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Current Perspectives on Pathobiology of the Ductus Arteriosus

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Abstract

The ductus arteriosus (DA) shunts blood away from the lungs during fetal life, but at birth this shunt is no longer needed and the vessel rapidly constricts. Postnatal persistence of the DA, patent ductus arteriosus (PDA), is predominantly a detrimental condition for preterm infants but is simultaneously a condition required to maintain systemic blood flow for infants born with certain severe congenital heart defects. Although PDA in preterm infants is associated with significant morbidities, there is controversy regarding whether PDA is truly causative. Despite advances in our understanding of the pathobiology of PDA, the optimal treatment strategy for PDA in preterm infants is unclear. Here we review recent studies that have continued to elucidate the fundamental mechanisms of DA development and pathogenesis.

Keywords: Patent ductus arteriosus; Vascular development

Introduction

During early embryogenesis, the paired dorsal aortae give rise to a set of symmetric aortic arch arteries. As development progresses, these arteries go through an elegant transformation to form the asymmetric mature vessels of great arteries, head vessels and proximal aorta. Many forms of congenital heart disease involve aberrant remodeling of these arteries [1]. The left sixth aortic arch artery persists to become the ductus arteriosus (DA), a unique blood vessel linking the pulmonary and systemic circulations *in utero*. The DA shunts blood away from the lungs during fetal life, but at birth this shunt is no longer needed and the vessel rapidly constricts. Postnatal persistence of the DA, patent ductus arteriosus (PDA), is predominantly a detrimental condition for preterm infants but is simultaneously a condition required to maintain systemic blood flow for infants born with certain severe congenital heart defects such as hypoplastic left heart syndrome.

Although PDA in preterm infants is associated with significant morbidities including intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC) and bronchopulmonary dysplasia (BPD) [2-4], there is intense controversy regarding whether PDA is truly causative [5]. The use of prophylactic indomethacin is effective in reducing symptomatic PDA and surgical PDA ligation but it does not improve mortality or reduce the incidence of BPD or NEC [6]. Prophylactic indomethacin does lower the incidence and severity of IVH [7-9]. Studies have demonstrated improved neurodevelopment outcomes at 4 years of age but not at 18 months or 12 years of age [7,10,11]. Despite advances in our understanding of the pathobiology of PDA, the optimal treatment strategy for PDA in very low birth weight preterm infants is unclear [5]. Is it possible indomethacin and other cyclooxygenase inhibitors are having detrimental effects on non-DA tissues and thus confounding the beneficial effects of closing the DA? Are there alternative pathways to target for treatment of PDA? The hope for future targeted therapies and for the prevention of PDA requires knowledge of the fundamental mechanisms controlling its development and pathogenesis. Here we review recent studies that expanded our understanding of the DA, building a foundation upon which to base future investigations that address some of these questions.

Genetic Considerations in PDA Pathobiology

PDA in the mouse model system

Elegant methods allowing targeted genetic manipulation have made the mouse an ideal system in which to investigate genes involved in closure of the DA. Initial studies that focused on prostaglandin pathway components were based upon both the important role of prostaglandin E_2 (PGE_2) in maintaining patency of the DA *in utero*, and the successful pharmacologic manipulation of postnatal PGE_2 levels to either maintain patency or induce closure of the DA. Several genes in the prostaglandin pathway have been investigated in knockout mice, as shown in Table 1.

Prostaglandin-endoperoxide synthase 1 (*Ptgs1*) and 2 (*Ptgs2*) encode bi-functional enzymes, with both cyclooxygenase (COX) and peroxidase activities, that catalyze the rate limiting step in the production of prostaglandins from arachidonic acid. Mice lacking both isoforms of *Ptgs* have a normal DA *in utero*, suggesting the presence of maternal and/or placental sources of PGE_2 [12]. Although the absence of Ptgs1 does not affect neonatal closure of the DA, 35-57% of *Ptgs2*-/- mice die shortly after birth as a result of a PDA [12,13]. Interestingly, mortality and incidence of PDA was increased in *Ptgs2*-/- mice when one copy of *Ptgs1* was also inactivated, suggesting compensation by Ptgs1 for loss of Ptgs2. Neonatal mice with combined deficiency of Ptgs1 and Ptgs2 have PDA and substantial perinatal mortality [12,14]. A Ptgs2 mouse model has been generated in which cyclooxygenase activity was inhibited without affecting peroxidase activity [13]. These mice showed normal DA closure after birth. The paradoxical persistent ductal patency seen in knockout mice following elimination of PGE_2 synthesis or signaling can be explained by the developmental role of PGE_2 in preparing the fetal DA for postnatal closure in response to increasing oxygen tension [15].

