4 mRNA in DASMCs, aortic SMCs (ASMCs), and pulmonary SMCs (PASMCs). n=6, **P<0.01. **E**, Immunostaining for elastin (red) and DNA (blue) of the EP4-or LacZ-overexpressing ASMCs treated with or without AE1-329. Activation of EP4 did not affect elastic fiber formation in LacZ-overexpressing ASMCs, whereas it decreased elastic fiber formation in EP4-overexpressing ASMCs. Each drug was used at 1 μmol/L. Scale bars, 20 μm.

(Figure 5F). Because several recent studies have demonstrated that PGE$_2$ promotes cancer cell migration via the EP4–c-Src signal pathway[31,32] and that c-Src plays a critical role in the phosphorylation of PLCγ in several cell types,[33,34] we hypothesized that the c-Src-PLCγ signal pathway may be involved. We found that ONO-AE1-329 significantly increased PLCγ1 phosphorylation (Figure 5G). In contrast, the Src-family kinase inhibitor PP2 significantly decreased PLCγ1 phosphorylation (Figure 5H and 5I) and restored the reduction in LOX protein induced by ONO-AE1-329 (Figure 5J and 5K). These results support our hypothesis that the c-Src-PLCγ signal pathway plays a primary role in the PGE$_2$-EP4-induced reduction in LOX protein.

EP4 Signaling Promotes LOX Degradation in Lysosomes

Although ONO-AE1-329 decreased the expression of LOX protein in DASMCs, we found that ONO-AE1-329 did not decrease the mRNA expression of LOX (Figure 6A). Active LOX is synthesized as a 50-kDa inactive LOX proenzyme (pro-LOX), which is secreted into the extracellular space. Pro-LOX is then processed by proteolysis into a functional 32 kDa enzyme LOX and an 18-kDa propeptide.[27,35] Using a pro-LOX–specific antibody, we found that the pro-LOX protein itself was not decreased by ONO-AE1-329 (Figure 6B and 6C), indicating that LOX was decreased post-translationally. BMP1 is a major protease that cleaves pro-LOX in the extracellular space.[27] However, ONO-AE1-329 did not change the expression of BMP1 mRNA or protein in DASMCs (Figure IVA and IVB in the online-only Data Supplement). Instead, we found that lysosomal degradation inhibitors, such as NH_4Cl and bafilomycin, eliminated the EP4-induced reduction in LOX protein (Figure 6D and 6E). These lysosomal degradation inhibitors also restored the PLC-mediated reduction in LOX protein (Figure 6F and 6G). Furthermore, we found that the clathrin-mediated endocytosis inhibitors chlorpromazine and phenylarsine oxide similarly restored the EP4-induced reduction in LOX protein (Figure 6H–6J). Administration of chlorpromazine also restored the PLC-induced reduction in LOX protein (Figure 6K and 6L). In comparison, the caveolar endocytosis inhibitor methyl-beta-cyclodextrin (MβCD), the macropinocytosis inhibitor ethylisopropylamiloride (EIPA), and the proteosomal inhibitor MG132 showed little or no effect on LOX protein reduction (Figure VA–VC in the online-only Data Supplement). These data suggest that PGE$_2$-EP4-PLC stimulation promotes the degradation of the LOX protein in lysosome through clathrin-mediated endocytosis.

