IHIと横浜市大との産学連携に関する協定

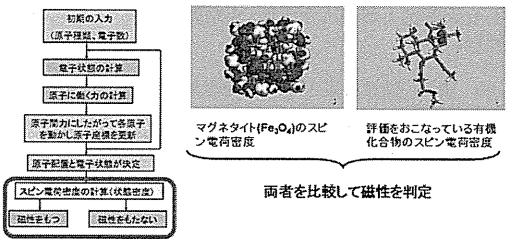
Agreement on industry-university cooperation with IHI and Yokohama City University

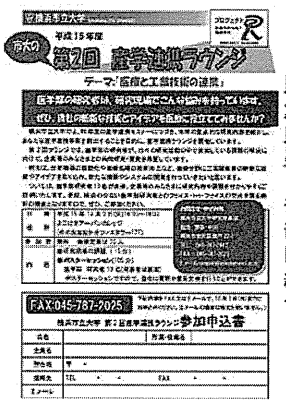
研究交流、地域貢献の目的で2003年6月25日に締結。

下記の新聞で本件が報道。

時事通信　2003/06/24
・企業「◎産学連携へ石川島播磨と基本協定＝横浜市大」
日本経済新聞　2003/06/25 朝刊　35面
読売新聞　2003/06/25 朝刊　33面[神奈川版]
朝日新聞　2003/06/26 朝刊　35面[神奈川版]
日刊工業新聞　2003/06/26 朝刊　39面
日本工業新聞　2003/06/26 朝刊　2面
神奈川新聞　2003/06/26 朝刊　7面

有機磁性体のスクリーニングおよび設計方法（特許第4279330号）

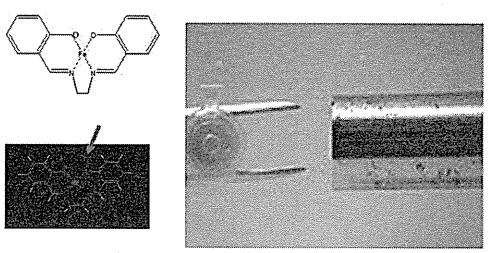




医学部研究者10名が直接、企業等のみなさまに研究内容や課題を分かりやすくご説明いたします。

普段、接点の少ない医学部研究者とのフェイス・トゥ・フェイスの交流を図る絶好の機会となりますので、ぜひ、ご参加ください。

磁性スクリーニング見出した有機磁性体



鉄サレン錯体

フォルスコリン誘導体の解析：
Analysis of forskolin derivative
アデニール酸シクラーゼサブタイプ選択性
Isoform-Selective Regulation of Adenylyl Cyclase
① 負の静電ポテンシャル（論文）
Negative electrostatic potential (paper)
② 福井関数（日本、米国登録特許）
Fukui Function (JP US issued patents)

EI236の本当の姿は何ですか？

What is the real picture of EI236?

予期しない出来事

Unexpected incidents

磁性体のスクリーニング

Screening of magnetic compounds

EI236の応用
Application of EI236

15:10～15:35　磁性医薬品を用いた神経膠芽腫に対する新規治療法の開発
大竹　誠（公立大学法人横浜市立大学　大学院医学研究科　脳神経外科）

15:35～16:00　講演　磁性医薬品を用いた舌癌に対する新規治療法の開発
佐藤　格（公立大学法人横浜市立大学　大学院医学研究科・助教）

謝辞
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- 山形大工　黒谷先生
- OIST　Kim先生
- 横国大　星野先生、井上先生
- 京大工　雨宮先生
- 愛知医大　佐藤元先生
- SPring-8 平田先生、山本先生
- ONRL D. J. Singh先生
- 東大物性研　益田先生
- 甲南大理工　山本先生
- 東北大WPI　谷垣先生
- 放医研　青木先生
- 横浜市立大学 生命医科学　佐藤衛先生

