Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Fibulin-1 Integrates Subendothelial Extracellular Matrices and Contributes to Anatomical Closure of the Ductus Arteriosus

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OBJECTIVE: The ductus arteriosus (DA) is a fetal artery connecting the aorta and pulmonary arteries. Progressive matrix remodeling, that is, intimal thickening (IT), occurs in the subendothelial region of DA to bring anatomic DA closure. IT is comprised of multiple ECMs (extracellular matrices) and migrated smooth muscle cells (SMCs). Because glycoprotein fibulin-1 binds to multiple ECMs and regulates morphogenesis during development, we investigated the role of fibulin-1 in DA closure.

APPROACH AND RESULTS: Fibulin-1–deficient (*Fbln1*$^{-/-}$) mice exhibited patent DA with hypoplastic IT. An unbiased transcriptome analysis revealed that EP4 (prostaglandin E receptor 4) stimulation markedly increased fibulin-1 in DA-SMCs via phospholipase C-NFκB (nuclear factor κB) signaling pathways. Fluorescence-activated cell sorting (FACS) analysis demonstrated that fibulin-1 binding protein versican was derived from DA-endothelial cells (ECs). We examined the effect of fibulin-1 on directional migration toward ECs in association with versican by using cocultured DA-SMCs and ECs. EP4 stimulation promoted directional DA-SMC migration toward ECs, which was attenuated by either silencing fibulin-1 or versican. Immunofluorescence demonstrated that fibulin-1 and versican V0/V1 were coexpressed at the IT of wild-type DA, whereas 30% of versican-deleted mice lacking a hyaluronan binding site displayed patent DA. Fibulin-1 expression was attenuated in the EP4-deficient mouse (*Ptger4*$^{-/-}$) DA, which exhibits patent DA with hypoplastic IT, and fibulin-1 protein administration restored IT formation. In human DA, fibulin-1 and versican were abundantly expressed in SMCs and ECs, respectively.

CONCLUSIONS: Fibulin-1 contributes to DA closure by forming an environment favoring directional SMC migration toward the subendothelial region, at least, in part, in combination with EC-derived versican and its binding partner hyaluronan.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: ductus arteriosus ■ extracellular matrix ■ fibulin ■ mice ■ neointima ■ vascular remodeling ■ versicans

The ductus arteriosus (DA), which bypasses the aorta and pulmonary arteries, is required for fetal circulation and closes after birth to adapt to neonatal circulation.[1] However, it has been reported that the DA failed to close in 67% of low-birth-weight infants, and medical treatment with COX (cyclooxygenase) inhibitors or surgical ligation was performed in such cases.[2] Patent DA (PDA) increases the mortality risk of premature infants.[3,4] Since the mid-1970s, COX inhibitors targeting smooth muscle cell (SMC) contraction represent the only pharmacological treatment for PDA, but >30% of these patients are still resistant to COX inhibitor therapy.[2] COX inhibitors have serious side effects on premature infants such

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For Sources of Funding and Disclosures, see page 2224.

Nonstandard Abbreviations and Acronyms

COX	cyclooxygenase
DA	ductus arteriosus
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
Epac	exchange protein activated by a cAMP
FACS	fluorescence-activated cell sorting
GFP	green fluorescence protein
HA	hyaluronic acid
IKK	IκB kinase
IT	intimal thickening
NFκB	nuclear factor κB
PC	phospholipase C
PC-PLC	phosphatidylcholine-specific phospholipase C
PDA	patent ductus arteriosus
PGE_2	prostaglandin E2
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
SMC	smooth muscle cell

Highlights

- Fibulin-1 deficient mice exhibited patent ductus arteriosus with poorly formed intimal thickening.
- EP4 (prostaglandin E receptor 4) stimulation increased Fibulin-1 production via PLC (phospholipase C)-PKC (protein kinase C)-noncanonical NFκB (nuclear factor κB) signaling pathway in ductus arteriosus-smooth muscle cells.
- Smooth muscle cell-derived fibulin-1 integrates ECMs (extracellular matrices) at the subendothelial region and promotes directional smooth muscle cell migration toward the internal lumen.
- Administration of fibulin-1 recombinant protein restored intimal thickening formation of ductus arteriosus in EP4-deficient mice.
- Fibulin-1 was abundantly expressed in smooth muscle cells of human ductus arteriosus tissues.

as renal failure and neonatal necrotizing enterocolitis.[5] Thus, COX inhibitors are not always effective and safe for premature infants.

In addition to SMC contraction induced by an increase in blood oxygen tension at birth, intimal thickening (IT) formation, which is the portion of the vessel wall lying internal to the internal elastic laminae and projecting into the lumen, is required to achieve anatomic closure of the DA.[6] In humans, IT begins to occur during the mid-to-late gestational periods.[1] Toda et al[7] performed an electron microscopy study on 25 human DA specimens from at least 15 weeks' gestation and found that DA-SMCs in the tunica media migrated into the subendothelial region through fragmented elastic laminae during the fetal periods. These histological changes are also observed in other mammals, including sheep, dogs, rats, and mice.[1,8,9] Hence, IT formation is a common characteristic feature in mammalian DAs.

Accumulation of a variety of ECMs (extracellular matrices) at the subendothelial region and directional migration of SMCs toward endothelial cells (ECs) are important processes for forming IT in the DA.[1,10] Hyaluronan is deposited in the area around the DA[10] and promotes DA-SMC migration.[9,11] Abundant chondroitin sulfate is present in the subendothelial region,[12] and positive immunostaining for fibronectin is also detected in the DA.[12] Each ECM may provide environments favoring cell migration, but the molecular mechanisms by which multiple ECMs are integrated at the subendothelial region and by which subsequent directional SMC migration occurs in the DA remain unknown.

The glycoprotein fibulin-1 is expressed in the developing vessels[13] and binds to chondroitin sulfate,[14] fibronectin,[15] and other ECMs.[16,17] Although the biological consequence of these fibulin-1-ECM interactions are not yet understood, it has been reported that fibulin-1 regulates directed cell migration during development.[18,19] In addition, the DA is largely derived from neural crest cells,[20] and fibulin-1 is reported to influence neural crest cells.[18] Based on these findings, we hypothesized that fibulin-1 forms an intermolecular bridge with EC- and SMC-derived ECM at the subendothelial region, thus providing an appropriate environment favoring directional SMC migration and colonization for SMCs to promote IT formation, which leads to anatomic closure of the DA.

METHODS

The authors declare that all supporting data are available within the article and its Data Supplement.

Animals

We used Wister rat fetuses from pregnant mothers on gestational day 21 (SLC, Inc, Shizuoka, Japan). Generation and phenotypes of EP4 (prostaglandin E receptor 4)-deficient (*Ptger4*$^{-/-}$) mice have been described previously.[21] These mice were kindly provided by Drs. Narumiya and Furuyashiki (Kyoto University School of Medicine and Kobe University School of Medicine, respectively). Neonatal mice (*Ptger4*$^{-/-}$, *Ptger4*$^{+/+}$) were obtained by crossing heterozygous C57BL/6 N background littermates (*Ptger4*$^{+/-}$). The generation of fibulin-1–deficient (*Fbln1*$^{-/-}$; 129P2/OlaHsd and C57BL/6J mixed background) and *Vcan*$^{\Delta 3/\Delta 3}$ (C57BL/6J background) mice was described previously.[18,22] *Fbln1*$^{-/-}$ and *Fbln1*$^{+/+}$ neonates were obtained by crossing heterozygous *Fbln1*$^{+/-}$ littermates. All animals were cared for in compliance with the guiding principles of the American Physiological Society.

DNA Microarray

Rat DA-SMCs were stimulated with ONO-AE1-329 (1 μmol/L, n=4) or were not stimulated (n=5) for 24 hours. RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. Total RNA was donated to the DNA microarray assay using the Gene 1.0 ST Array System (RaGene-1_0-st-v1; Affymetrix, Santa Clara, CA). Of the 27 342 genes on the microarray, we analyzed their differential expression between ONO-AE1-329 and control. For analysis, we selected the genes that had more than a 3.0-fold change (≥3.0; Table I in the Data Supplement).

Fluorescence-Activated Cell Sorting

DA-endothelial cells (DA-ECs) were isolated by fluorescence-activated cell sorting (FACS).[23] DA tissues from ≈30 rat fetuses on gestational day 21 were subjected to FACS to obtain one sample. We obtained 6 samples for quantitative reverse transcription polymerase chain reaction. Dispersed cells by collagenase enzyme mixture were subjected to FACS analysis. Fluorescein isothiocyanate (FITC)–conjugated anti-CD31 antibody (Abcam, Cambridge, United Kingdom) and APC/Cy7–conjugated anti-CD45 antibody (BioLegend, San Diego, MA) were used as cell surface markers for ECs and hematopoietic derivation cells, respectively. $CD31^+/CD45^-$ and $CD31^-/CD45^-$ were defined as DA-ECs and DA-non-ECs. This analysis was conducted using BD FACS AriaII (Becton Dickinson, San Jose, CA).

SMC Migration Assay (Silicon Culture-Insert Method)

To investigate the contribution of ECs to rat DA-SMC migration, we performed a migration assay using culture-inserts made of silicon (Ibidi, Munich, Germany). Rat DA-SMCs and EA. hy926 cells were separately seeded in this insert on a glass slide. The gap between SMCs and ECs was 500 μm. To identify ECs during the migration assay, EA. hy926 cells were infected with lentiviral vector particles containing GFP (green fluorescence protein; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) or treated with PKH67 Green Fluorescent Cell Linker Midi Kit for General Cell Membrane (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions.

Cells were kept in FBS free overnight, and rat DA-SMCs were incubated with or without ONO-AE1-329 (1 μmol/L) for 48 hours before removal of the insert. When cells were transfected with siRNAs (small interfering RNAs), transfection was performed before ONO-AE1-329 (or fibulin-1C/1D recombinant proteins, 1.10 μg/mL) treatment for 24 hours. After the removal of the insert, the culture medium was exchanged with or without ONO-AE1-329 (1 μmol/L). Then, the glass slide was placed in the temperature-controlled incubator (5% CO_2 at 37°C), and the path areas of rat DA-SMCs were measured by time-lapse microscopy. Images were recorded at 1-hour intervals for up to 96 hours. In rescue experiments, fibulin-1D recombinant proteins (1.10 μg/mL) were added to medium at the removal of the insert.

Amounts of fibulin-1C and fibulin-1D recombinant proteins used in a migration assay and organ culture were determined with reference to the amount of endogenous fibulin-1 proteins secreted by 72-hour EP4 stimulation in DA-SMCs (2×10^6 cells).

Tissue Staining

Paraffin-embedded blocks containing DA tissues were analyzed. The structural characterization of the elastic fiber was evaluated by elastica van Gieson staining.