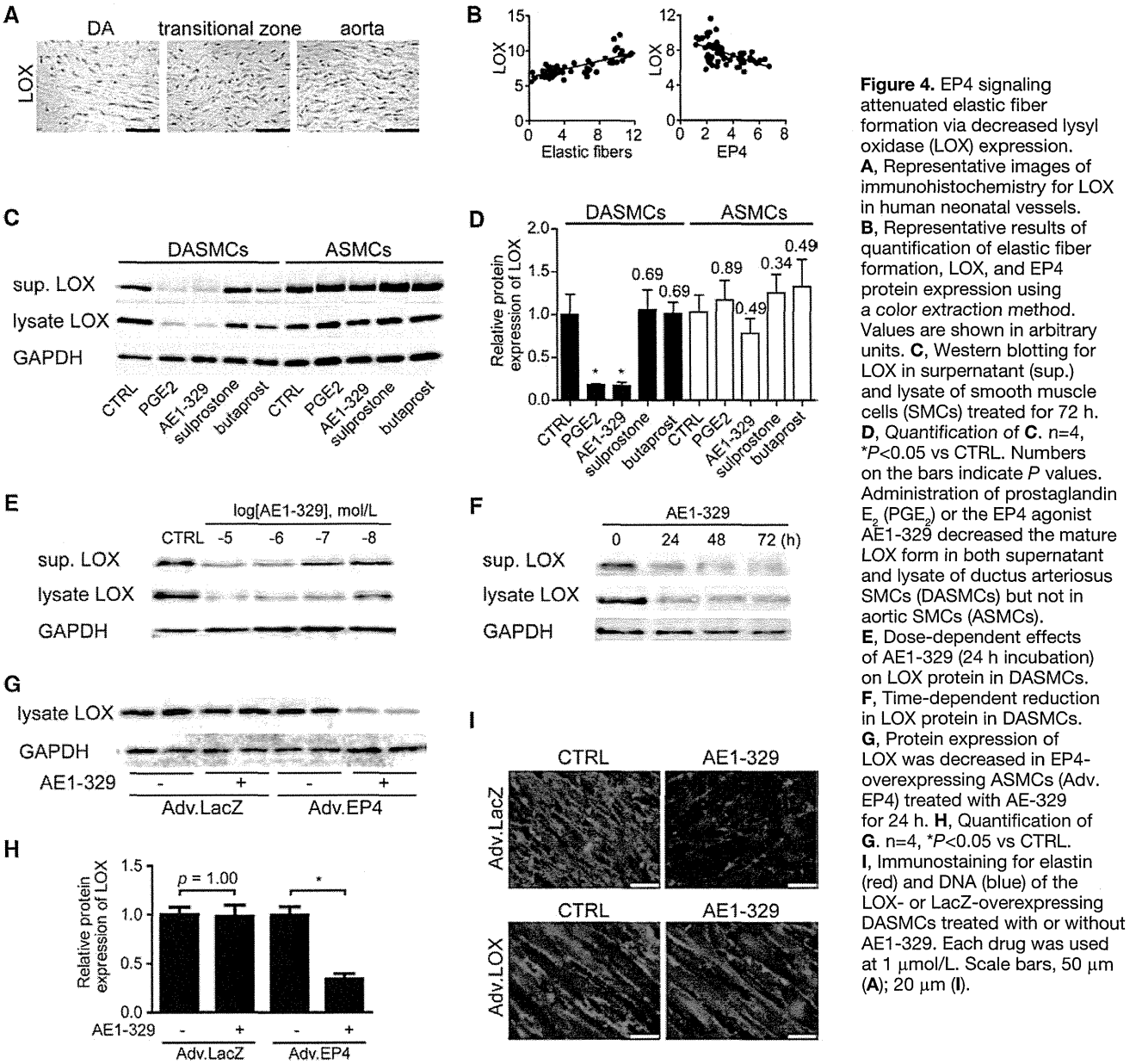

Figure 4. EP4 signaling attenuated elastic fiber formation via decreased lysyl oxidase (LOX) expression. **A**, Representative images of immunohistochemistry for LOX in human neonatal vessels. **B**, Representative results of quantification of elastic fiber formation, LOX, and EP4 protein expression 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

