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Chronic activation of the prostaglandin receptor EP4 promotes hyaluronan-mediated neointimal formation in the ductus arteriosus

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PGE, a potent vasodilator, plays a primary role in maintaining the patency of the ductus arteriosus (DA). Genetic disruption of the PGE-specific receptor EP4, however, paradoxically results in fatal patent DA (PDA) in mice. Here we demonstrate that EP4-mediated signals promote DA closure by hyaluronic acid–mediated (HA-mediated) intimal cushion formation (ICF). Chronic EP4 stimulation by ONO-AE1-329, a selective EP4 agonist, significantly enhanced migration and HA production in rat DA smooth muscle cells. When HA production was inhibited, EP4-mediated migration was negated. Activation of EP4, adenylyl cyclase, and PKA all increased HA production and the level of HA synthase 2 (HAS2) transcripts. In immature rat DA explants, ICF was promoted by EP4/PKA stimuli. Furthermore, adenovirus-mediated *Has2* gene transfer was sufficient to induce ICF in *EP4*-disrupted DA explants in which the intimal cushion had not formed. Accordingly, signals through EP4 have 2 essential roles in DA development, namely, vascular dilation and ICF. The latter would lead to luminal narrowing, helping adhesive occlusion and permanent closure of the vascular lumen. Our results imply that HA induction serves as an alternative therapeutic strategy for the treatment of PDA to the current one, i.e., inhibition of PGE signaling by cyclooxygenase inhibitors, which might delay PGE-mediated ICF in immature infants.

Introduction

The ductus arteriosus (DA), a fetal arterial connection between the pulmonary artery and the descending aorta, is indispensable for fetal life. PGE_2, the most potent vasodilatory lipid mediator in the DA, principally maintains the open status of the DA during fetal life (1). A rapid decline in the level of circulating PGE_2 is a primary trigger of DA closure at the time of birth. Although the DA closes immediately after birth, it remains open in some infants, a condition known as patent DA (PDA). PDA is a frequent problem affecting premature infants, with a prevalence greater than 40% in infants with a birth weight of 1,500 g or less (2). PDA is responsible for significant morbidity and mortality in premature infants and is associated with increased risks for intraventricular hemorrhage, bronchopulmonary dysplasia, and necrotizing enterocolitis (2, 3). COX inhibitors including indomethacin, which principally inhibit the production of prostaglandins, have been widely used for treatment of PDA (1, 4, 5). However, the efficacy of COX inhibitors may be limited in a substantial number of cases, and surgical ligation must be conducted, with its attendant morbidities (4, 5). Therefore, an alternative strategy for PDA treatment is required.

Although PGE_2 plays a primary role in maintaining the patency of DA, previous studies have demonstrated that genetic disruption of the PGE-specific receptor *EP4* paradoxically results in fatal PDA in mice (6, 7). In addition, double mutant mice in which *COX-1* and *COX-2* are disrupted also exhibit PDA (8). Furthermore, several clinical studies showed that maternal administration of COX inhibitors during pregnancy increases the incidence of PDA in newborns, especially in premature infants (9, 10). However, a biological rationale behind these paradoxical results has not been satisfactorily provided.

DA closure occurs in 2 phases. During the first few hours after birth in term newborns, there is acute and functional closure as a result of smooth muscle contraction of the DA, which is triggered by an increase in oxygen tension and a decline in levels of circulating PGE_2 (1). Importantly, prior to this, anatomical luminal narrowing develops through intimal cushion formation (ICF) that occludes the vascular lumen and results in permanent closure after birth (11, 12). The intimal cushion of DA is formed by many cellular processes, such as an increase in SMC migration and proliferation, production of hyaluronic acid (HA) under the endothelial layer, and decreased elastin fiber assembly (1, 11–14). In this regard, ICF is poorly developed in human PDA patients and animal models of PDA (15–17). Since DA is exposed to a large amount of PGE_2 during gestation and the PGE/EP4 signal cascade has been shown to regulate vascular remodeling (18–20), PGE/EP4 signaling may regulate DA development. Therefore, we hypothesized that, in addi-

Nonstandard abbreviations used: DA, ductus arteriosus; HA, hyaluronic acid; HAS, HA synthase; ICF, intimal cushion formation; PDA, patent DA.

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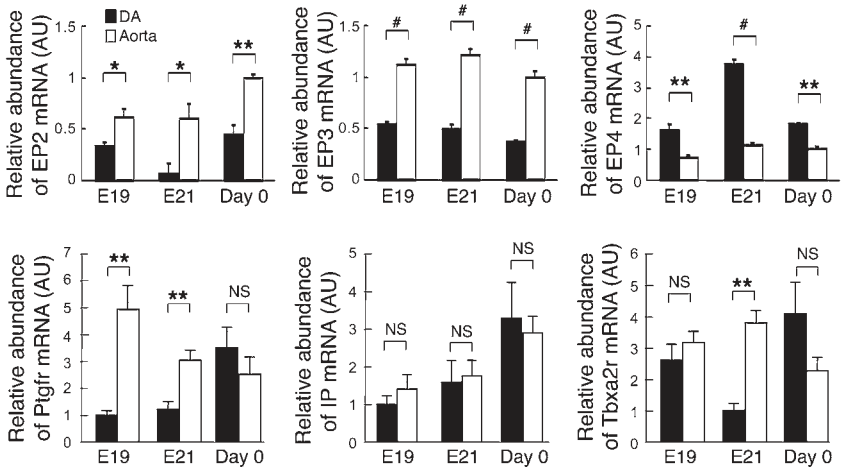

Figure 1
The expression of prostanoid receptors in the rat DA and aorta during development. Quantitative RT-PCR analyses of EP isoforms (EP2, EP3, and EP4) and other prostanoid receptors ($PGF_{2\alpha}$ receptor [Ptgfr], PGI_2 receptor [IP], and thromboxane A_2 receptor [Tbxa2r]) in the rat DA and aorta. The expression of EP4 mRNA was significantly higher in DA than in the aorta (upper right). The expression level of EP4 mRNA was maximal at E21 in the DA. $^*P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$.

tion to its vasodilatory effect, PGE_2/EP4 signaling plays a primary role in promoting HA-mediated ICF in the DA and that this is essential for its anatomical closure, in utero prior to birth.

Results

Chronic EP4 stimuli promoted DA SMC migration accompanied by HA accumulation. We found that the expression of EP4 mRNA in rats was significantly higher in the DA than in the aorta, whereas the expression levels of EP2 and EP3 mRNAs were significantly lower in the DA than in the aorta (Figure 1). The expression level of EP4 mRNA was maximal at E21 in the DA. Our data indicate that EP4 is a DA-specific PGE_2 receptor in rat, as previously demonstrated in other species (21–24). Other prostanoid receptors for $PGF_{2\alpha}$, PGI_2, and thromboxane A_2 were not predominantly expressed in the DA (Figure 1).