Immunohistochemistry staining was conducted as described previously.[24] Briefly, mouse and human fibulin-1 antigen were activated by trypsin and citric acid, respectively. Immunohistochemistry for the human versican glycosaminoglycan β (GAGβ) domain was performed without antigen activation. To detect versican V0 and V1 core protein, samples were treated with chondroitinase ABC (0.5 U/mL diluted in 60 mmol/L sodium acetate and 50 mmol/L Tris-HCl pH 8.0 buffer) for 1 hour at 37°C before incubation with a primary antibody. Tissue sections were incubated with anti-fibulin-1 antibody (1:200 dilution [mouse], 1:2000 dilution [human]) and anti-versican GAGβ antibody (1:500 dilution). After overnight incubation at 4°C, slides were incubated with streptavidin peroxidase at room temperature for 30 min and visualized by diaminobenzidine chromogen substrate solution, following counterstaining with Mayer hematoxylin.

Immunofluorescent staining was conducted as described previously.[25] All antigens were activated by EDTA for immunofluorescent. Tissue sections were incubated with anti-fibulin-1 and anti-versican GAGβ domain antibodies (1:100 dilution). Chondroitinase ABC treatment was also performed before incubation with a primary antibody. After a 48-hour incubation at 4°C, tissues were incubated with Alexa Flour 488 anti-goat IgG and Alexa Flour 594 anti-rabbit IgG (Invitrogen, Carlsbad, CA) for 1 hour. DNA was stained using Hoechst 33342 solution (Invitrogen, Carlsbad, CA). Images were obtained by FV1000 confocal laser microscope (Olympus, Tokyo, Japan).

Scoring of IT

We defined the degree of IT of the DA as follows: grade 0 (score 0), no SMCs having migrated into the inner space of the internal elastic lamina; grade 1 (score 1), a single layer of SMCs having migrated into the inner space of the internal elastic lamina; and grade 2 (score 2), ≥2 layers of SMCs having migrated into the inner space of the internal elastic lamina. Each elastica van Gieson–stained DA tissue was evenly divided into 4 parts, and an individual score was determined for all four parts in a blinded manner. The total score of each DA was the sum of the scores for each of the parts (maximum score, 8).

Organ Culture

Arteries of preterm *Ptger4*$^{-/-}$ neonates (day 0), including the DA, aorta, and pulmonary trunk were removed from the thoracic cavity. Adhesive connective tissues surrounding arteries and lung tissues were carefully removed. DA tissues were incubated with ONO-AE1-329 (1 μmol/L) for 72 hours in DMEM containing 0.5% FBS. To investigate the effect of fibulin-1 on IT formation, fibulin-1D recombinant protein (1.10 μg/mL) was added to the medium, and the medium was refreshed at 24-hour intervals. Tissues were incubated in the incubator at 37°C in 5% CO_2-95% ambient mixed air. After a 72-hour incubation, tissues were fixed in 10% buffered formalin and embedded in paraffin. The sectioned segments in the middle portion of the DA were analyzed. Elastica van Gieson–stained DA tissues were scored to evaluate the degree of IT.

Human Samples

Human samples resected from patients with congenital heart disease were obtained from Yokohama City University Hospital and Kanagawa Children's Medical Center at the time of operation (Table II in the Data Supplement).

Statistics

The data were expressed as the mean±SEM of independent experiments. A Kruskal-Wallis test followed by Fisher least significant difference post hoc test was used to generate a multiple group comparison. A Mann-Whitney *U* test was used when conducting a comparison between 2 groups. A value of $P<0.05$ was considered significant.

Study Approval

Animal study was conducted with the approval of the Institutional Animal Care and Use Committees of the Yokohama City University School of Medicine (Reference number: F-A-16-010). The study using human samples was conducted with the approval of the human subject committees of the Yokohama City University School of Medicine (reference number: B150305001) and Kanagawa Children's Medical Center (reference number: 1502-05). Written informed consent was obtained.

RESULTS

Patency of the DA in Neonatal Fbln1$^{-/-}$ Mice

To investigate the role of fibulin-1 in DA closure, we obtained *Fbln1*$^{-/-}$ neonates[18] from at least 6 hours after birth because mouse DA generally closes within 4 hours. The DA remained open in all *Fbln1*$^{-/-}$ mice among the 7 mice from 3 different littermates examined, while all wild-type DAs were closed (Figure 1A). Abundant expression of fibulin-1 was observed in the tunica media of wild-type DAs but was not present in *Fbln1*$^{-/-}$ mice (Figure 1A). The formation of IT, in which SMCs migrated across the internal elastic laminae to internal lumen, was significantly less developed in *Fbln1*$^{-/-}$ mice compared with wild-type mice (Figure 1B). Among 4 fibulin-1 variants (fibulin-1A/B/C/D), fibulin-1C and -1D are commonly expressed in humans, mice, and rats.[26] Preferential expression of total fibulin-1 mRNAs (fibulin-1C and -1D) was observed in the rat DAs compared with the aorta (Figure 1C). These results suggested that fibulin-1 in DA-SMCs contributes to IT formation and subsequent DA anatomic closure.

Fibulin-1 Expression Was Increased by Prostaglandin E_2-EP4 Signaling Pathways

Mice deficient in PGE_2 (prostaglandin E_2) receptor EP4 are known to die shortly after birth and display PDA with poor IT formation.[9,21,27] We then investigated whether PGE_2-EP4 signaling regulated fibulin-1 expression. A DNA microarray analysis using rat DA-SMCs revealed that the most upregulated gene by the EP4 agonist ONO-AE1-329 was *Fbln1* (Table I in the Data Supplement). Quantitative real-time polymerase chain reaction showed that PGE_2 and ONO-AE1-329 markedly increased total fibulin-1 mRNA expression in DA-SMCs, which was inhibited by the EP4 antagonist ONO-AE3-208 (Figure 2A). An EP1/3 agonist and an EP2 agonist did not affect fibulin-1 expression (Figure 2A). The EP4-mediated increase in fibulin-1 mRNAs in aortic SMCs, which have lower EP4 expression, was much smaller than that in DA-SMCs[9] (Figure 2B). Similar effects of these stimulations on fibulin-1 mRNA were observed in protein levels of fibulin-1 (Figure 2C and 2D). Dose- and time-dependency of EP4-induced fibulin-1 upregulation was observed in mRNAs and proteins in DA-SMCs (Figure 2E through 2H). The administration of actinomycin D inhibited the EP4 agonist–mediated increase in fibulin-1 mRNA expression (Figure 2I). These data suggested that fibulin-1 was transcriptionally upregulated by PGE_2-EP4 signaling. The robust increase in fibulin-1 expression likely results from especially high levels of EP4 in DA-SMCs.

Phospholipase C–PKC–Noncanonical Nuclear Factor κB Signaling Pathway Plays a Primary Role in the EP4-Mediated Increase in Fibulin-1

EP4 belongs to the G-protein–coupled receptors, and cAMP is the major second messenger of EP4.[28] However, the nonselective, PKA (protein kinase A)-selective, or exchange protein activated by a cAMP (Epac [exchange protein activated by a cAMP])-selective cAMP analogs did not increase fibulin-1 expression (Figure 3A). The EP4-mediated increase in fibulin-1 mRNA was significantly attenuated by inhibition of the PLC (phospholipase C) by U73122 or D609, the PKC (protein kinase C) inhibitors GO6983 or Calphostin C, or the NFκB (nuclear factor κB) inhibitors IKK (IκB kinase) 16 or SN50 (Figure 3B). Similar effects of these inhibitors were observed in fibulin-1 protein levels (Figure 3C and 3D). EP4 stimulation promoted p100 degradation but not IκBα (nuclear factor kappa-B inhibitor, alpha) degradation in the cytosolic fraction of DA-SMCs (Figure 3E through 3G). These results indicated that the PLC-PKC–noncanonical NFκB signaling pathway plays a primary role in the EP4-mediated increase in fibulin-1. Expression levels of PLC isoforms did not differ between rat DA-SMCs and aortic SMCs (Figure I in the Data Supplement).

Fibulin-1 Promoted DA-SMC Migration

SMC migration into the subendothelial region is critical in IT formation. Based on the histology of *Fbln1*$^{-/-}$ DAs, we hypothesized that fibulin-1 promoted SMC migration. EP4 stimulation promoted DA-SMC migration, and this