厚生労働科学研究委託費（革新的がん医療実用化研究事業）
委託業務成果報告（業務項目）

口腔がんに対する磁性抗がん治療薬の実用化に関する研究（臨床試験の検討）

担当責任者　浦野勉　横浜市立大学大学院医学研究科　客員教授

研究要旨

口腔がんに対する磁性抗がん剤治療薬の実用化において、磁性抗がん剤の有用性を検証した。

A. 研究目的

医薬品開発は、抗がん剤、とりわけ本申請研究のように口腔がんなどの希少がんを対象にした場合も同様である。抗がん剤としての効力を裏付ける非臨床薬理試験は、抗がん剤としての有効性や作用機序などについて、ヒトによらない非臨床レベルでの探索を中心として検討する。これに対して、ＰＭＤＡをはじめとする諸国の医薬品の承認審査では、ヒトを用いた臨床試験結果に基づく有効性評価を評価の基準としながらも、薬剤としての作用機序などからその効力が裏付けられているかどうか、いわゆるプルーフオブコンセプトという観点から、ヒトによらない非臨床薬理試験の評価を行っている。

B. 研究方法

本研究計画では磁性抗がん剤の開発を目的とするが、磁性抗がん剤においても、一般の薬剤と変わることはなく、抗がん剤の承認審査時に必要とされる非臨床薬理試験の範囲は、抗がん剤としての作用機序であり、がん治療においてはそれぞれの適応とされる癌種に応じた有効性の検討が必要である。本年度の研究においては、口腔がんとも関連の深い皮膚がんにおける磁性抗がん剤の治療効果を基礎実験的に検討し、その結果を評価した。

C. 研究結果

抗がん剤の非臨床薬理試験の現状と承認審査時における評価の考え方については、抗がん作用検討とともに、適応癌種に対する有効性の検討が重要である。磁性抗がん剤においては、本年度に受理された論文に記載されているように、皮膚がんを中心として薬理効果が検討され、高い治療効果が証明された。さらに磁性抗がん剤としての磁性特性が確認されたことにより、磁場誘導による抗がん効果を動物実験においても証明することができた。

D. 考察

抗がん剤としての効力を裏付ける非臨床試験において、磁性抗がん剤の薬理作用と、磁性特性を併用した治療効果が動物実験で検討された。治療効果が顕著に増強したことにより、磁性抗がん剤が、今後の実験において口腔がん治療においても有用であることが推測された。

E. 結論

磁性抗がん剤は、我が国独自の抗がん剤であり、口腔がんをはじめとした難治性の癌に対して治療効果を示すことが推測された。今後薬事申請に必要な非臨床薬理試験が追加で必要と考えられるが、新規メカニズムによる抗がん剤の開発は、我が国の薬事行政にとっても極めて意義深いと考えられる。

F. 健康危険情報

特記事項なし

G. 研究発表

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H. 知的財産の出願・登録状況(予定を含む)