SMC migration from vascular media into the endothelial layer is an important vascular remodeling process during ICF (12, 25). Since the DA is chronically exposed to a high dose of PGE_2 during gestation, we hypothesized that chronic exposure to EP4 stimuli may alter DA SMC migration. We found that chronic treatment with an EP4 agonist, ONO-AE1-329 (10^{-6} M) for long exposures (>2 days) increased SMC migration in a time-dependent manner (Figure 2A, black bars). We also observed similar increases in DA SMC migration after exposure to PGE_2 (10^{-6} M) (data not shown). Since HA-rich matrices are important for cell migration and proliferation (26, 27), we measured HA production in culture media. We found that an increase in HA production in culture media was accompanied by an increase in SMC migration (Figure 2A, filled circles). To clarify whether an increase in HA production was responsible for the effect of ONO-AE1-329 on SMC migration, HA was removed from the culture media every day by replacing condition medium with fresh medium or by adding hyaluronidase (0.02 and 0.05 mg/ml) to the media to digest the secreted HA. The removal of HA from the culture medium significantly decreased the stimulatory effects of ONO-AE1-329 (Figure 2B). In addition, we investigated whether ONO-AE1-329's effect was inhibited when HA synthase (HAS) was inhibited. We found that HAS2 was largely responsible for ONO-AE1-329–mediated HA production in DA, although HA is synthesized by 3 isoforms of HAS, namely HAS1, HAS2, and HAS3 (28). We tested 2 different siRNAs for HAS2 for their capacity to decrease the expression of HAS2 mRNA and HA production in DA SMCs. We found that transfection of one siRNA for HAS2 significantly inhibited ONO-AE1-329–mediated HAS2 expression (~90% decrease) and thus the DA SMC migration, whereas that of a nontargeting negative control siRNA did not (Figure 2B). Moreover, HA supplementation (50 ng/ml) in the absence of ONO-AE1-329 increased DA SMC migration to the same level as treatment

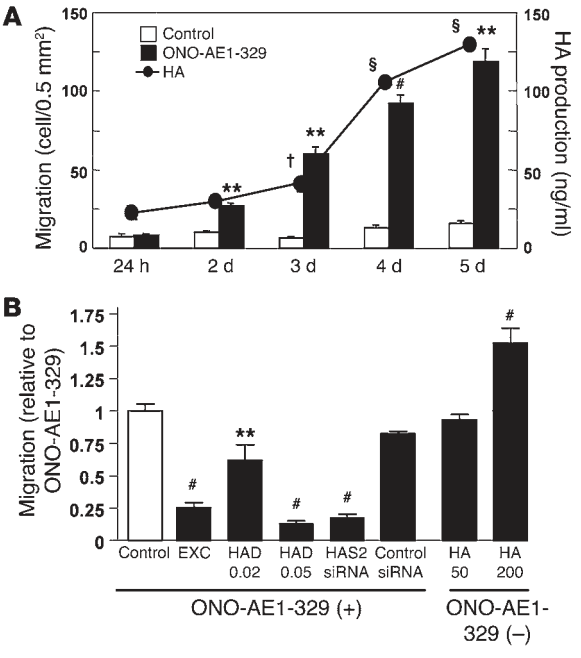

Figure 2
Effects of chronic EP4 stimulation on DA SMC migration. (**A**) Cell migration and HA production in response to long exposures (>24 hours) to ONO-AE1-329 (10^{-6} M). SMC migration (bar graph) and HA secretion into the media (line graph) are shown. An increase in HA production in culture media was accompanied by the time-dependent increase in SMC migration. $n = 4$–8; $^{**}P < 0.01$, $^{\#}P < 0.001$ compared with control. $^{\dagger}P < 0.05$, $^{\S}P < 0.001$ compared with HA production at 24 hours. (**B**) ONO-AE1-329–induced HA production is responsible for increased migration. The ONO-AE1-329–induced SMC migration was significantly attenuated by daily exchange of culture media (EXC); addition of hyaluronidase (HAD) (0.02 mg/ml: HAD 0.02; 0.05 mg/ml: HAD 0.05) to culture media; or transfection with rat HAS2 siRNA (100 pmol). HA (50 ng/ml: HA 50; 200 ng/ml: HA 200) alone was sufficient to stimulate DA SMC migration to the same or a higher level as was ONO-AE1-329 (10^{-6} M). $n = 4$; $^{**}P < 0.01$, $^{\#}P < 0.001$ compared with control (10^{-6} M ONO-AE1-329).

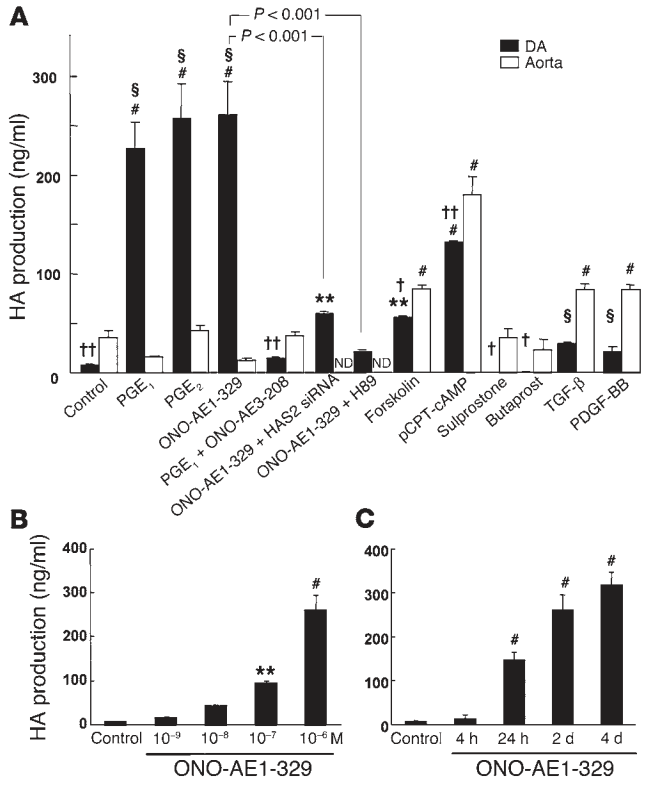

Figure 3

HA production is mediated by PGE/EP4/PKA signal in DA SMCs. (**A**) HA production after 48-hour stimulation with agents. PGE_1, PGE_2, and ONO-AE1-329 at 10^{-6} M dramatically increased HA production in the DA but not in the aorta. ONO-AE3-208 (10^{-6} M), an EP4 antagonist, almost abolished the effect of PGE_1 (10^{-6} M). The ONO-AE1-329–induced HA production was significantly attenuated by transfection with rat HAS2 siRNA (100 pmol) or H89. Forskolin (10^{-5} M) and pCPT-cAMP (10^{-4} M) significantly increased HA production in both the DA and aortic SMCs. Sulprostone (10^{-6} M), an EP1/3-selective agonist, and butaprost (10^{-6} M), an EP2-selective agonist, had little effect on HA secretion in DA SMCs. TGF-β (10 ng/ml) and PDGF-BB (10 ng/ml) significantly increased HA production in the aorta but not in the DA. n = 4–8; $^{**}P < 0.01$, $^{\#}P < 0.001$ compared with control. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\S}P < 0.001$ compared with aorta. (**B**) Dose-dependent HA production after 48-hour stimulation with ONO-AE1-329. (**C**) Time-dependent HA production stimulated by 10^{-6} M ONO-AE1-329. n = 4; $^{**}P < 0.01$, $^{\#}P < 0.001$ compared with control. ND, not done.

with ONO-AE1-329 alone (10^{-6} M) (Figure 2B). These findings indicate that EP4-induced HA production plays a critical role in SMC migration in DA.