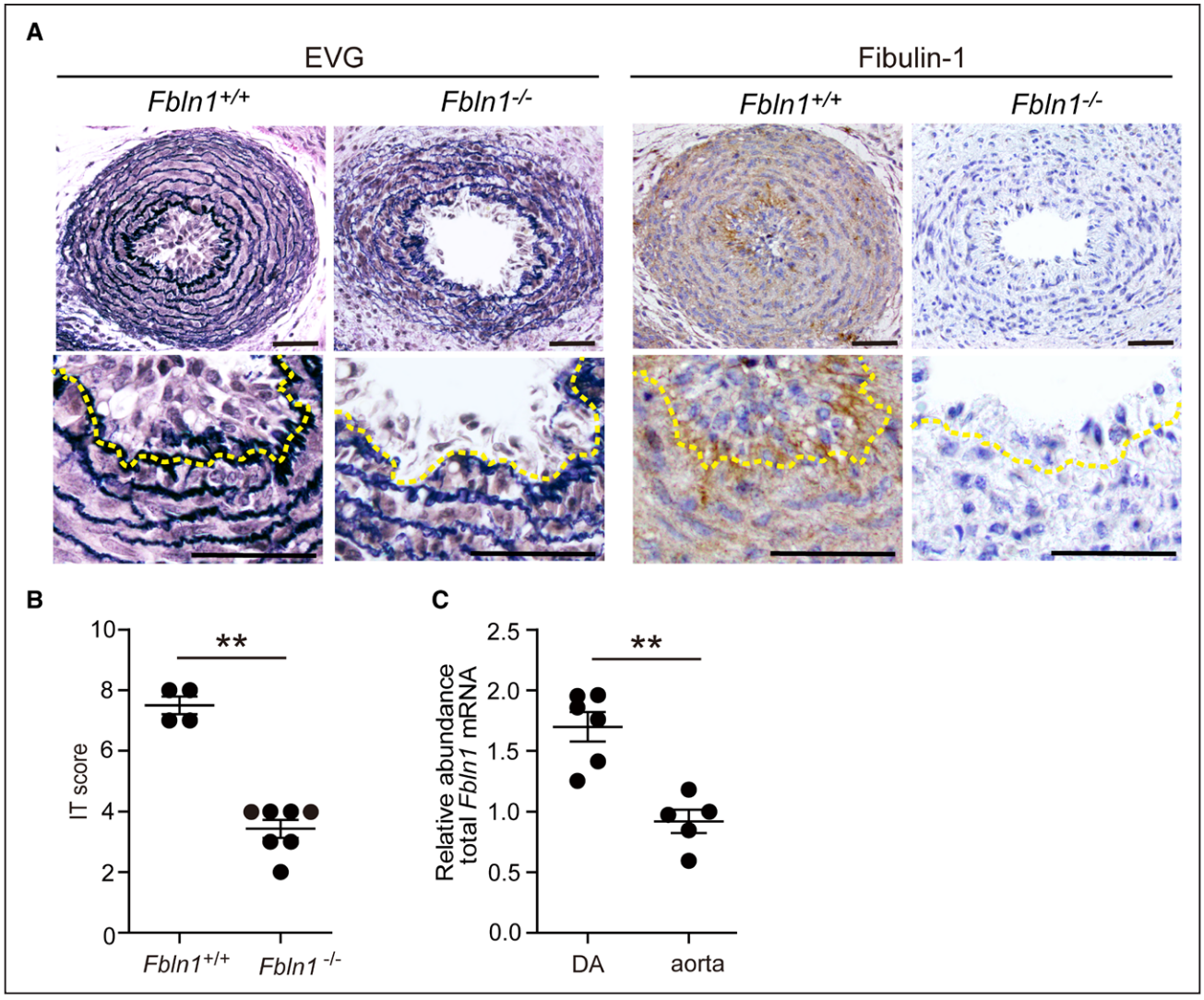

Figure 1. Patency of the ductus arteriosus (DA) in neonatal Fbln1$^{-/-}$ mice.
A, Elastica van Gieson (EVG) staining and immunohistochemistry for fibulin-1 in the DA tissues from *Fbln1*$^{+/+}$ and *Fbln1*$^{-/-}$ neonates (day 0). Dotted yellow lines indicate internal elastic lamina. Scale bars, 50 μm. **B**, Scoring of the degree of intimal thickening (IT) in the DAs of *Fbln1*$^{+/+}$ and *Fbln1*$^{-/-}$ mice. n=4–7, $^{**}P<0.01$. Data are presented as mean±SEM. **C**, Total fibulin-1 (fibulin-1C and -1D) mRNA expression in the DA and aortic tissues of rats on gestational day 21. n=5–6, $^{**}P<0.01$. Data are presented as mean±SEM.

effect was significantly attenuated by 2 kinds of fibulin-1–targeted siRNAs, in which both fibulin-1–targeted siRNAs significantly decreased the expression levels of fibulin-1 mRNAs and proteins but not EP4 mRNAs (Figure IIA through IID in the Data Supplement). These data suggested that fibulin-1 per se promoted DA-SMC migration.

Fibulin-1 Promoted Directional SMC Migration Toward ECs in Combination With Versican

To examine whether fibulin-1 contributed to the directional SMC migration toward ECs, we performed a coculture of SMCs and ECs in which DA-SMCs and ECs were seeded with a gap of 500 μm (Figure 4A). DA-SMCs cocultured with ECs rarely migrated to the EC region under basal conditions (Figure 4B). However, when ONO-AE1-329–stimulated DA-SMCs were cocultured with ECs, DA-SMCs markedly migrated toward ECs (Figure 4B and 4C, Movies I and II in the Data Supplement). EP4 stimulation increased expression levels of both fibulin-1C and-1D mRNAs to a similar extent in DA-SMCs (Figure IIE and IIF in the Data Supplement). Recombinant proteins of fibulin-1C or -1D promoted DA-SMC migration towards ECs even under the absence of EP4 stimulation, and fibulin-1D–induced migration was greater than that caused by fibulin-1C (Figure 4D). These data suggested that both SMC-derived fibulin-1 and EC-derived factor(s) were involved in directional SMC migration.

Versican is one of the binding partners of fibulin-1 and has the ability to promote cell migration.[29] Next, we obtained rat DA-ECs and non-EC cells that were mostly composed of SMCs using FACS analysis of rat

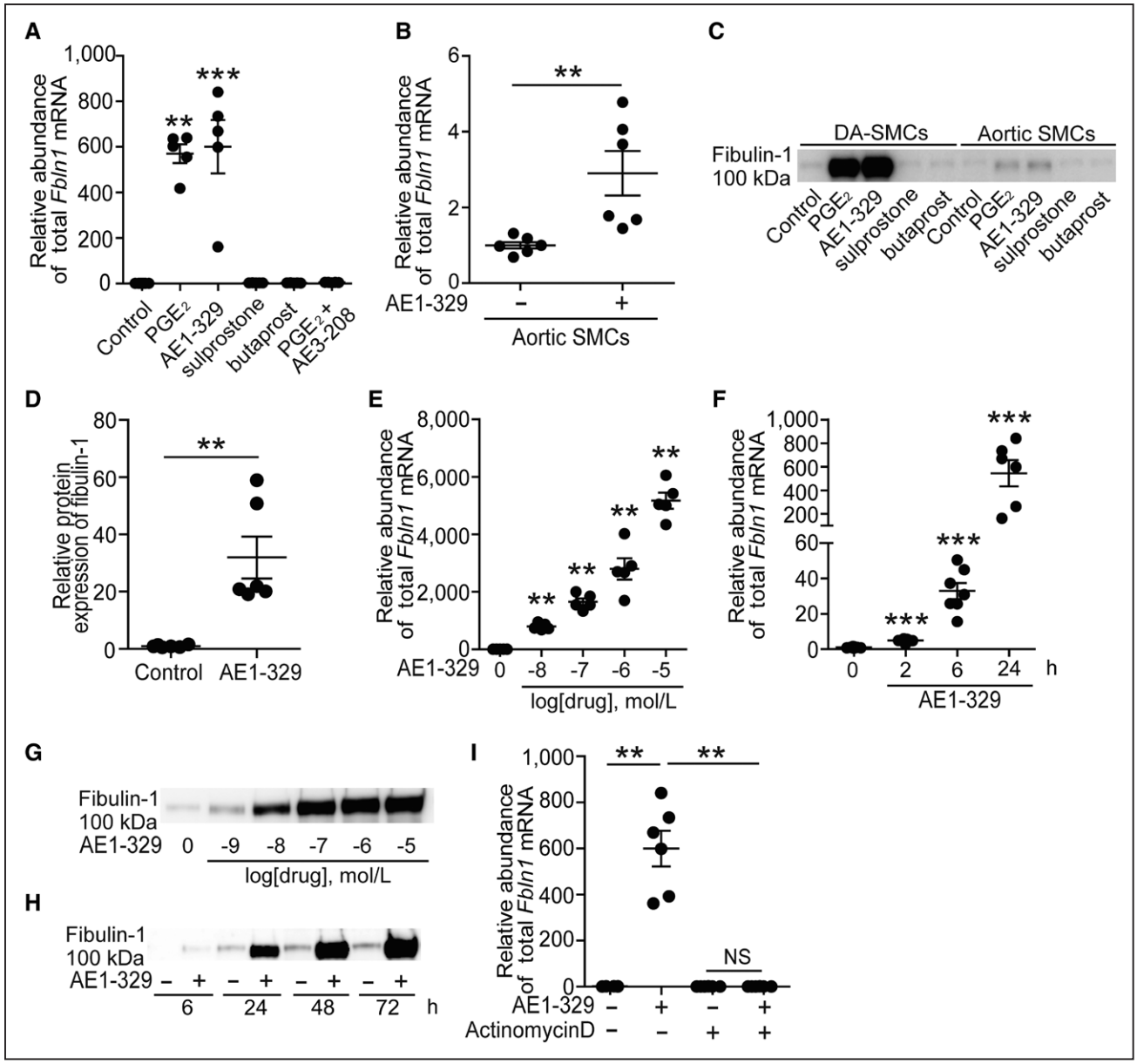

Figure 2. Fibulin-1 expression was increased by PGE2 (prostaglandin E2)-EP4 (prostaglandin E receptor 4) signaling pathways. **A**, Expression of total fibulin-1mRNA in rat ductus arteriosus (DA)–smooth muscle cell (SMCs) treated for 24 h by PGE_2 (1 μmol/L), ONO-AE1-329 (AE1-329: EP4 agonist, 1 μmol/L), ONO-AE3-208 (AE3-208: EP4 antagonist, 1 μmol/L), sulprostone (EP1/3 agonist, 1 μmol/L), or butaprost (EP2 agonist, 1 μmol/L). n=5–9, $^{**}P<0.01$, $^{***}P<0.001$ vs control. Data are presented as mean±SEM. **B**, Relative expression of total fibulin-1 (fibulin-1C and-1D) mRNA in aortic SMCs stimulated with ONO-AE1-329 (AE1-329, 1 μmol/L) for 24 h. n=6, $^{**}P<0.01$. Data are presented as mean±SEM. **C**, Protein expression of fibulin-1 in the supernatant of rat DA-SMCs and aortic SMCs cultured with each drug as indicated for 72 h. The same dose as (**A**) was used for each drug. **D**, Quantification of **C**. n=6, $^{**}P<0.01$. Data are presented as mean±SEM. **E**, Dose-dependent total fibulin-1 mRNA expression after 24 h stimulation with ONO-AE1-329. n=5, $^{**}P<0.01$ vs control. Data are presented as mean±SEM. **F**, Time-dependent fibulin-1 mRNA expression after stimulation by ONO-AE1-329 (AE1-329, 1 μmol/L). n=6–9, $^{***}P<0.001$ vs control. Data are presented as mean±SEM. **G**, Dose-dependent fibulin-1 protein production in supernatant of DA-SMCs treated for 72 h by ONO-AE1-329. **H**, Time-dependent fibulin-1 protein production in supernatant of DA-SMCs treated with ONO-AE1-329 (AE1-329, 1 μmol/L). **I**, EP4-induced total fibulin-1 mRNA expression in rat DA-SMCs treated with or without actinomycin D (5 μg/mL) for 24 h. n=6, $^{**}P<0.01$. Data are presented as mean±SEM. NS indicates not significant.

DA tissues and found that versican V0 and V1 mRNAs were expressed primarily in DA-ECs compared with DA-SMCs (Figure 4E and 4F). Similar findings were observed in immunofluorescent staining showing that fibulin-1 was present in DA-SMCs, and versican V0/V1 detected by anti-GAGβ domain antibody was primarily expressed in DA-ECs (Figure IIIA in the Data Supplement). Unlike fibulin-1, there was no difference in versican expression between the rat DA and aorta (Figure IIIB in the Data Supplement).