特記事項なし

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学会等発表実績

様式第１９

学　会　等　発　表　実　績

委託業務題目「　口腔がんに対する磁性抗がん治療薬の実用化に関する研究　　」
公立大学法人横浜市立大学

１．学会等における口頭・ポスター発表

口頭

発表した成果 （発表題目）	発表者氏名	発表した場所 （学会等名）	発表した時期	国内・外の別
進行口腔癌に対する逆公行性超選択的動注化学放射線療法は頸部リンパ節転移に有効か?	光藤健司，小泉敏之，飯田昌樹，大屋貴志，岩井俊憲，中島英行，小栗千里，來生　知，廣田　誠，藤内　祝	第38回日本頭頸部癌学会，東京	2014	■国内 □国外
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Extracellular matrix remodeling is critical for closure of the ductus arteriosus.	Minamisawa S, Yokoyama U	The 91th Annual Meeting of the Physiological Society of Japan, Kagoshima	2014	■国内 □国外
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口腔内病変の切除マージンを正確に決定するための口腔内メジャーの使用経験	小栗千里，岩井俊憲，松井義郎，中島英行，廣田　誠，來生　知，光藤健司，藤内　祝	第38回日本頭頸部癌学会，東京	2014	■国内 □国外
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Prostaglandin E2 receptor EP4 signaling in vascular smooth muscle cells decreases aortic elasticity.	Ichikawa Y, Yokoyama U, Ishikawa Y	Experimental Biology Annual Meeting, San Diego, USA	2014	□国内 ■国外
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A novel treatment for triple-negative breast cancer using intrinsic magnetized paclitaxel.	Masanari Umemura, Ayako Makino, Itaru Sato, Xianfeng Feng, Maki Iwai, Kayoko Ito, Akiyoshi Miyajima, Makoto Otake, Akane Nagasako, Kosuke Matsuo, Haruki Eguchi, and Yoshihiro Ishikawa	The 105th American Association for Cancer Research Annual meeting, San Diego, USA	2014	□国内 ■国外
A novel paclitaxel with intrinsic magnetism.	Ayako Makino, Masanari Umemura, Itaru Sato, Kayoko Oda, Makoto Otake, Akane Nagasako, Haruki Eguchi, Yoshihiro Ishikawa	第73回日本癌学会学術総会，横浜	2014	■国内 □国外
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Vidarabine, an anti-herpesvirus agent, prevents catecholamine-induced atrial fibrillation in mice.	Sujita K, Fujita T, Cai W, Hidaka Y, Jin H, Jin M, Okumura S, Ishikawa Y	ESC (European society of cardiorogy) Congress, Barcelona, Spain	2014	□国内 ■国外
EPAC1 plays an important role in regulation of renin and AQP2 expression in the kidney of mice.	Jin H, Fujita T, Cai W, Hidaka Y, Sagara S, Kubota K, Suita K, Ishikawa Y	ESC (European society of cardiorogy) Congress, Barcelona, Spain	2014	□国内 ■国外
A novel treatment for triple-negative breast cancer using intrinsic magnetized paclitaxel.	Masanari Umemura, Ayako Makino, Itaru Sato, Xianfeng Feng, Maki Iwai, Kayoko Ito, Akiyoshi Miyajima, Makoto Otake, Akane Nagasako, Kousuke Matsuo, Haruki Eguchi and Yoshihiro Ishikawa	AACR Annual Meeting 2014, San Diego, USA	2014	□国内 ■国外
Development of thermochemothrapy using cisplatin and ferucarbotran (Resovist) in head and neck cancer.	Itaru Sato, Masanari Umemura, Kenji Mitsudo, Xianfeng Feng, Hideyuki Nakashima, Mitomu Kioi, Haruki Eguchi, Iwai Tohnai and Yoshihiro Ishikawa	AACR Annual Meeting 2014, San Diego, USA	2014	□国内 ■国外
A novel paclitaxel with intrinsic magnetism.	Ayako Makino, Masanari Umemura, Itaru Sato, Kayoko Oda, Makoto Otake, Akane Nagasako, Haruki Eguchi and Yoshihiro Ishikawa	第73回日本癌学会学術総会、横浜	2014	■国内 □国外

A novel methotrexate derivative with intrinsic magnetism.	Mayumi Katsumata, Masanari Umemura, Itaru Sato, Makoto Ohtake, Kayoko Oda, Akane Nagasako, Ayako Makino, Haruki Aoyama, Haruki Eguchi and Yoshihiro Ishikawa	第92回日本生理学会大会、神戸	2015	■国内 □国外
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発表論文

掲載した論文 (発表題目)	発表者氏名	発表した場所 (学会誌・雑誌等名)	発表した時期	国内・外の別
Prostaglandin E2 inhibits elastogenesis in the ductus arteriosus via EP4 signaling.	Yokoyama U, Minamisawa S, Shioda A, Ishiwata R, Jin MH, Masuda M, Asou T, Sugimoto Y, Aoki H, Nakamura T, and Ishikawa Y	Circulation 129:487-96	2014	□国内 ■国外
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研究成果の刊行物・別刷