EP4/PKA signaling profoundly increased HA synthesis in DA. We then performed an in-depth analysis of the role of PGE/EP4 signaling in HA production using cultured embryonic SMCs. PGE_1, PGE_2, and ONO-AE1-329 at 10^{-6} M dramatically increased (≥200-fold) the secretion of HA into culture media of SMCs from the DA but not from the aorta (Figure 3A). The PGE_1-mediated increase in HA production, however, was almost abolished in the presence of ONO-AE3-208 (10^{-6} M), an EP4-specific antagonist. In addition, we found that the siRNA for HAS2 also significantly inhibited the ONO-AE1-329–mediated HA production ($P < 0.001$), although the level of HA production still remained higher when compared with control (Figure 3A); however, the siRNAs for HAS1 or a nontargeting negative control siRNA did not inhibit the ONO-AE1-329–mediated HA production, although the expression of HAS1 mRNA was significantly decreased, by approximately 90% (data not shown). These data indicated that the HAS2 isoform was largely responsible for ONO-AE1-329–mediated HA production in DA SMCs. The result was consistent with previous studies demonstrating that HAS2 is a major isoform in vascular SMCs (29, 30). At 10^{-6} M, sulprostone, an EP1/3-selective agonist, and butaprost, an EP2-selective agonist, had little effect on HA secretion in DA SMCs (Figure 3A). HA production was increased by ONO-AE1-329 in a dose- and time-dependent manner (Figure 3, B and C, respectively), indicating that EP4 was responsible for PGE-mediated HA production in the DA.

We further investigated the role of EP4 downstream signaling that regulates HA production. Forskolin (10^{-5} M), a direct activator of adenylyl cyclase that increases intracellular levels of cAMP, and a cAMP-dependent PKA activator, pCPT-cAMP (10^{-4} M), significantly increased HA production in DA, although they increased HA production to a greater degree in aortic SMCs than in the DA (Figure 3A). Furthermore, H89 (10^{-5} M), a PKA-specific inhibitor, markedly reduced the ONO-AE1-329–mediated increase in HA production; it was decreased to a comparable level in the absence of ONO-AE1-329 in DA SMCs (Figure 3A). Taken together, the data indicate that PKA plays a primary role in EP4-mediated HA production in the DA.

TGF-β and PDGF-BB have been known as potent stimulators of HA production (12, 31). We found that TGF-β (10 ng/ml) or PDGF-BB (10 ng/ml) increased HA production in the DA and aortic SMCs, although the stimulatory effect of TGF-β or PDGF-BB was much weaker in the DA than that of PGE_2/EP4 (Figure 3A).

EP4 stimuli increased HAS transcripts in DA. We also examined which of the 3 HAS isoforms is/are affected by EP4 stimuli. Quantitative RT-PCR analyses revealed that ONO-AE1-329 (10^{-6} M) markedly increased the expression of Has1 and Has2 mRNAs, whereas Has3 mRNA expression slightly increased in a time-dependent manner in DA SMCs (Figure 4A).

We then investigated the role of PKA in the EP4-mediated increase in Has2 transcripts. H89 (10^{-5} M) markedly attenuated the expression level of HAS2 mRNA induced by ONO-AE1-329 after a 24-hour incubation (Figure 4B). In addition, pCPT-cAMP (10^{-4} M) significantly increased the expression level of Has2 mRNA in DA SMCs (Figure 4B). These data suggested that PKA directly regulates the expression of Has2 mRNA and that EP4/PKA signaling plays a primary role in HA synthesis in DA SMCs, at least in part, by increasing the level of Has2 transcripts. TGF-β (10 ng/ml) and PDGF-BB (10 ng/ml) also increased the expression of Has2 mRNA, although they produced much lower levels of Has2 transcripts than ONO-AE1-329 after 24-hour stimulation (Figure 4B).

EP4 stimuli are essential for ICF in DA explants. ICF, a characteristic developmental remodeling process in the DA, is required for postnatal DA closure (12, 32). We found developing ICF in mature rat DA at E21, while immature DA at E19 lacked such ICF (Figure 5A). We found that when immature rat DA was exposed to ONO-AE1-329 for 48 hours in organ culture, ICF was fully developed in DA explants and was accompanied by increased HA deposition (Figure 5B) and HAS2 transcript levels (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI28639DS1). ONO-AE1-329 also increased the number of Ki-67–positive cells in DA explants, suggesting that chronic EP4 stimuli also promoted cell proliferation (Figure 5B). In the absence of ONO-AE1-329, however,

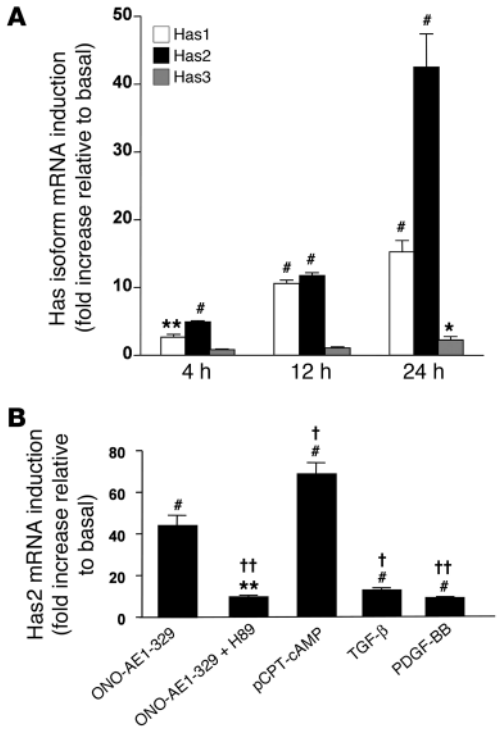

Figure 4
EP4/PKA stimuli profoundly increased the level of Has2 transcripts in the DA. (**A**) Time-dependent HAS isoform mRNA induction by ONO-AE1-329 (10^{-6} M). $n = 4–8$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$ compared with control. (**B**) Fold increase in HAS2 mRNA expression after 24-hour stimulation with ONO-AE1-329 (10^{-6} M) in the absence or presence of H89 (10^{-5} M), pCPT-cAMP (10^{-4} M), TGF-β (10 ng/ml), and PDGF-BB (10 ng/ml). $n = 4–8$. $^{**}P < 0.01$, $^{\#}P < 0.001$ compared with control. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with ONO-AE1-329.

rat DA remained immature, with visible poor ICF (Figure 5B). The intimal thickening was 2.4-fold greater in DA incubated with ONO-AE1-329 ($n = 6$) than that in control ($n = 5$; $P = 0.025$). Promotion of intimal cushion formation by ONO-AE1-329 was absent in DA explants from *EP4*-disrupted mouse embryos (Supplemental Figure 2), indicating that the effect of ONO-AE1-329 was specific for EP4.

In addition, ONO-AE1-329–mediated ICF was significantly inhibited by H89 (10^{-5} M) (Figure 5B). H89 also decreased ONO-AE1-329–mediated HA deposition and the number of Ki-67–positive cells. Furthermore, when *PKA* was overexpressed in immature rat DA explants using an adenovirus vector, in the absence of ONO-AE1-329, the neointimal cushion became thicker (Figure 5B), with an increase in HA deposition ($n = 3$), than when *GFP* was overexpressed (Figure 6A). The intimal thickening was 2.0-fold greater in *PKA*-overexpressing DA than that in *GFP*-overexpressing DA ($P = 0.025$). These data indicated that EP4/PKA stimuli promote ICF.