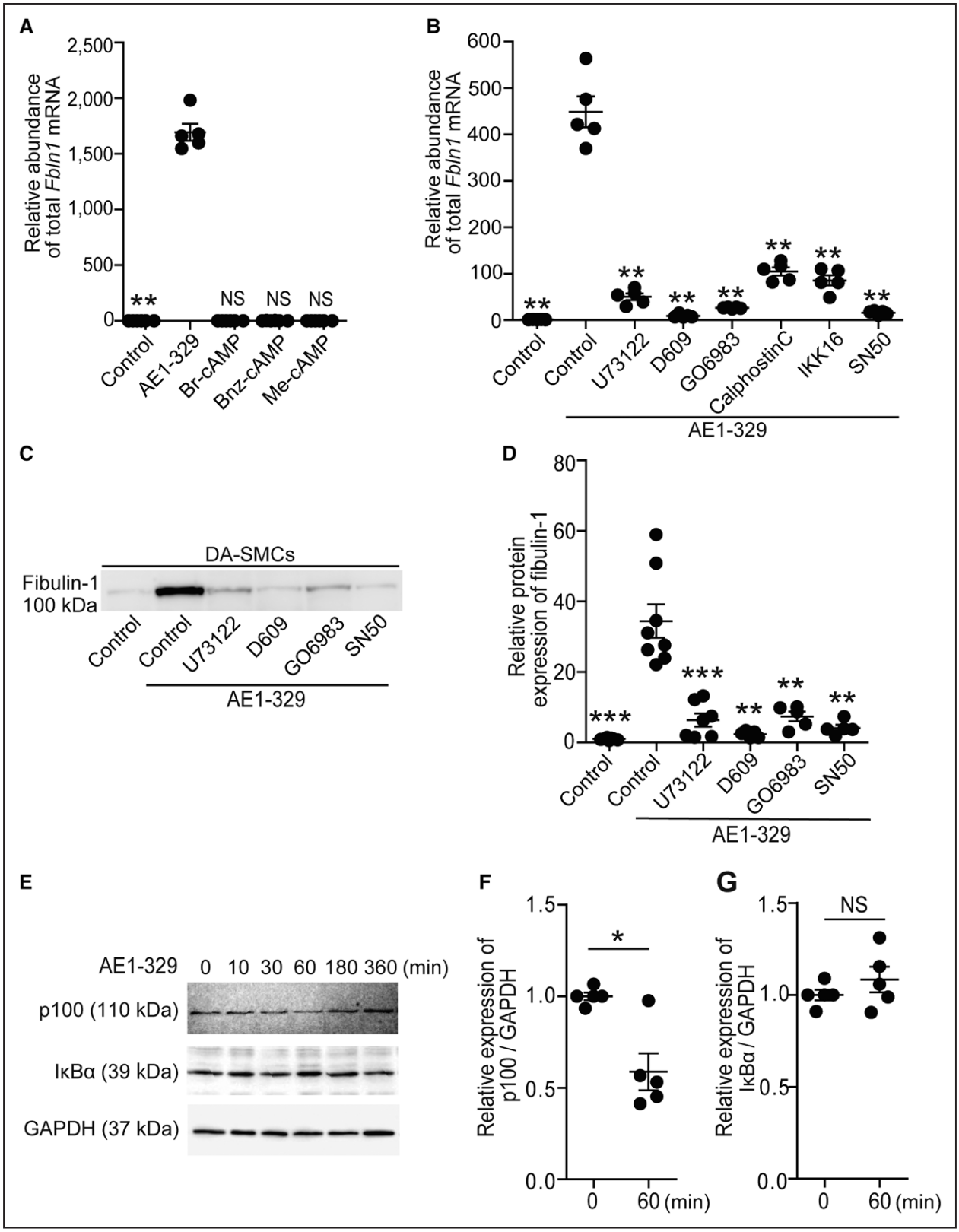

Figure 3. The PLC (phospholipase C)-PKC (protein kinase C)–noncanonical NFκB (nuclear factor κB) signaling pathway plays a primary role in the EP4 (prostaglandin E receptor 4)-mediated increase in fibulin-1.

A, Fibulin-1 mRNA expression in ductus arteriosus (DA)–smooth muscle cells (SMCs) treated for 24 h with ONO-AE1-329 (AE1-329, 1 μmol/L) or cAMP analogs (50 μmol/L): 8-bromo-cAMP (Br-cAMP), nonselective; N6-benzoyladenosine-cAMP (Bnz-cAMP), PKA (protein kinase A)-selective; and 8-p-methoxyphenylthon-2'-O-methyl-cAMP (Me-cAMP), Epac (exchange protein activated by a cAMP)-selective cAMP analogs. n=5–6, $^{**}P<0.01$. Not significant (NS) vs control. Data are presented as mean±SEM. **B**, Fibulin-1 mRNA expression in DA-SMCs after 24-h stimulation by ONO-AE1-329 (AE1-329, 1 μmol/L) with or without PLC inhibitors, (*Continued*)

Based on these expression data, we investigated whether SMC-derived fibulin-1 and EC-derived versican were associated with directional cell migration. EP4 stimulation promoted directional migration of DA-SMCs toward ECs when DA-SMCs and ECs were transfected with negative siRNAs, whereas either silencing of fibulin-1 in DA-SMCs or that of versican in ECs (Figure IIIC in the Data Supplement) attenuated EP4-induced DA-SMC directional migration (Figure 4G and 4H, Movies III through VI in the Data Supplement). Administration of fibulin-1D recombinant proteins increased migration in DA-SMCs treated with *Fbln1*-targeted siRNA (Figure 4G and 4H, Movie VII in the Data Supplement). These data suggest that SMC-derived fibulin-1 was required for directional migration of DA-SMCs toward ECs. EC-derived versican was suggested to be involved in the directional migration.

Fibulin-1-Versican Complex and Versican-Hyaluronic Acid Complex Are Involved in IT Formation of the DA in Mice

Immunofluorescent stain demonstrated that fibulin-1 proteins were expressed in both the tunica media and IT area, and immunoreaction for versican was prominent in the IT area of the wild-type mouse DA (Figure 5A, upper). Fibulin-1 and versican proteins were colocalized in the IT region in the wild-type mouse DA (Figure 5A, upper). As reported previously, *Ptger4*$^{-/-}$ neonatal mice exhibited PDA with poor IT formation. Fibulin-1 protein expression was markedly decreased in *Ptger4*$^{-/-}$ DA (Figure 5A, lower). Administration of fibulin-1D recombinant proteins in the organ culture of *Ptger4*$^{-/-}$ DA significantly promoted IT formation (Figure 5B and 5C).

It has been demonstrated that impaired IT formation in *Ptger4*$^{-/-}$ was, at least partially, attributed to EP4-mediated hyaluronic acid (HA) production in DA-SMCs.[9] We examined the importance of HA in association with versican. *Vcan*$^{\Delta 3/\Delta 3}$ neonatal mice were deleted in the A-subdomain of the versican G1 domain, which is an HA binding site.[22] Three of the 9 DA isolated from *Vcan*$^{\Delta 3/\Delta 3}$ neonatal mice displayed PDA, whereas all 6 of the DA in wild-type mice were closed with prominent IT (Figure 5D). In both *Vcan*$^{\Delta 3/\Delta 3}$ and wild-type DAs, there was no difference in the protein expression of fibulin-1 and versican (Figure 5D). These results suggested that the versican-HA complex partly participates in HA-mediated IT formation in the DA.

Human DA Tissues Show a Similar Distribution to Rodents in Fibulin-1 and Versican

In human DAs isolated from 9 patients (Table II in the Data Supplement), EP4 and fibulin-1 mRNAs were similarly expressed in both the tunica media and IT, whereas preferential expression of versican in IT was observed (Figure 6A through 6C) in a manner similar to rodents. In accordance with the data of transcriptional analysis, immunohistochemistry demonstrated that fibulin-1 proteins were abundantly expressed in the tunica media and IT, and protein expression of versican V0 and V1 seems to be greater in the IT area than in the tunica media in human DA tissues (Figure 6D).

DISCUSSION

The findings of rodent and human SMCs and tissues of the DA in the present study suggest that SMC-derived fibulin-1 plays a major role in forming an intermolecular bridge in the subendothelial region in the DA, at least, in part, in combination with EC-derived chondroitin sulfate versican. Fibulin-1–mediated integration of ECMs serves as an appropriate environment favoring migration and colonization with SMCs derived from inner tunica media, resulting in IT formation and DA closure (Figure IVA in the Data Supplement).

An emerging body of evidence has demonstrated that EC-SMC interaction contributes to vascular remodeling.[30] EC-derived factors, including growth factors, extracellular vesicles, and microRNAs, have been demonstrated to affect SMC migration and proliferation,[30] which are important cellular functions for IT formation. A recent study demonstrated that EC-derived C-X-C motif chemokine 12 and other factors increased forkhead box M1 expression in SMCs, which lead to SMC proliferation and pulmonary hypertension in mice.[31] As shown in these studies regarding vascular remodeling, the effects of EC-derived factors on migration and proliferation of adjacent SMCs have been extensively studied. However, it is largely uncertain how the subendothelial region is expanded to provide an environment favorable for SMC migration during vascular remodeling. Although it is recognized that both ECs and SMCs synthesize and secrete a variety of ECMs and the roles of individual ECM has been investigated,[32] to the best of our knowledge, the molecular mechanisms of integration between multiple ECMs and forming IT have not been clarified. We demonstrate that IT formation was

Figure 3 Continued. U73122 (5 μmol/L) or D609 (50 μmol/L); PKC inhibitors, GO6983 (10 μmol/L) or Calphostin C (0.2 μmol/L); or NFκB inhibitors, IKK (IκB kinase) 16 (3 μmol/L) or SN50 (20 μmol/L). n=5, ***P*<0.01 vs control with ONO-AE1-329. Data are presented as mean±SEM. **C**, Protein expression of fibulin-1 in supernatant of rat DA-SMCs treated for 72 h by ONO-AE1-329 (AE1-329, 1 μmol/L) combined with PLC inhibitors, U73122 (5 μmol/L) or D609 (50 μmol/L); PKC inhibitor, GO6983 (10 μmol/L); or NFκB inhibitor, SN50 (20 μmol/L). **D**, Quantification of (C). n=5-8, ***P*<0.01, ****P*<0.001 vs control with ONO-AE1-329. Data are presented as mean±SEM. **E**, Protein expression of p100, IκBα (nuclear factor kappa-B inhibitor, alpha), and GAPDH at 0–360 min in DA-SMCs stimulated by ONO-AE1-329 (AE1-329, 1 μmol/L). **F** and **G**, Quantification of **E** at 0 min and 60 min. n=5, **P*<0.05. Data are presented as mean±SEM.