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

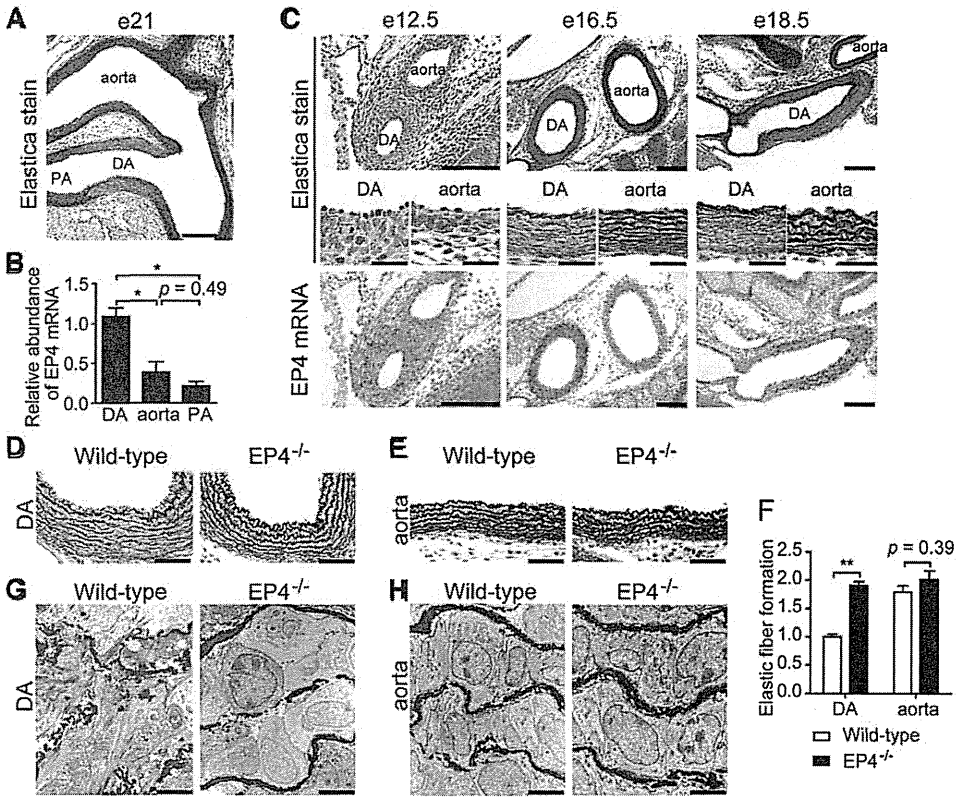

Figure 1. EP4 signaling attenuated elastic fiber formation in vivo. **A**, Elastica van Gieson stain (elastica stain) of rat fetus on day 21 of gestation (e21). **B**, Expression of EP4 mRNA of the rat ductus arteriosus (DA), aorta, and pulmonary artery (PA) on day 21 of gestation. n=6. **C**, Developmental 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%