We then investigated whether EP4 signals play a critical role in ICF. The present study established that ICF was completely absent in DA from *EP4*-disrupted neonatal mice (Figure 5C), which was not well characterized in the previous studies (6, 7). Moreover, a marked reduction in HA production was found in *EP4*-disrupted DA, whereas a thick layer of HA deposit was present in wild-type DA (Figure 5C, lower panels). Thus, the features of *EP4*-disrupted DA (Figure 5C) were quite similar to those of immature rat DA (Figure 5A). Results from these complementary experiments (Figure 5, B and C) suggest that EP4 signaling is essential for ICF in the DA. In addition to the well-known vasodilatory role of PGE_2, our findings suggest that chronic PGE_2/EP4 stimulation promotes ICF via HA production, narrowing the vascular lumen, and thus disruption of *EP4* results in failure of permanent closure of the DA.

Has2 gene transfer was sufficient for ICF in DA explants. We then examined whether induction of HA production is sufficient for ICF in DA. When *Has2* was overexpressed in immature rat DA explants using an adenovirus vector as previously described (33), the neointimal cushion became thicker (Figure 6B), with strong HA deposition, than when *GFP* was overexpressed (Figure 6A). The size of the vascular lumen, accordingly, was significantly smaller in *Has2*-overexpressing DA (Figure 6C). The number of Ki-67–positive cells was increased in *Has2*-overexpressing DA explants (Supplemental Figure 3). When indomethacin (10^{-5} M), a COX inhibitor, was administered after *Has2* or *GFP* gene transfer, the size of the vascular lumen was further decreased, whereas the thickness of intimal cushion remained unchanged (Figure 6, B and C). Since indomethacin inhibits endogenous PGE_2 production, it is most likely that the DA was constricted after the withdrawal of the vasodilatory effect of PGE_2. In addition, when bovine testicular hyaluronidase (0.05 mg/ml) was added in culture media, EP4-stimulated ICF, shown in Figure 5B, was significantly reduced (Figure 6, D and E).

Finally, we performed similar experiments using DA from *EP4*-disrupted embryos. In *EP4*-disrupted embryos, *Has2* gene transfer induced prominent ICF, with strong HA production, in the DA compared with control (Figure 6F). The intimal cushion thickness became significantly greater in *Has2*-overexpressing DA (Figure 6G). Accordingly, these findings suggest that HA played a critical role in ICF and that *Has2* gene transfer was sufficient to rescue impaired ICF in premature DA in rat embryos as well as in genetically defective DA in *EP4*-disrupted mice.

Discussion

In addition to the well-known vasodilatory role of PGE, our findings indicate that chronic PGE stimulation via EP4 during gestation induces vascular remodeling of the DA to promote neointimal cushion formation and structural closure of the vascular lumen. Activation of the EP4/cAMP/PKA pathway increased transcription of the HAS2 gene and thus HA production. The EP4-mediated HA accumulation promoted DA SMC migration into the endothelial layer, resulting in the promotion of neointimal cushion formation. Interestingly, our findings are also reminiscent of the results in mice with disrupted *Has2* gene, which failed to form similar subintimal cushions for heart valves (34).

The physiological process of neointimal cushion formation in DA closely resembles the pathological process of neointimal thickening caused by vascular injury or atherosclerosis in adult arteries (35). In this sense, Fischer's group has demonstrated that PGE_2-EP2–mediated HAS2 induction plays an important role in neointimal thickening during atherosclerosis (29) and during failure of venous bypass grafts (36). The present study and these data indicate that PGE_2 activates the cAMP/PKA pathway via EP4 in the DA or EP2 in adult arterial SMCs, respectively, resulting in the promotion of neointimal formation accompanied by HA accumulation. We propose here that the PGE_2/EP4/PKA signal promotes ICF in the DA from late gestation and that the DA remains open by the counteracting action of EP4, i.e., vasodilation. Withdrawal of EP4 stimulation at the time of birth, by a rapid decrease in levels of