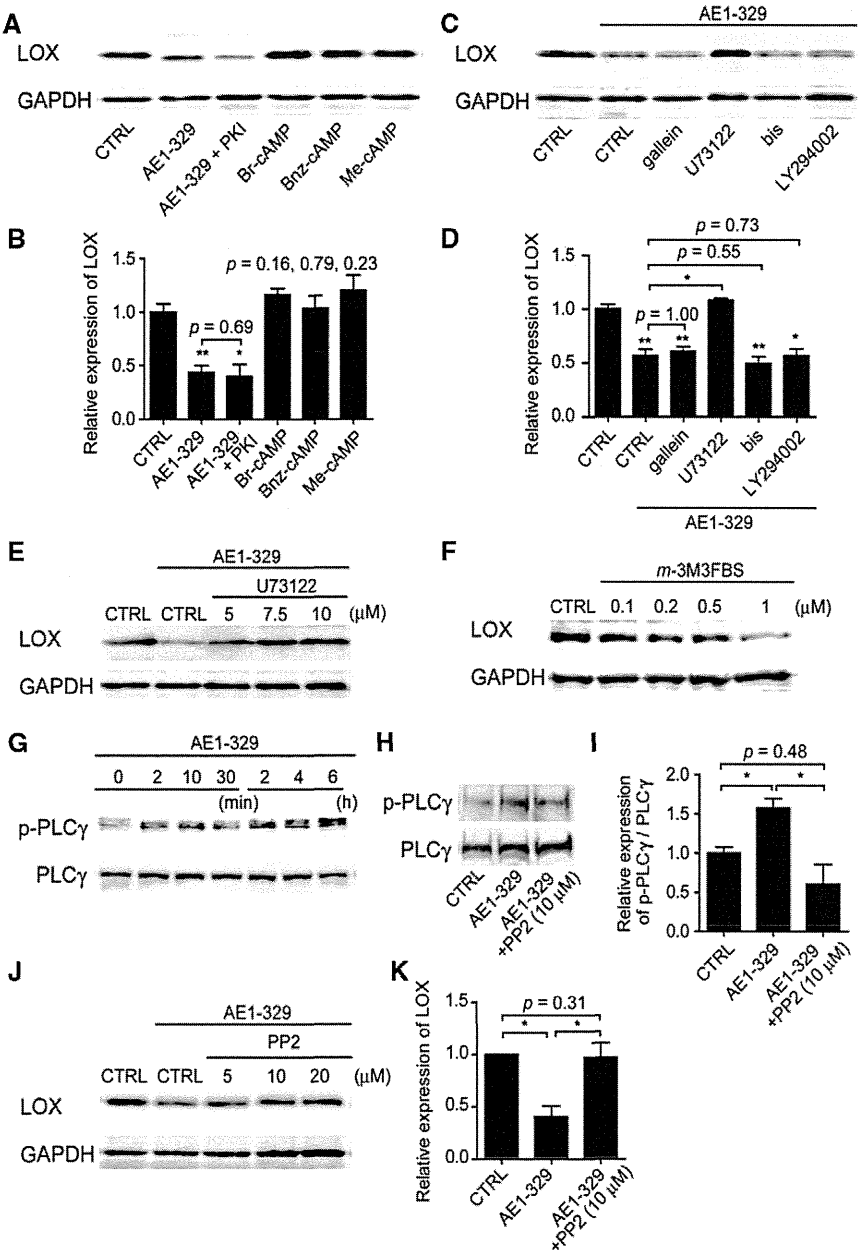

Figure 5. EP4 signaling decreased lysyl oxidase (LOX) protein via the c-Src-PLCγ pathway in ductus arteriosus smooth muscle cells (DASMCs). **A**, LOX protein expression in DASMCs treated with nonselective (Br-cAMP, 50 μmol/L), protein kinase A (PKA)-selective (Bnz-cAMP, 50 μmol/L) or Epac-selective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

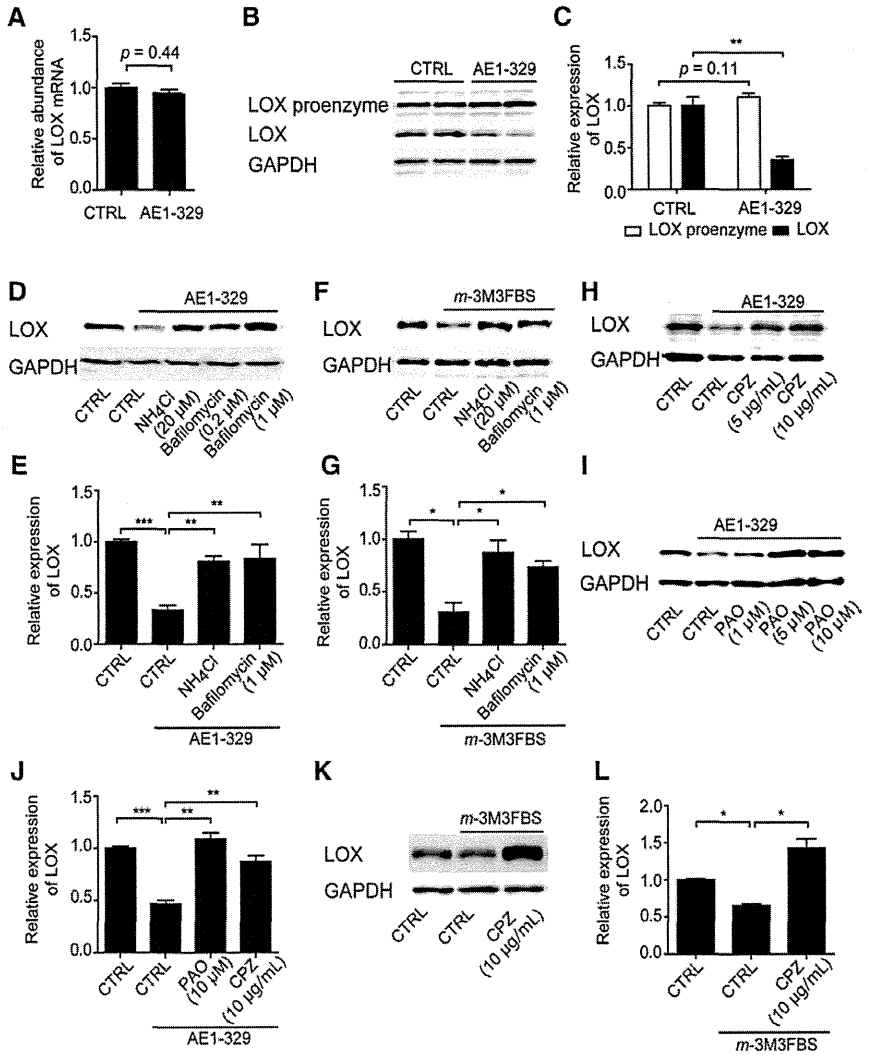

Figure 6. EP4 signaling decreased lysyl oxidase (LOX) protein through lysosomal degradation. **A** and **B**, Expression of LOX mRNA, pro-LOX, and LOX protein in ductus arteriosus smooth muscle cells (DASMCs) treated with AE1-329. mRNA of LOX and pro-LOX protein were not decreased by EP4 agonist in DASMCs.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

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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. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$