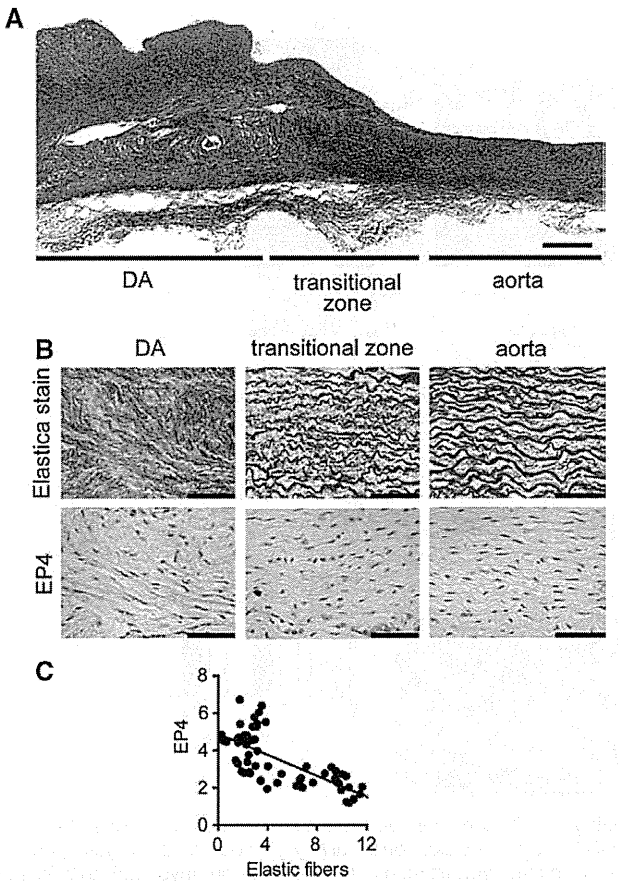

Figure 2. Human vascular tissues show a negative correlation between elastic fibers and EP4 expression. **A**, A representative image of the elastica stain of a human neonatal vessel. **B**, Elastica stain and immunohistochemistry for EP4 in human neonatal vessels. **C**, Representative results of quantification 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

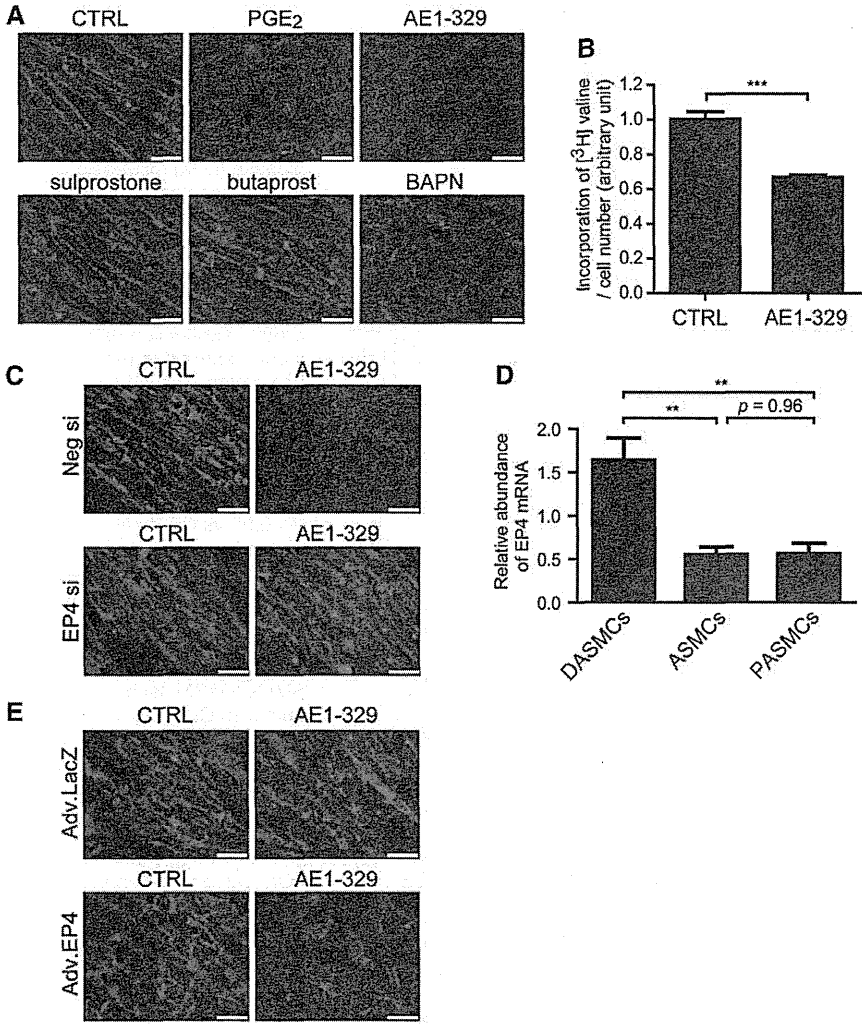

Figure 3. EP4 signaling attenuated elastic fiber formation in vitro. **A** and **C**, Immunostaining for elastin (red) and DNA (blue) of ductus arteriosus smooth muscle cells (DASMCs) treated with each drug indicated or EP4-targeted siRNA (EP4 si). AE1-329 indicates ONO-AE1-329; CTRL, control; and Neg 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.