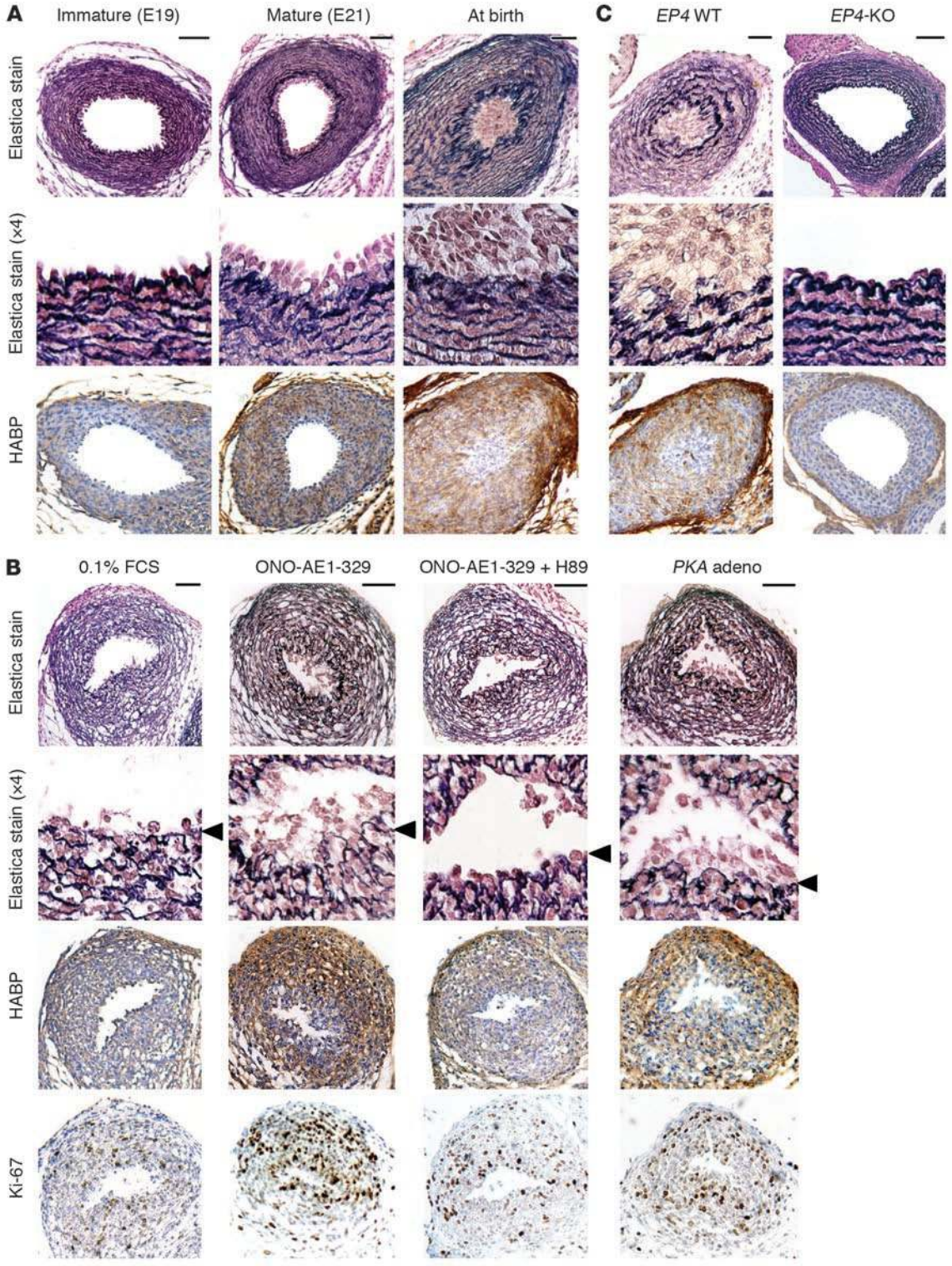

Figure 5

EP4 signaling is essential for ICF in the DA. (**A**) Developmental changes in ICF and HA production in rat DA. ICF was poor, with very little HA production at E19, whereas it became apparent with increased HA production at E21. At birth, the vascular lumen was filled by intimal cushion, and the DA was completely closed. DA intimal layers are also shown at higher magnification (middle row). HA production was visualized by by staining for HA-binding protein (HABP) (bottom row). Scale bars: 100 μm. (**B**) EP4/PKA stimuli promoted ICF in rat DA explants. ICF was fully developed and HABP -and Ki-67–positive staining was increased in DA explants in the presence of ONO-AE1-329 (10^{-6} M) or by adenovirus-mediated *PKA* gene transfer (*PKA* adeno). ONO-AE1-329–mediated ICF was suppressed by adding H89 (10^{-5} M). Arrows indicate the basement membrane of DA. (**C**) Wild-type (left) and *EP4*-KO (right) DA at birth. ICF of the vascular lumen was absent in neonatal *EP4*-KO DA. A marked reduction in HA production was found in *EP4*-KO DA, whereas a thick layer of HA deposit was present in wild-type DA.

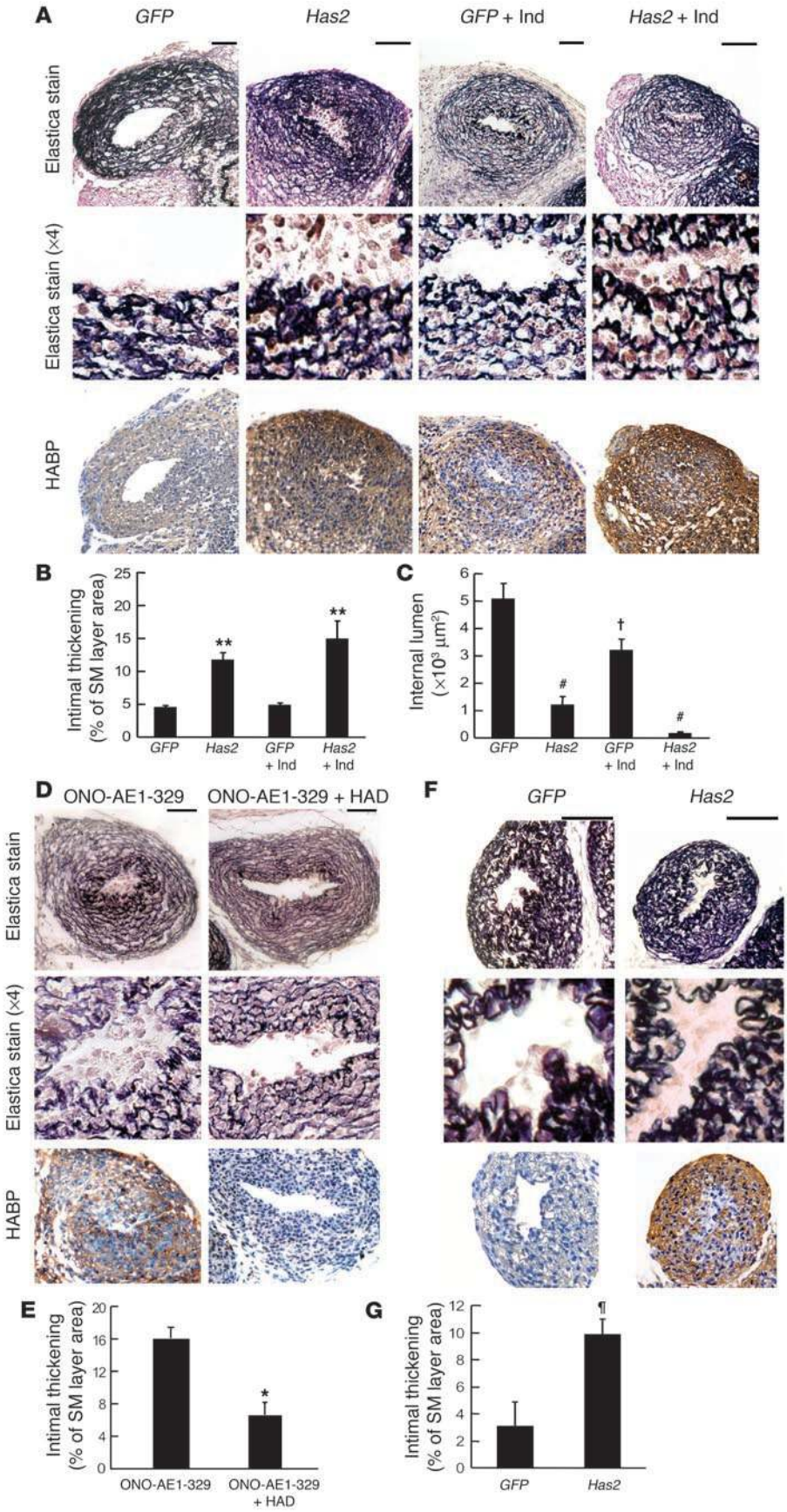

Figure 6

Has2 gene transfer promotes ICF in the DA. (**A**) Immature rat DA overexpressing *GFP* or *Has2*. Indomethacin (10^{-5} M) was administered 72 hours after *Has* infection (+Ind). Scale bars: 100 μm. (**B**) Intimal cushion thickening was expressed as the percent of whole smooth muscle layer. (**C**) The size of the vascular lumen was compared in the same experiments. The neointimal cushion became thicker and the size of vascular lumen was smaller in *Has2*-overexpressing DA. When indomethacin (10^{-5} M), a COX inhibitor, was administered 72 hours after *Has2* or *GFP* gene transfer, the size of the vascular lumen was further decreased, whereas the thickness of intimal cushion remained unchanged. $n = 5–10$; $^{**}P < 0.01$, $^{\#}P < 0.001$ compared with GFP. $^{\dagger}P < 0.05$ with indomethacin versus without indomethacin. (**D**) Similar images of immature rat DA explants at E19 that were incubated with ONO-AE1-329 (10^{-6} M) in the absence (left) or presence (right) of hyaluronidase (0.05 mg/ml). Scale bars: 100 μm. (**E**) Changes in intimal cushion thickening expressed as percent of whole smooth muscle layer in ONO-AE1-329–stimulated DA in the absence or presence of HAD. EP4-mediated ICF was significantly attenuated in DA explants in the presence of HAD. $n = 3$; $^{*}P = 0.041$. (**F**) Similar images of *EP4*-KO DA explants overexpressing *GFP* or *Has2* at E18.5. Scale bars: 100 μm. (**G**) Changes in intimal cushion thickening in *Has2*-overexpressing *EP4*-KO DA. ICF was significantly increased in DA explants by *HAS2* gene transfer. $n = 3$; $^{¶}P = 0.035$. SM, smooth muscle.