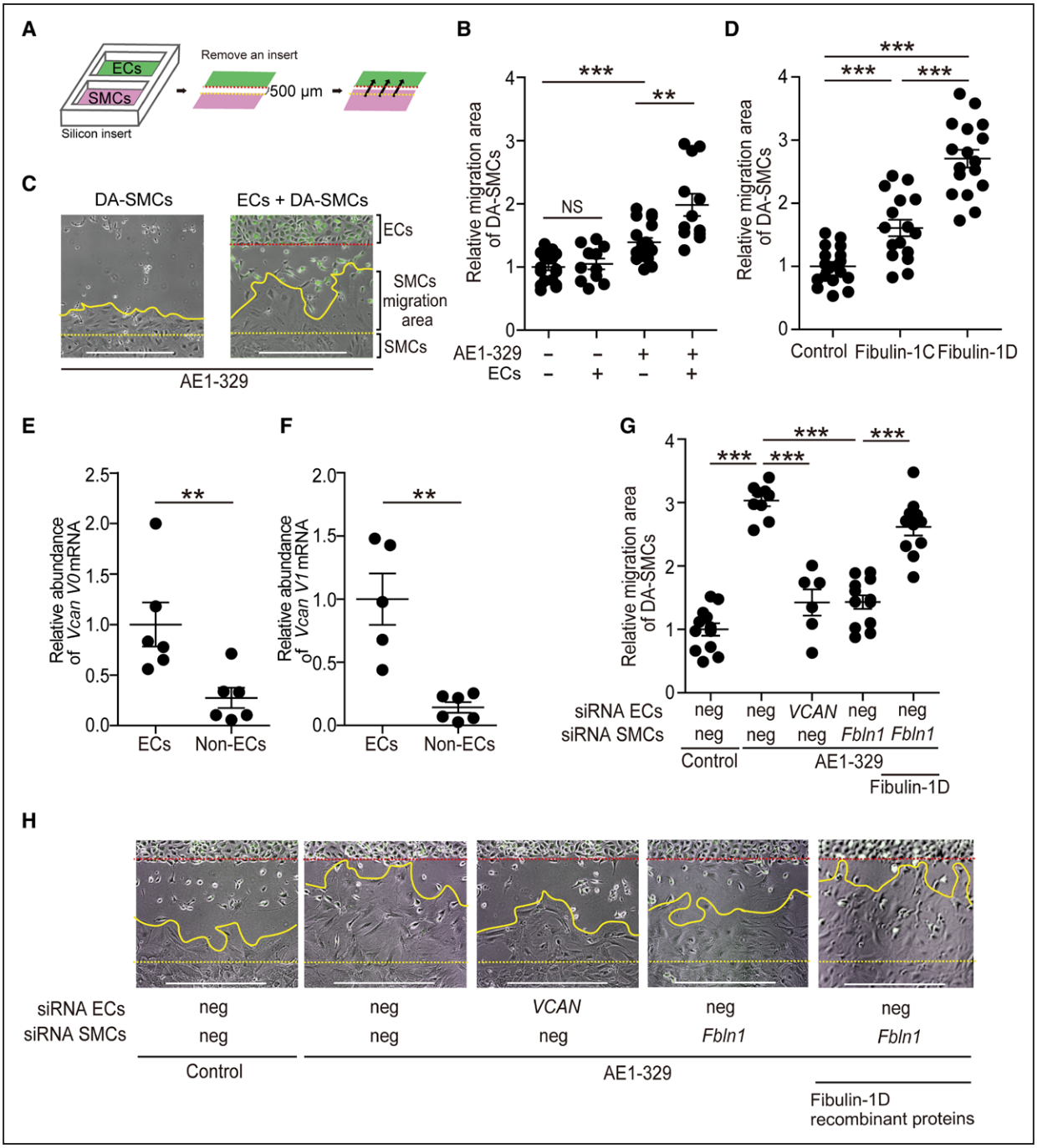

Figure 4. Fibulin-1 in cooperation with versican promoted directional smooth muscle cell (SMC) migration toward endothelial cells (ECs).

A, A method of coculture using a silicon-insert is shown as a schematic representation. EA. hy926 cells (ECs) were labeled with GFP (green fluorescence protein) or PKH67 Green Fluorescent Cell Linker Midi Kit. Migration areas for 96 h were quantified. Dotted red lines, boundary of ECs; dotted yellow lines, start line of ductus arteriosus (DA)-SMCs. **B**, Quantification of DA-SMC migration area. DA-SMCs were pretreated with or without ONO-AE1-329 (AE1-329, 1 μmol/L) before removal of the silicon-insert. DA-SMCs were cultured with or without ECs. n=11–19, $^{**}P<0.01$, $^{***}P<0.001$. Data are presented as mean±SEM. **C**, Representative images of **B**. Dotted red lines, boundary of ECs; dotted yellow lines, start line of DA-SMCs; solid yellow lines, 96-h migrated lines of DA-SMCs. **D**, Quantification of DA-SMC migration area. DA-SMCs were pretreated with fibulin-1–targeted siRNA (small interfering RNA) and cultured with ECs under stimulation of fibulin-1C or fibulin-1D recombinant proteins for 24 h. The amounts of fibulin-1C and fibulin-1D were equal (1.10 μg/mL). n=16–18, $^{***}P<0.001$. Data are presented as mean±SEM. **E** and **F**, Expression of versican V0 and V1 mRNAs in rat DA-ECs and non-ECs that were isolated by fluorescence-activated cell sorting (FACS). n=5–6, $^{**}P<0.01$. Data are presented as mean±SEM. **G**, Migration areas of DA-SMCs treated with or without *Fbln1*-targeted siRNA. EA. hy926 cells (ECs) were treated with or without *VCAN*-targeted siRNA. DA-SMCs were treated with or without ONO-AE1-329 (AE1-329, 1 μmol/L) after siRNA transfection. Fibulin-1D recombinant proteins were added after silencing of *Fbln1* in DA-SMCs. n=6-12, $^{***}P<0.001$. Data are presented as mean±SEM. **H**, Representative images of **G**. Dotted red lines, boundary of ECs; dotted yellow lines, start line of DA-SMCs; and solid yellow lines, 96-h migrated lines of DA-SMCs. Scale bars, 500 μm. Neg indicates negative control siRNA; and NS, not significant.

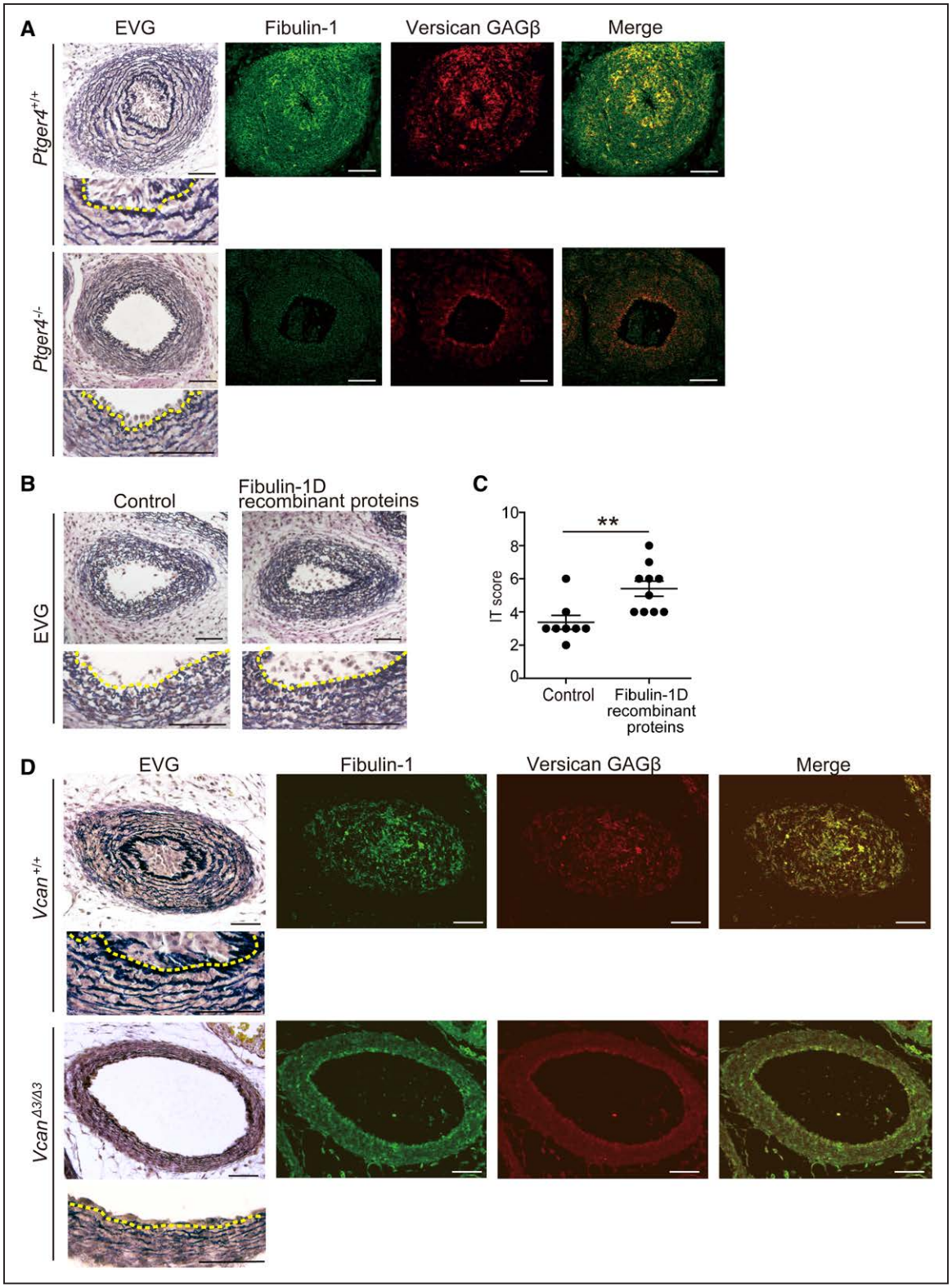

Figure 5. Fibulin-1-versican complex and fibulin-1-versican–hyaluronic acid **complex promote intimal thickening (IT) formation of the** ductus arteriosus **(DA) in mice.**

A, Elastica van Gieson staining (EVG) and immunofluorescent staining for fibulin-1 (green) and versican glycosaminoglycan β (GAGβ) domain (red) in the DA of EP4 (prostaglandin E receptor 4)-deficient (*Ptger4*$^{-/-}$) and wild-type (*Ptger4*$^{+/+}$) neonatal mice (day 0). Dotted yellow lines indicate internal elastic lamina. Scale bars, 50 μm. **B**, Effect of fibulin-1D recombinant proteins (1.10 μg/mL) on IT formation of organ-cultured *Ptger4*$^{-/-}$ DAs. Dotted yellow lines show internal elastic lamina. Scale bars, 50 μm. **C**, IT formation of **B** was evaluated by IT score. n=8-10, $^{**}P<0.01$. Data are presented as mean±SEM. **D**, EVG and immunofluorescent staining for fibulin-1 (green) and versican GAGβ domain (red) in the DAs from versican G1 A-subdomain deleted (*Vcan*$^{\Delta 3/\Delta 3}$) and wild-type (*Vcan*$^{+/+}$) neonatal mice (day 0). Dotted yellow lines indicate internal elastic lamina. Scale bars, 50 μm.