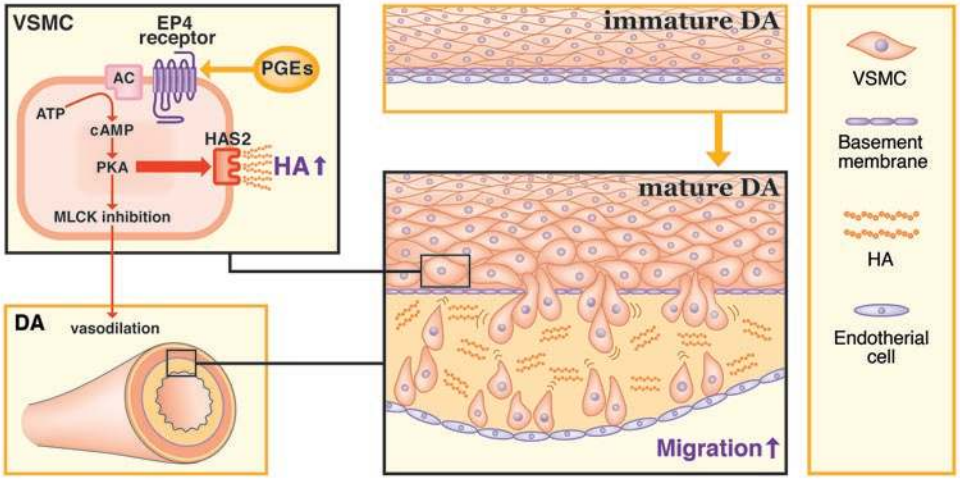

Figure 7

A schematic model of chronic PGE_2/EP4 stimulation in DA. The PGE_2/EP4/cAMP/PKA signal promotes HA production and thus ICF in the DA at late gestation, and the DA remains open by the counteracting action of EP4, i.e., vasodilation. Withdrawal of EP4 stimulation at the time of birth, by a rapid decrease in levels of circulating PGE_2, would reduce its vasodilatory action on the DA, leading to rapid and complete closure of the DA. Accumulated HA still remains active and promotes SMC migration, leading to complete closure of DA. Dual consequences of EP4 stimulation during a perinatal period are thus integrated to prepare for postnatal closure of the DA. AC, adenylyl cyclase; MLCK, myosin light chain kinase.

circulating PGE_2, would reduce its vasodilatory action on the DA, leading to rapid and functional closure of the DA. Accumulated HA still remains active and promotes SMC migration, leading to complete closure of the DA. The dual consequences of EP4 stimulation during a perinatal period are thus integrated to prepare for postnatal closure of the DA (Figure 7). When a defect or developmental delay occurs in EP4-mediated ICF, it may cause PDA, as shown in *EP4*-knockout mice (6, 7) and premature infants (16, 37). In this regard, mice with disruption of both *COX-1* and *COX-2* also exhibit PDA (8). The present study may thus provide a rationale to explain the paradoxical phenotype found in both *EP4*- and *COX-1/COX-2*-knockout mice.

We found that the number of Ki-67–positive cells was increased in both EP4-stimulated and *Has2*-overexpressing DA explants. Previous studies have demonstrated that SMC proliferation is an important cellular process for ICF (1, 14, 38). Since a considerable number of studies have demonstrated that HA promotes cell proliferation (36), accumulated HA is likely to promote DA SMC proliferation. Using a [^{3}H]thymidine incorporation assay, however, we found that neither ONO-AE1-329 nor HA itself increased [^{3}H]thymidine incorporation in rat DA SMCs (Supplemental Figure 4). Therefore, cell proliferation did not influence the increase in ONO-AE1-329–mediated migration, at least at a cellular level. We currently cannot explain the discrepancy between the results of the in vitro and in vivo experiments. HA may affect other types of cells in ex vivo DA that secrete a proliferating factor. We need to further investigate the role of HA in SMC proliferation in the DA.

The potential outcome of the current results is the emergence of an alternative strategy for PDA treatment. COX inhibitors including indomethacin, which principally inhibit the production of prostaglandins, have been widely used (4, 5). However, our data indicate that chronic PGE inhibition by COX inhibitors may prevent the anatomical DA remodeling. In fact, it is known that pregnant mothers taking indomethacin have a higher incidence of delivering infants with PDA (9, 10). Long-term use of COX inhibitors for PDA treatment might have a potent adverse effect on ICF as well. Our results from adenovirus-mediated *Has2* gene transfer, instead, suggest that ICF may be promoted by HA production and does not necessarily require EP4 stimulation thereafter. These results imply that HAS-mediated ICF may be achieved even after birth when EP4 signals are decreased. Importantly, Mason et al. have demonstrated that preventing fibronectin-dependent ICF would be a feasible manipulation to cause PDA as a mode of treatment of congenital heart diseases (32). It should be noted that fibronectin and HA are not the sole constituents of extracellular matrix in DA intimal cushion (12, 38). For example, versican, an HA-binding proteoglycan, plays an important role in proliferation and migration of vascular SMCs (26). Tenascin, a hexameric glycoprotein, also has been known to regulate vascular SMC proliferation (39). Therefore, it remains to be determined whether HA induction alone is sufficient to form mature and functional cushion in the human infant. Nevertheless, the present study suggests that HAS activation in combination with PGE_2 inhibition might be an effective strategy to treat PDA, especially in premature infants.

Methods

Animals and materials. We used Wistar rat embryos from timed-pregnant mothers (SLC). Generation and phenotypes of *EP4*-knockout mice have been described previously (6). All fetal 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, Kyoto University, and the Medical University of South Carolina. ONO-AE1-329 and ONO-AE3-208 were provided by ONO Pharmaceutical Co. PGE_2, sulprostone, butaprost, pCPT-cAMP, forskolin, elastase type II-A, trypsin inhibitor type I-S, bovine serum albumin V, penicillin-streptomycin solution, DMEM, and HBSS were obtained from Sigma-Aldrich. PGE_1, H89, and biotinylated HA-binding protein were from Calbiochem. HA, hyaluronidase (from bovine testis), PDGF-BB, indomethacin, and 10% buffered formalin were from Wako. Cyto Quick, Mayer's hematoxylin, and htica van Gieson stain were from Muto Pure Chemicals Co. Latex-labeled HA-binding protein was from Fujirebio Inc. TGF-β was from Immuno-Biological Laboratories Co. Anti–Ki-67 antibodies, peroxidase blocking reagent, and streptavidin peroxidase were from Dako. Collagenase II was from Worthington Biochemical Corp. Collagenase/dispase was from Roche Diagnostics. Fetal calf serum was from Biological Industries. siRNAs for HAS1 and HAS2 (HP GenomeWide siRNA) and PCR primers for HAS3 (QuantiTect Primer Assay) were from QIAGEN. TaqMan Gene Expression Assays and TaqMan Rodent GAPDH control reagents kits were from Applied Biosystems.

Table 1
Oligonucleotides for quantitative RT-PCR

GeneGenBank accession no.						Size (bp)
		Forward (5′–3′)		Reverse (5′–3′)		
Ptgfr	NM_013115	TGCCCACTTTTCCTGGGCAGT	494–514	TGCTTGGATTTGATAATCTCGGT	654–676	183
IP	XM_218457	CTCGTTTGTGCCGACCTCTCG	398–418	ACAGACAACACAACCAGAACT	640–660	236
Tbxa2r	NM_017054	GTGGCCTTCGGGCTCATGTTT	958–978	CCTACGAGCTGAACCATCAT	1,102–1,121	164
		Primer name				
EP2	NM_031088	TaqMan Gene Expression Assays (Rn00579419_m1)				Unknown
EP3	NM_012704	TaqMan Gene Expression Assays (Rn00579419_m1)				Unknown
EP4	NM_032076	TaqMan Gene Expression Assays (Rn00583420_m1)				Unknown
Has1	NM_172323	TaqMan Gene Expression Assays (Rn00597231_m1)				Unknown
Has2	NM_013153	TaqMan Gene Expression Assays (Rn00565774_m1)				Unknown
Has3	AB097569	Quanti Tect Primer Assay (QT00192857)				Unknown

Ptgfr, *$PGF_{2\alpha}$ receptor*; *IP*, *PGI_2 receptor*; *Tbxa2r*, *thromboxane A_2 receptor*.

Quantitative RT-PCR. Total RNA was isolated from pooled tissues of 1 littermate of Wistar rat embryos, and generation of cDNA and RT-PCR analysis were done as described previously (40, 41). The primers for PCR amplification were designed based on the rat nucleotide sequences of prostanoid receptors and HAS isoforms (Table 1).

Primary culture of rat DA SMCs. Vascular SMCs in primary culture were obtained from the DA and aorta of Wistar rat embryos at E21. Minced tissues were transferred to 800 μl of collagenase/dispase enzyme mixture as described previously (42).

Transfection of DA SMCs with siRNA. Two double-stranded 21-bp siRNAs to the selected region of HAS1 cDNA or HAS2 cDNA were purchased from QIAGEN. The antisense siRNA sequences targeting HAS1 were 5′-UAGAAGAGCCUCAGCACCGdTdG-3′ and 5′-UUGAAGGCUACCCAGUAUCdTdT-3′. The antisense siRNA sequences targeting HAS2 were 5′-UUGGUUACCCAUGAAUUCCdTdG-3′ and 5′-UAUGAAAUUAACGACAAUGdGdT-3′. AllStars Negative Control siRNA (QIAGEN) was used as a control nonsilencing siRNA. DA SMCs were serum deprived and transfected with siRNA (100 pmol) using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the instructions of the manufacturer. Cells were kept serum free for an additional 24 hours before the initiation of the experiments.

SMC migration assay. The migration assay was performed using 24-well Transwell culture inserts with polycarbonate membranes (8-μm pores; Corning Inc.) without any coating materials. The DA SMCs were harvested with trypsin-EDTA, resuspended in serum-free DMEM, and distributed at a density of 1×10^5 cells/100 μl in the inserts. The cells were allowed to settle in serum-free DMEM for 1 hour before the addition of agents in the lower chamber. Under basal conditions, the lower chambers were filled with 600 μl serum-free DMEM. Then SMCs were allowed to migrate to the underside of the insert's membrane at 37°C/5% CO_2. At the end of the experiment, the cells were fixed in 10% buffered formalin. SMCs were stained with Cyto Quick (Muto Pure Chemicals), and cells on the upper surface of the membrane were mechanically removed with a cotton swab. Cells that migrated onto the lower surface of the membrane were manually counted from 3 different fields (0.5 mm^2/field) under a microscope.

Quantitation of HA. The amount of HA in the cell culture supernatant was measured by a latex agglutination method based on the specific interaction of HA with the latex-labeled HA-binding protein from bovine cartilage (Fujirebio Inc.). HA was quantified in duplicate according to the instructions of the manufacturer using 2.5-μl aliquots of the conditioned cell culture medium using the HITACHI 7070 analysis system (Hitachi) at an 800-nm wavelength.

Cell proliferation assays. Cell proliferation was measured in DA SMCs using [^{3}H]thymidine incorporation assays as described previously (42).

Adenovirus construction. Recombinant adenovirus, driving expression of murine *Has2*, was prepared as described previously (33). For construction of adenoviral vectors, full-length cDNA-encoding mouse *alpha 1 catalytic subunit of PKA* was cloned into the shuttle vector for construction of the adenoviral vector harboring *PKA* using an Adeno-X adenovirus construction kit (Clontech).

Organ culture. Fetal arteries including the DA and the aortic arch arteries were removed from the thoracic cavity. Adhesive connective tissues surrounding arteries were carefully removed. Cut segments were then incubated with ONO-AE1-329 (10^{-6} M) for 48 hours in DMEM containing 0.1% fetal calf serum. For *Has2* or *PKA* adenovirus infection, cut segments were infected with 1.2×10^7 PFU/ml of each adenovirus for 2 hours in DMEM containing 0.5% fetal calf serum. After infection, the segments were cultured up to 6 days with humidified 5% CO_2 and 95% ambient mixed air at 37°C. Explants were then fixed in 10% buffered formalin and embedded in paraffin. The sectioned segments in the middle portion of the DA were analyzed histochemically. Morphometric analyses were performed using Win ROOF software version 5.0 (Mitani Corp.). ICF was defined as (neointima area/media area) × 100%. The average of at least 3 sections was used as the value for each tissue.

Tissue staining and immunohistochemistry. Paraffin-embedded blocks containing DA tissues were prepared as previously described (42). HA staining was done as previously described (43). Briefly, the specimens were deparaffinized, rehydrated, and incubated for 5 minutes in peroxidase blocking reagent (Dako) to inactivate endogenous peroxidases. Tissue sections were incubated with biotinylated HA-binding protein (Calbiochem; 8 μg/ml) or anti–Ki-67 antibody (1:200 dilution; Dako) at room temperature for 2 hours or at 4°C for 16 hours, respectively. The slides were sequentially incubated with streptavidin peroxidase at room temperature for 30 minutes and DAB chromogen substrate solution (Nichirei). The slides were counterstained with Mayer's hematoxylin.

Statistics. Data are presented as mean ± SEM of independent experiments. Statistical analysis was performed between 2 groups by unpaired 2-tailed Student's *t* test or unpaired *t* test with Welch correction and among multiple groups by 1-way ANOVA followed by Neuman-Keuls multiple comparison test. A *P* value of less than 0.05 was considered significant.