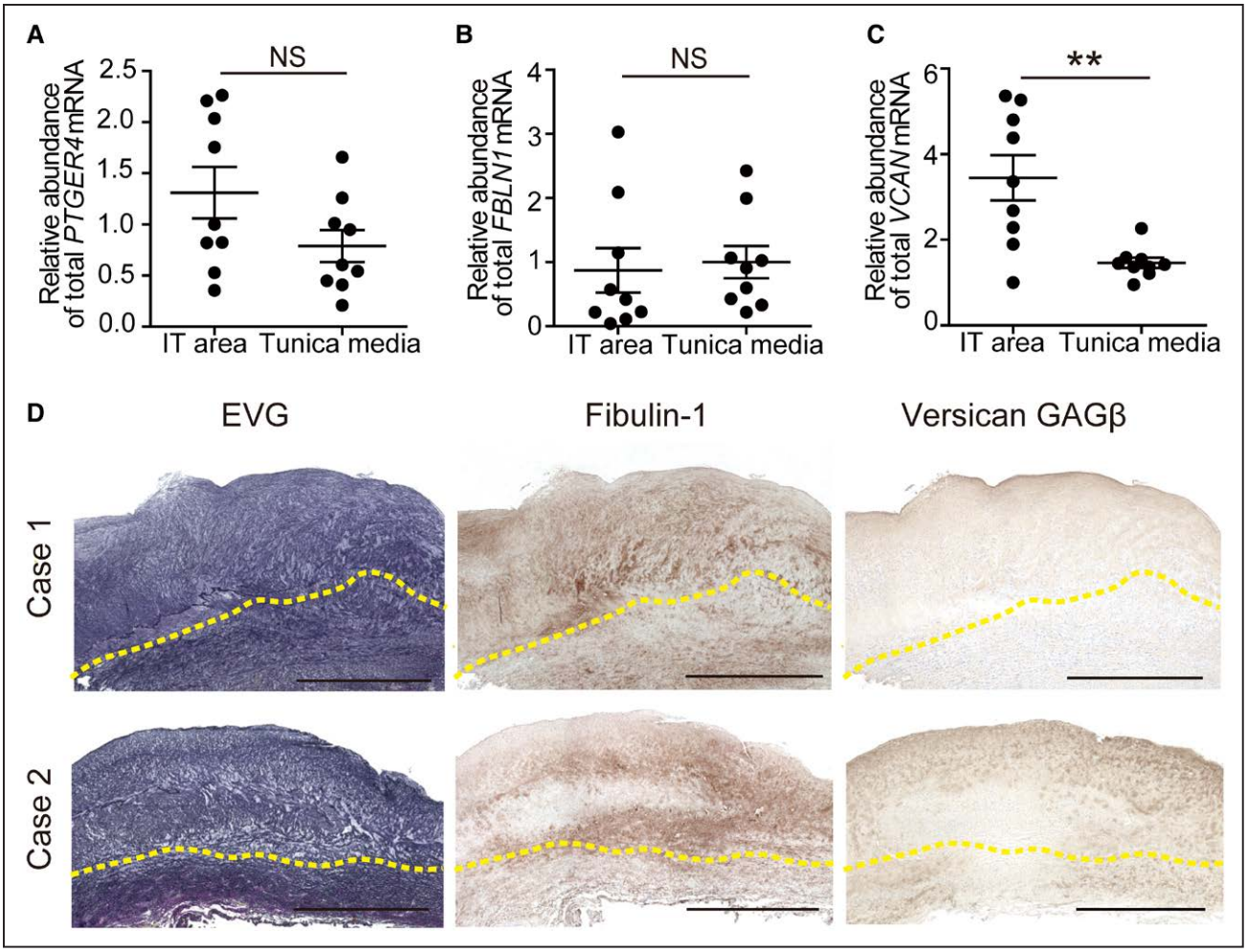

Figure 6. Human ductus arteriosus (**DA) tissues show a similar distribution to rodents in fibulin-1 and versican.**
A–**C**, Expression of EP4 (prostaglandin E receptor 4; *PTGER4*), total fibulin-1 (*FBLN1*), and total versican (*VCAN*) mRNAs in the intimal thickening (IT) area and the tunica media of human DA tissues. n=9, $^{**}P<0.01$. Data are presented as mean±SEM. **D**, Elastica van Gieson (EVG) staining and immunohistological analysis of fibulin-1 and versican glycosaminoglycan β (GAGβ) domain in human DA tissues. Dotted yellow lines show internal elastic lamina. Case 1, patient no. 10; case 2, patient no. 11 in Table II in the Data Supplement. Scale bars, 1 mm. NS indicates not significant.

induced by integrating multiple ECMs (fibulin-1-versican-HA complex).

Fibulin-1 is a glycoprotein that stabilizes ECM integrity through interactions with other ECMs.[14,17] Fibulin-1 has been shown to bind versican, aggrecan, fibronectin, laminin, nidogen, and the coagulation protein fibrinogen.[14,17] In addition, fibulin-1 is capable of self-association.[17] Fibulin-1 is composed of amino-terminal anaphylatoxin-like modules followed by 9 EGF (epidermal growth factor)-like modules and a carboxyl terminus.[17] A fibulin-1 self-association site and a fibronectin-binding site are localized to EGF-like module 5 and 6.[17] The fibulin-1 EGF-like domain also contributes to binding with the versican C-terminal G3 domain.[14] These interactions could account for the matrix assembly in tissues of physiological and pathological conditions. It was demonstrated that fibulin-1 was expressed throughout the vessel wall of rat adult aorta, including the tunica media and the tunica adventitia.[14] In the same study, versican expression was restricted to ECs and the subintimal layer of the tunica media, but no positive immunoreaction for versican was observed in the tunica media on the adventitial side and in the tunica adventitia.[14] These data suggest that SMC-derived fibulin-1 and EC-derived versican complex may be formed in the arterial subendothelial region, which is consistent with the findings of the present study.

It was reported in the early 1990s that chondroitin sulfate proteoglycan was abundantly expressed in the subendothelial region of the human DAs,[12] but this proteoglycan had not been determined until now. Our study identifies the chondroitin sulfate proteoglycan as versican. The present study also demonstrated that V0/V1 was preferentially expressed in the DA IT region and suggested that versican contributes to DA IT formation as a binding partner of fibulin-1. Versican is a multidomain aggregating proteoglycan and a major component of the ECMs involved in migration and differentiation.[29,33] Among 4 isoforms of versican, the V0 and V1 isoforms

contain the GAGβ domain, which plays a role in cell migration.[29] In addition to our data showing that fibulin-1 per se promotes SMC migration, versican may further assist in SMC migration. Based on our finding that fibulin-1 deficiency causes PDA, fibulin-1 is suggested to play a major role in the integration of ECMs at the subendothelial lesion.

Our data demonstrated that versican was involved in SMC directional migration and suggest a potential interaction of fibulin-1 and versican. However, the presence of versican does not seem to be a prerequisite for DA closure. Fanhchaksai et al[34] generated mice in which versican was specifically deleted in ECs (VE-cadherin [vascular endothelial cadherin]-Cre;*Vcan*$^{flox/flox}$) by mating mice with a flox allele for *Vcan* to mice with an EC-targeted expression of cre recombinase and found that a deficiency of versican displayed normal DA closure and development (S. Makino, unpublished data, 2020). In addition to ECs, versican is produced by arterial SMCs.[35,36] To investigate the roles of SMC-derived versican in DA closure, we generated mice in which versican was specifically deleted in SMCs (*SM22-Cre;Vcan*$^{flox/flox}$) and found that a deficiency of versican displayed normal DA closure (unpublished observation). In this study, we used the versican-expressing EC cell line EA. hy926, instead of DA-ECs, because the sorted DA-ECs were not viable enough in coculture with SMCs to examine directional cell migration for 96 hours, which is a limitation of the present study. Similar to DA-SMCs, DA-ECs have a distinct gene expression profile compared with those of adjacent arteries.[37,38] In our coculture system, silencing of versican in ECs significantly attenuated SMC directional migration toward ECs, but other ECMs that are highly expressed in DA-ECs would contribute to form a migration-favoring environment at the subendothelial region of the DA. Based on these observations in genetically modified mice and the study limitation, in addition to versican, multiple ECMs would contribute to DA IT formation.

SMCs of the ascending aorta and the DA originate from the neural crest,[39] and ECs of the pulmonary trunk and the DA are derived from the second heart field.[40] Although the DA shares the same cell lineage with adjacent arteries, the DA acquires distinct characteristics. A systematic review of transcriptional profiling in the rodent and human DA in comparison to the aorta demonstrated that a higher expression of EP4 was observed commonly in the rodent and human DA.[41] The differential EP4 expression was observed in mice and humans at embryonic day 14 and 21 weeks of gestation, respectively,[25,41] and EP4 expression increases toward term.[9,25,42,43] The present study identified fibulin-1 as the gene regulated by EP4 signaling, and fibulin-1 expression may be upregulated in accordance with that of EP4 expression. Investigating developmental changes in fibulin-1 expression would be of interest in DA and cardiovascular system research.

The present study demonstrated a potential interaction between SMC-derived fibulin-1 and EC-derived versican. It has been reported that several genes were differentially upregulated in DA-ECs compared with ECs in the aorta.[37,38] Although known direct binding partners of fibulin-1 have not been reported as DA-EC specific genes, DA-EC-derived ECM would contribute to SMC phenotypes and IT formation and subsequent DA closure. Further study is required to clarify the contribution of specific DA-EC molecules in DA remodeling.

A previous study demonstrated that PGE_2-EP4–induced hyaluronan promoted SMC migration and IT formation of the DA, suggesting the importance of hyaluronan in DA closure.[9] Versican is anchored to the hyaluronan meshwork through its N-terminal G1 domain with relatively high affinity.[44] Abundant expressions of versican and hyaluronan were observed in human restenotic arteries and of the neointima in balloon-injured rat carotid arteries.[45] Because *Vcan*$^{\Delta 3/\Delta 3}$ neonatal mice partly exhibited poor IT formation and PDA, versican-mediated accumulation of hyaluronan in the subendothelial region seems to promote directional SMC migration.

Versican has a core protein surrounded by a network of glycosaminoglycan chains. The glycosaminoglycans are negatively charged and are key to the water retention. Glycosaminoglycan hyaluronan is also a highly hydrophilic molecule that attracts large amounts of water, forming a highly hydrated gel and thereby promoting expansion of the extracellular spaces and facilitating cell migration.[46] The hygroscopic properties of versican and hyaluronan might cause a retention of water, which could help widen the subendothelial region, creating an environment that is well suited for directional SMC migration into the subendothelial region.