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1. Smith, G.C. 1998. The pharmacology of the ductus arteriosus. *Pharmacol. Rev.* **50**:35–58.
2. Cotton, R.B., Stahlman, M.T., Kovar, I., and Catterton, W.Z. 1978. Medical management of small preterm infants with symptomatic patent ductus arteriosus. *J. Pediatr.* **92**:467–473.
3. Gomez, R., et al. 1980. Management of patent ductus arteriosus in preterm babies. *Ann. Thorac. Surg.* **29**:459–463.
4. Friedman, W.F., Hirschklau, M.J., Printz, M.P., Pitlick, P.T., and Kirkpatrick, S.E. 1976. Pharmacologic closure of patent ductus arteriosus in the premature infant. *N. Engl. J. Med.* **295**:526–529.
5. Heymann, M.A., Rudolph, A.M., and Silverman, N.H. 1976. Closure of the ductus arteriosus in premature infants by inhibition of prostaglandin synthesis. *N. Engl. J. Med.* **295**:530–533.
6. Segi, E., et al. 1998. Patent ductus arteriosus and neonatal death in prostaglandin receptor EP4-deficient mice. *Biochem. Biophys. Res. Commun.* **246**:7–12.
7. Nguyen, M., et al. 1997. The prostaglandin receptor EP4 triggers remodelling of the cardiovascular system at birth. *Nature.* **390**:78–81.
8. Loftin, C.D., et al. 2001. Failure of ductus arteriosus closure and remodeling in neonatal mice deficient in cyclooxygenase-1 and cyclooxygenase-2. *Proc. Natl. Acad. Sci. U. S. A.* **98**:1059–1064.
9. Norton, M.E., Merrill, J., Cooper, B.A., Kuller, J.A., and Clyman, R.I. 1993. Neonatal complications after the administration of indomethacin for preterm labor. *N. Engl. J. Med.* **329**:1602–1607.
10. Hammerman, C., et al. 1998. Indomethacin tocolysis increases postnatal patent ductus arteriosus severity. *Pediatrics.* **102**:E56.
11. Gittenberger-de Groot, A.C. 1977. Morphology of the normal human ductus arteriosus. In *The ductus arteriosus, report of the 75th Ross Conference on Pediatric Research*. M.A. Heymann and A.M. Rudolph, editors. Ross Laboratories. Columbus, Ohio, USA. 3–9.
12. Rabinovitch, M. 1996. Cell-extracellular matrix interactions in the ductus arteriosus and perinatal pulmonary circulation. *Semin. Perinatol.* **20**:531–541.
13. Hinek, A., Mecham, R.P., Keeley, F., and Rabinovitch, M. 1991. Impaired elastin fiber assembly related to reduced 67-kD elastin-binding protein in fetal lamb ductus arteriosus and in cultured aortic smooth muscle cells treated with chondroitin sulfate. *J. Clin. Invest.* **88**:2083–2094.
14. Slomp, J., et al. 1992. 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.* **93**:25–39.
15. Gittenberger-de Groot, A.C., van Ertbruggen, I., Moulaert, A.J., and Harinck, E. 1980. The ductus arteriosus in the preterm infant: histologic and clinical observations. *J. Pediatr.* **96**:88–93.
16. Gittenberger-de Groot, A.C., Strengers, J.L., Mentink, M., Poelmann, R.E., and Patterson, D.F. 1985. Histologic studies on normal and persistent ductus arteriosus in the dog. *J. Am. Coll. Cardiol.* **6**:394–404.
17. Tada, T., et al. 1985. Human ductus arteriosus. A histological study on the relation between ductal maturation and gestational age. *Acta Pathol. Jpn.* **35**:23–34.
18. Wong, S.T., et al. 2001. Adenylyl cyclase 3 mediates prostaglandin E(2)-induced growth inhibition in arterial smooth muscle cells. *J. Biol. Chem.* **276**:34206–34212.
19. Fujino, T., et al. 2002. Effects of the prostanoids on the proliferation or hypertrophy of cultured murine aortic smooth muscle cells. *Br. J. Pharmacol.* **136**:530–539.
20. Bulin, C., et al. 2005. Differential effects of vasodilatory prostaglandins on focal adhesions, cytoskeletal architecture, and migration in human aortic smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **25**:84–89.
21. Smith, G.C., Coleman, R.A., and McGrath, J.C. 1994. Characterization of dilator prostanoid receptors in the fetal rabbit ductus arteriosus. *J. Pharmacol. Exp. Ther.* **271**:390–396.
22. Narumiya, S., Sugimoto, Y., and Ushikubi, F. 1999. Prostanoid receptors: structures, properties, and functions. *Physiol. Rev.* **79**:1193–1226.
23. Leonhardt, A., et al. 2003. Expression of prostanoid receptors in human ductus arteriosus. *Br. J. Pharmacol.* **138**:655–659.
24. Waleh, N., et al. 2004. Prostaglandin E2-mediated relaxation of the ductus arteriosus: effects of gestational age on G protein-coupled receptor expression, signaling, and vasomotor control. *Circulation.* **110**:2326–2332.
25. Murphy-Ullrich, J.E. 2001. The de-adhesive activity of matricellular proteins: is intermediate cell adhesion an adaptive state? *J. Clin. Invest.* **107**:785–790.
26. Evanko, S.P., Angello, J.C., and Wight, T.N. 1999. Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **19**:1004–1013.
27. Toole, B.P., Wight, T.N., and Tammi, M.I. 2002. Hyaluronan-cell interactions in cancer and vascular disease. *J. Biol. Chem.* **277**:4593–4596.
28. Itano, N., et al. 1999. Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J. Biol. Chem.* **274**:25085–25092.
29. Sussmann, M., et al. 2004. Induction of hyaluronic acid synthase 2 (HAS2) in human vascular smooth muscle cells by vasodilatory prostaglandins. *Circ. Res.* **94**:592–600.
30. Evanko, S.P., et al. 2001. Platelet-derived growth factor stimulates the formation of versican-hyaluronan aggregates and pericellular matrix expansion in arterial smooth muscle cells. *Arch. Biochem. Biophys.* **394**:29–38.
31. Papakonstantinou, E., Karakiulakis, G., Roth, M., and Block, L.H. 1995. Platelet-derived growth factor stimulates the secretion of hyaluronic acid by proliferating human vascular smooth muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* **92**:9881–9885.
32. Mason, C.A., et al. 1999. Gene transfer in utero biologically engineers a patent ductus arteriosus in lambs by arresting fibronectin-dependent neointimal formation. *Nat. Med.* **5**:176–182.
33. Ghatak, S., Misra, S., and Toole, B.P. 2005. Hyaluronan constitutively regulates ErbB2 phosphorylation and signaling complex formation in carcinoma cells. *J. Biol. Chem.* **280**:8875–8883.
34. Camenisch, T.D., Schroeder, J.A., Bradley, J., Klewer, S.E., and McDonald, J.A. 2002. Heart-valve mesenchyme formation is dependent on hyaluronan-augmented activation of ErbB2-ErbB3 receptors. *Nat. Med.* **8**:850–855.
35. Newby, A.C., and Zaltsman, A.B. 2000. Molecular mechanisms in intimal hyperplasia. *J. Pathol.* **190**:300–309.
36. Van den Boom, M., et al. 2006. Differential regulation of hyaluronic acid synthase isoforms in human saphenous vein smooth muscle cells: possible implications for vein graft stenosis. *Circ. Res.* **98**:36–44.
37. Gittenberger-de Groot, A.C. 1977. Persistent ductus arteriosus: most probably a primary congenital malformation. *Br. Heart J.* **39**:610–618.
38. Slomp, J., et al. 1997. Differentiation, dedifferentiation, and apoptosis of smooth muscle cells during the development of the human ductus arteriosus. *Arterioscler. Thromb. Vasc. Biol.* **17**:1003–1009.
39. Cowan, K.N., Jones, P.L., and Rabinovitch, M. 2000. Elastase and matrix metalloproteinase inhibitors induce regression, and tenascin-C antisense prevents progression, of vascular disease. *J. Clin. Invest.* **105**:21–34.
40. Minamisawa, S., et al. 2003. Atrial chamber-specific expression of sarcolipin is regulated during development and hypertrophic remodeling. *J. Biol. Chem.* **278**:9570–9575.
41. Tadano, M., et al. 2005. Congenital semilunar valvulogenesis defect in mice deficient in phospholipase C epsilon. *Mol. Cell. Biol.* **25**:2191–2199.
42. Yokoyama, U., et al. 2006. 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.* **290**:H1660–H1670.
43. Lokeshwar, V.B., et al. 2001. Stromal and epithelial expression of tumor markers hyaluronic acid and HYAL1 hyaluronidase in prostate cancer. *J. Biol. Chem.* **276**:11922–11932.