In the present study, we found that the *Vcan*$^{\Delta 3/\Delta 3}$ DA has a thinner vascular wall. Coexpression of versican and hyaluronan is observed during organogenesis.[47] The interaction of these 2 molecules further creates a pericellular open space due to their water retention ability, which allows cells to migrate and proliferate.[47] In the cardiovascular system, versican and hyaluronan are similarly expressed in cardiac jelly to support endocardial cushion morphogenesis and subsequent cardiac development.[48] Global deficiency of the gene for either versican or hyaluronan causes embryonic lethality due to failure of the heart to form.[22,48] The failure of binding versican and hyaluronan in *Vcan*$^{\Delta 3/\Delta 3}$ mice caused embryonic lethality or ventricular septal defect.[22] These data suggest the importance of the interaction of versican and hyaluronan in cardiovascular morphogenesis. Failure of the interaction between versican and hyaluronan may affect vascular wall development in the DA.

We revealed a PGE_2-EP4–mediated robust increase in fibulin-1 expression through newly identified intracellular downstream signaling of EP4 (Figure IVB in the Data Supplement).

Expression levels of fibulin-1 mRNA and protein were increased through the PLC-PKC–noncanonical NFκB pathway. EP4 belongs to the G protein-coupled receptors, and cAMP is the major second messenger of EP4.[28] However, none of the non-, PKA-, or Epac-selective cAMP analogs affect fibulin-1 expression, whereas PLC is involved in this signaling pathway. Fibulin-1 expression was inhibited by D609 administration, and D609 actions are mainly attributed to inhibiting PC-PLC (phosphatidylcholine-specific phospholipase C).[49] PC-PLC hydrolyzes PC to generate phosphocholine and 1,2-diacylglycerol.[49] Therefore, 1,2-diacylglycerol–mediated PKA activation seems to play a role in the downstream signaling of EP4. Among PKC isoforms, classical PKCs (α, β1, β2, γ) and novel PKCs (δ, ε, η, θ) have both C1A and C1B domains in their regulatory domain, and these isoforms can bind 1,2-diacylglycerol.[50] Classical PKCs or novel PKCs, but not atypical PKCs, are predicted to be involved in EP4-mediated fibulin-1 upregulation.

Noncanonical NFκB activation is an additional pathway that plays an important role in immune systems.[51] In the noncanonical NFκB pathway, translational activation occurs through κIKKα complex activation by NFκB-inducing kinase and leads to the phosphorylation and processing of p100.[52] To the best of our knowledge, there is only one report of the involvement of the noncanonical NFκB pathway in vascular remodeling, which demonstrated that the noncanonical NFκB signaling pathway-activated S-phase kinase–associated protein 2 promoted wire injury–induced IT formation.[53] The noncanonical NFκB pathway may be involved in both physiological and pathological IT formation.

Current COX inhibitor therapy would suppress IT formation because it inhibits PGE_2 production and subsequent fibulin-1 production in the DA. In addition to vasocontraction therapy, understanding the PGE_2-EP4–mediated downstream signaling pathways responsible for fibulin-1 upregulation may provide the basis for therapeutic strategies for inducing IT in the DA. Anti-HIV therapy using PKC agonists targeted to NFκB signaling has been investigated.[54] It was also reported that methylprednisolone is able to increase the NFκB-inducing kinase expression in human skeletal muscle.[55] Further studies are required for the new IT-inducing pharmacological therapy for patients with PDA.

Human fibulin-1 gene (*FBLN1*) is located in 22q13.31.[56] Deletion 22q13 syndrome includes a wide range of deletion sizes from 100 kb to 9 Mb, and total *FBLN1* gene deletion was reported in some cases with a larger deletion.[57] The core phenotypic features, such as neonatal hypotonia, absent or delayed speech, and moderate to profound developmental delay are thought to be caused by haploinsufficiency of SH3 and multiple ankyrin repeat domains 3 (*SHANK3*),[58] which is located ≈5 Mb away from *FBLN1*. It has been reported that congenital heart disease, including PDA, atrial septal defect, and total anomalous pulmonary return, occur in >25% of individuals.[58] Jeffries et al[57] investigated 30 cases of deletion 22q13 syndrome and found that 4 cases who have larger deletion in size had congenital heart disease, 2 with PDA and 2 with total anomalous pulmonary venous return. These reports did not assess the association between *FBLN1* deletion and PDA, and most reports of deletion 22q13 syndrome have not focused on cardiovascular anomalies.[59] Based on these case reports and our experimental data, however, lower expression of fibulin-1 may contribute to the patency of the DA in humans.

In conclusion, PGE_2-EP4–mediated fibulin-1 integrates ECMs at the subendothelial region and promotes directional SMC migration toward the internal lumen, resulting in IT formation and DA closure. In addition to current vasocontraction therapy, targeting fibulin-1 upregulation may provide the basis for therapeutic strategies for inducing anatomic closure of the DA.

ARTICLE INFORMATION

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Disclosures

None.

REFERENCES

1. Yokoyama U. Prostaglandin E-mediated molecular mechanisms driving remodeling of the ductus arteriosus. Pediatr Int. 2015;57:820–827. doi: 10.1111/ped.12769
2. Mitra S, Florez ID, Tamayo ME, Mbuagbaw L, Vanniyasingam T, Veroniki AA, Zea AM, Zhang Y, Sadeghirad B, Thabane L. Association of placebo, indomethacin, ibuprofen, and acetaminophen with closure of hemodynamically significant patent ductus arteriosus in preterm infants: a systematic review and meta-analysis. JAMA. 2018;319:1221–1238. doi: 10.1001/jama.2018.1896
3. Ito S, Matsuda T, Usuda H, Watanabe S, Kitanishi R, Hanita T, Watanabe T, Adachi O. Surgical ligation for patent ductus arteriosus in extremely premature infants: strategy to reduce their risk of neurodevelopmental impairment. Tohoku J Exp Med. 2016;240:7–13. doi: 10.1620/tjem.240.7

4. Noori S, McCoy M, Friedlich P, Bright B, Gottipati V, Seri I, Sekar K. Failure of ductus arteriosus closure is associated with increased mortality in preterm infants. Pediatrics. 2009;123:e138–e144. doi: 10.1542/peds.2008-2418
5. Clyman RI. Ibuprofen and patent ductus arteriosus. N Engl J Med. 2000;343:728–730. doi: 10.1056/NEJM200009073431009
6. Clyman RI. Mechanisms regulating the ductus arteriosus. Biol Neonate. 2006;89:330–335. doi: 10.1159/000092870
7. Toda T, Tsuda N, Takagi T, Nishimori I, Leszczynski D, Kummerow F. Ultrastructure of developing human ductus arteriosus. J Anat. 1980;131(pt 1):25–37.
8. Ito S, Yokoyama U, Saito J, Sato S, Usuda H, Watanabe S, Kitanishi R, Miura Y, Saito M, Hanita T, et al. Attenuation of ductus arteriosus intimal thickening in preterm sheep twins compared with singletons. J Physiol Sci. 2017;67:723–729. doi: 10.1007/s12576-017-0565-5
9. Yokoyama U, Minamisawa S, Quan H, Ghatak S, Akaike T, Segi-Nishida E, Iwasaki S, Iwamoto M, Misra S, Tamura K, et al. Chronic activation of the prostaglandin receptor EP4 promotes hyaluronan-mediated neointimal formation in the ductus arteriosus. J Clin Invest. 2006;116:3026–3034. doi: 10.1172/JCI28639
10. De Reeder EG, Girard N, Poelmann RE, Van Munsteren JC, Patterson DF, Gittenberger-De Groot AC. Hyaluronic acid accumulation and endothelial cell detachment in intimal thickening of the vessel wall. The normal and genetically defective ductus arteriosus. Am J Pathol. 1988;132:574–585.
11. Yokoyama U, Minamisawa S, Katayama A, Tang T, Suzuki S, Iwatsubo K, Iwasaki S, Kurotani R, Okumura S, Sato M, et al. Differential regulation of vascular tone and remodeling via stimulation of type 2 and type 6 adenylyl cyclases in the ductus arteriosus. Circ Res. 2010;106:1882–1892. doi: 10.1161/CIRCRESAHA.109.214924
12. Slomp J, van Munsteren JC, Poelmann RE, de Reeder EG, Bogers AJ, Gittenberger-de Groot AC. Formation of intimal cushions in the ductus arteriosus as a model for vascular intimal thickening. An immunohistochemical study of changes in extracellular matrix components. Atherosclerosis. 1992;93:25–39. doi: 10.1016/0021-9150(92)90197-o
13. Hungerford JE, Owens GK, Argraves WS, Little CD. Development of the aortic vessel wall as defined by vascular smooth muscle and extracellular matrix markers. Dev Biol. 1996;178:375–392. doi: 10.1006/dbio.1996.0225
14. Aspberg A, Adam S, Kostka G, Timpl R, Heinegård D. Fibulin-1 is a ligand for the C-type lectin domains of aggrecan and versican. J Biol Chem. 1999;274:20444–20449. doi: 10.1074/jbc.274.29.20444
15. Balbona K, Tran H, Godyna S, Ingham KC, Strickland DK, Argraves WS. Fibulin binds to itself and to the carboxyl-terminal heparin-binding region of fibronectin. J Biol Chem. 1992;267:20120–20125.
16. Sasaki T, Kostka G, Göhring W, Wiedemann H, Mann K, Chu ML, Timpl R. Structural characterization of two variants of fibulin-1 that differ in nidogen affinity. J Mol Biol. 1995;245:241–250. doi: 10.1006/jmbi.1994.0020
17. Tran H, VanDusen WJ, Argraves WS. The self-association and fibronectin-binding sites of fibulin-1 map to calcium-binding epidermal growth factor-like domains. J Biol Chem. 1997;272:22600–22606. doi: 10.1074/jbc.272.36.22600
18. Cooley MA, Kern CB, Fresco VM, Wessels A, Thompson RP, McQuinn TC, Twal WO, Mjaatvedt CH, Drake CJ, Argraves WS. Fibulin-1 is required for morphogenesis of neural crest-derived structures. Dev Biol. 2008;319:336–345. doi: 10.1016/j.ydbio.2008.04.029
19. Kostka G, Giltay R, Bloch W, Addicks K, Timpl R, Fässler R, Chu ML. Perinatal lethality and endothelial cell abnormalities in several vessel compartments of fibulin-1-deficient mice. Mol Cell Biol. 2001;21:7025–7034. doi: 10.1128/MCB.21.20.7025-7034.2001
20. Ivey KN, Sutcliffe D, Richardson J, Clyman RI, Garcia JA, Srivastava D. Transcriptional regulation during development of the ductus arteriosus. Circ Res. 2008;103:388–395. doi: 10.1161/CIRCRESAHA.108.180661
21. Segi E, Sugimoto Y, Yamasaki A, Aze Y, Oida H, Nishimura T, Murata T, Matsuoka T, Ushikubi F, Hirose M, et al. Patent ductus arteriosus and neonatal death in prostaglandin receptor EP4-deficient mice. Biochem Biophys Res Commun. 1998;246:7–12. doi: 10.1006/bbrc.1998.8461
22. Hatano S, Kimata K, Hiraiwa N, Kusakabe M, Isogai Z, Adachi E, Shinomura T, Watanabe H. Versican/PG-M is essential for ventricular septal formation subsequent to cardiac atrioventricular cushion development. Glycobiology. 2012;22:1268–1277. doi: 10.1093/glycob/cws095
23. Saito J, Yokoyama U, Nicho N, Zheng YW, Ichikawa Y, Ito S, Umemura M, Fujita T, Ito S, Taniguchi H, et al. Tissue-type plasminogen activator contributes to remodeling of the rat ductus arteriosus. PLoS One. 2018;13:e0190871. doi: 10.1371/journal.pone.0190871
24. Yokoyama U, Minamisawa S, Adachi-Akahane S, Akaike T, Naguro I, Funakoshi K, Iwamoto M, Nakagome M, Uemura N, Hori H, et al. Multiple transcripts of Ca2+ channel alpha1-subunits and a novel spliced variant of the alpha1C-subunit in rat ductus arteriosus. Am J Physiol Heart Circ Physiol. 2006;290:H1660–H1670. doi: 10.1152/ajpheart.00100.2004
25. Yokoyama U, Minamisawa S, Shioda A, Ishiwata R, Jin MH, Masuda M, Asou T, Sugimoto Y, Aoki H, Nakamura T, et al. Prostaglandin E2 inhibits elastogenesis in the ductus arteriosus via EP4 signaling. Circulation. 2014;129:487–496. doi: 10.1161/CIRCULATIONAHA.113.004726
26. Timpl R, Sasaki T, Kostka G, Chu ML. Fibulins: a versatile family of extracellular matrix proteins. Nat Rev Mol Cell Biol. 2003;4:479–489. doi: 10.1038/nrm1130
27. Nguyen M, Camenisch T, Snouwaert JN, Hicks E, Coffman TM, Anderson PA, Malouf NN, Koller BH. The prostaglandin receptor EP4 triggers remodelling of the cardiovascular system at birth. Nature. 1997;390:78–81. doi: 10.1038/36342
28. Yokoyama U, Iwatsubo K, Umemura M, Fujita T, Ishikawa Y. The prostanoid EP4 receptor and its signaling pathway. Pharmacol Rev. 2013;65:1010–1052. doi: 10.1124/pr.112.007195
29. Arslan F, Bosserhoff AK, Nickl-Jockschat T, Doerfelt A, Bogdahn U, Hau P. The role of versican isoforms V0/V1 in glioma migration mediated by transforming growth factor-beta2. Br J Cancer. 2007;96:1560–1568. doi: 10.1038/sj.bjc.6603766
30. Li M, Qian M, Kyler K, Xu J. Endothelial-vascular smooth muscle cells interactions in atherosclerosis. Front Cardiovasc Med. 2018;5:151. doi: 10.3389/fcvm.2018.00151
31. Dai Z, Zhu MM, Peng Y, Jin H, Machireddy N, Qian Z, Zhang X, Zhao YY. Endothelial and smooth muscle cell interaction via FoxM1 signaling mediates vascular remodeling and pulmonary hypertension. Am J Respir Crit Care Med. 2018;198:788–802. doi: 10.1164/rccm.201709-1835OC
32. Wagenseil JE, Mecham RP. Vascular extracellular matrix and arterial mechanics. Physiol Rev. 2009;89:957–989. doi: 10.1152/physrev.00041.2008
33. Landolt RM, Vaughan L, Winterhalter KH, Zimmermann DR. Versican is selectively expressed in embryonic tissues that act as barriers to neural crest cell migration and axon outgrowth. Development. 1995;121:2303–2312.
34. Fanhchaksai K, Okada F, Nagai N, Pothacharoen P, Kongtawelert P, Hatano S, Makino S, Nakamura T, Watanabe H. Host stromal versican is essential for cancer-associated fibroblast function to inhibit cancer growth. Int J Cancer. 2016;138:630–641. doi: 10.1002/ijc.29804
35. Wight TN. Arterial remodeling in vascular disease: a key role for hyaluronan and versican. Front Biosci. 2008;13:4933–4937. doi: 10.2741/3052
36. Evanko SP, Angello JC, Wight TN. Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 1999;19:1004–1013. doi: 10.1161/01.atv.19.4.1004
37. Liu NM, Yokota T, Maekawa S, Lü P, Zheng YW, Tei I, Taniguchi H, Yokoyama U, Kato T, Minamisawa S. Transcription profiles of endothelial cells in the rat ductus arteriosus during a perinatal period. PLoS One. 2013;8:e73685. doi: 10.1371/journal.pone.0073685
38. Bökenkamp R, van Brempt R, van Munsteren JC, van den Wijngaert I, de Hoogt R, Finos L, Goeman J, Groot AC, Poelmann RE, Blom NA, et al. Dlx1 and Rgs5 in the ductus arteriosus: vessel-specific genes identified by transcriptional profiling of laser-capture microdissected endothelial and smooth muscle cells. PLoS One. 2014;9:e86892. doi: 10.1371/journal.pone.0086892
39. Bergwerff M, Verberne ME, DeRuiter MC, Poelmann RE, Gittenberger-de Groot AC. Neural crest cell contribution to the developing circulatory system: implications for vascular morphology? Circ Res. 1998;82:221–231. doi: 10.1161/01.res.82.2.221
40. Wang X, Chen D, Chen K, Jubran A, Ramirez A, Astrof S. Endothelium in the pharyngeal arches 3, 4 and 6 is derived from the second heart field. Dev Biol. 2017;421:108–117. doi: 10.1016/j.ydbio.2016.12.010
41. Yarboro MT, Durbin MD, Herington JL, Shelton EL, Zhang T, Ebby CG, Stoller JZ, Clyman RI, Reese J. Transcriptional profiling of the ductus arteriosus: Comparison of rodent microarrays and human RNA sequencing. Semin Perinatol. 2018;42:212–220. doi: 10.1053/j.semperi.2018.05.003
42. Yokoyama U, Sato Y, Akaike T, Ishida S, Sawada J, Nagao T, Quan H, Jin M, Iwamoto M, Yokota S, et al. Maternal vitamin A alters gene profiles and structural maturation of the rat ductus arteriosus. Physiol Genomics. 2007;31:139–157. doi: 10.1152/physiolgenomics.00007.2006
43. Jin MH, Yokoyama U, Sato Y, Shioda A, Jiao Q, Ishikawa Y, Minamisawa S. DNA microarray profiling identified a new role of growth hormone in vascular remodeling of rat ductus arteriosus. J Physiol Sci. 2011;61:167–179. doi: 10.1007/s12576-011-0133-3
44. LeBaron RG, Zimmermann DR, Ruoslahti E. Hyaluronate binding properties of versican. J Biol Chem. 1992;267:10003–10010.

45. Riessen R, Wight TN, Pastore C, Henley C, Isner JM. Distribution of hyaluronan during extracellular matrix remodeling in human restenotic arteries and balloon-injured rat carotid arteries. Circulation. 1996;93:1141–1147. doi: 10.1161/01.cir.93.6.1141
46. Lee SA, Flowers MR, Oliver WF, Rupprecht A, Lindsay SM. Brillouin-scattering study of hyaluronic acid: dynamic coupling with the water of hydration and phase transitions. Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics. 1993;47:677–683. doi: 10.1103/physreve.47.677
47. Wight TN. Provisional matrix: a role for versican and hyaluronan. Matrix Biol. 2017;60-61:38–56. doi: 10.1016/j.matbio.2016.12.001
48. Camenisch TD, Spicer AP, Brehm-Gibson T, Biesterfeldt J, Augustine ML, Calabro A Jr, Kubalak S, Klewer SE, McDonald JA. Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. J Clin Invest. 2000;106:349–360. doi: 10.1172/JCI10272
49. Adibhatla RM, Hatcher JF, Gusain A. Tricyclodecan-9-yl-xanthogenate (D609) mechanism of actions: a mini-review of literature. Neurochem Res. 2012;37:671–679. doi: 10.1007/s11064-011-0659-z
50. Mukherjee A, Roy S, Saha B, Mukherjee D. Spatio-temporal regulation of PKC isoforms imparts signaling specificity. Front Immunol. 2016;7:45. doi: 10.3389/fimmu.2016.00045
51. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 2004;25:280–288. doi: 10.1016/j.it.2004.03.008
52. Hayden MS, Ghosh S. NF-κB, the first quarter-century: remarkable progress and outstanding questions. Genes Dev. 2012;26:203–234. doi: 10.1101/gad.183434.111
53. Song P, Wang S, He C, Wang S, Liang B, Viollet B, Zou MH. AMPKα2 deletion exacerbates neointima formation by upregulating Skp2 in vascular smooth muscle cells. Circ Res. 2011;109:1230–1239. doi: 10.1161/CIRCRESAHA.111.250423
54. Jiang G, Dandekar S. Targeting NF-κB signaling with protein kinase C agonists as an emerging strategy for combating HIV latency. AIDS Res Hum Retroviruses. 2015;31:4–12. doi: 10.1089/AID.2014.0199
55. Fry CS, Nayeem SZ, Dillon EL, Sarkar PS, Tumurbaatar B, Urban RJ, Wright TJ, Sheffield-Moore M, Tilton RG, Choudhary S. Glucocorticoids increase skeletal muscle NF-kappaB inducing kinase (NIK): links to muscle atrophy. Physiol Rep. 2016;4:e13014. doi: 10.14814/phy2.13014
56. Korenberg JR, Chen XN, Tran H, Argraves WS. Localization of the human gene for fibulin-1 (FBLN1) to chromosome band 22q13.3. Cytogenet Cell Genet. 1995;68:192–193. doi: 10.1159/000133911
57. Jeffries AR, Curran S, Elmslie F, Sharma A, Wenger S, Hummel M, Powell J. Molecular and phenotypic characterization of ring chromosome 22. Am J Med Genet A. 2005;137:139–147. doi: 10.1002/ajmg.a.30780
58. Phelan K, McDermid HE. The 22q13.3 Deletion Syndrome (Phelan-McDermid Syndrome). Mol Syndromol. 2012;2:186–201. doi: 10.1159/000334260
59. Kolevzon A, Angarita B, Bush L, Wang AT, Frank Y, Yang A, Rapaport R, Saland J, Srivastava S, Farrell C, et al. Phelan-McDermid syndrome: a review of the literature and practice parameters for medical assessment and monitoring. J Neurodev Disord. 2014;6:39. doi: 10.1186/1866-1955-6-39