Genes encoding other components of the prostaglandin

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pathway have also been studied. Mice lacking *Ptger4* (encoding the prostaglandin E receptor 4) have PDAs resulting in neonatal death [16-18]. In addition, stimulation of EP4 promotes DA closure by enhancing intimal thickening [19]. Thus, PGE_2 binding to EP4 may have two opposite effects on closure of the DA: vascular smooth muscular relaxation and intimal thickening. *Slco2a1*-/- mice, in which the prostaglandin transporter gene has been deleted, fail to close the DA after birth and die prior to postnatal day 2 [20]. Finally, deletion of the *Hpgd* gene, which encodes hydroxyprostaglandin dehydrogenase 15-(NAD), attenuates the normal postnatal decrease in PGE levels, resulting in PDA and neonatal death [21,22]

Genes important in vascular smooth muscle development have also been studied using knockout mice. Smooth muscle myosin heavy chain (encoded by *Myh11*) contracts in response to the postnatal rise in oxygen levels. *Myh11*-/- mice have delayed closure of the DA, but full closure does occur [23]. Closure of the DA in these mice suggests that DA smooth muscle cells may contract using non-muscle myosin components. Myocardin is a transcriptional coactivator that is important in both cardiovascular development and adaptation of the cardiovascular system to hemodynamic stress. The selective deletion of the myocardin gene in neural crest derived smooth muscle cells populating the cardiac outflow tract and great arteries resulted in mice that are born alive, but die before postnatal day 3 secondary to a PDA [24]. Selective deletion of *Jag1* in smooth muscle cells resulted in mice that are alive at birth but become cyanotic in the early neonatal period [25]. The mortality of these mice was 50% on day 1 and 100% by day 2. Histologic examination exhibited a significant defect in DA smooth muscle cell differentiation and 95% of the mice had an identifiable PDA on postmortem examination. Deletion of Brahma-related gene 1 (*Brg1*- a component of the chromatin-remodeling complex) in smooth muscle cells resulted in death from cardiovascular anomalies, including PDA and ventricular septal defects, in one third of *Brg1*-/- offspring [26]. Finally, *Tcfap2b* encodes a transcription factor expressed in the neural crest cells, from which the DA originates. The first study of *Tcfap2b*-/- mice found that they were born alive, but died in the first 24 hours of life from what was attributed to renal abnormalities [27]. Ductal anatomy was not examined in these mice. Interestingly, the morphology of the fetal DA after mid-gestation is not altered in *Tcfap2b*- /- mice compared to wild type mice [28]. However, *Tcfap2b*-/- mice die within the first day of life with a PDA and signs of pulmonary over-circulation [29]. *Tcfap2b*-/- mice exhibit decreased DA expression of calponin and hypoxia-inducible factor 2α, which are markers of differentiated smooth muscle cells. Thus, Tcfap2b in mice appears to play a role in maturation of the muscular layer of the DA. In addition, transcription factor AP-2 beta regulates the expression of both EPAS1 (also known as hypoxia inducible factor 2 alpha), which is involved in oxygen sensing, and endothelin-1, which is a potent vasoconstrictor of DA smooth muscle [28].

These studies highlight two critical pathways involved in permanent closure of the DA, smooth muscle formation and regulation of muscle contraction. Many genes are involved in the development of the various smooth muscle layers in the DA. The control of DA closure also requires a complex set of regulators. Shortly after birth, when the pulmonary vascular resistance drops dramatically, the DA constricts to the point of complete luminal closure. Identifying and characterizing the regulatory networks controlling this unique behavior remains an area of active research.

PDA in human infants

PDA can be divided into 2 groups: 1) a relatively rare condition seen in term infants that can exist as part of a constellation of other physical anomalies (syndromic PDA) or as an isolated finding (non-syndromic PDA); and 2) a common condition present in very preterm infants in which the vast majority of PDA cases are non-syndromic and have a significant developmental component, i.e., the PDA would likely not be present if the preterm infant had been born at term.

Term infants

Several genetic studies have focused on PDA associated with syndromes in small cohorts of subjects, often excluding preterm infants. A syndrome of thoracic aortic aneurysm and PDA [30,31] has been linked to a region of chromosome 16p12.2-13.13 [32]. Mutations in *MYH11*, a gene encoding smooth muscle myosin heavy chain 11 (located at 16p13.11) have been identified as one cause for this syndrome [33]. Missense mutations in the smooth muscle α-actin gene (*ACTA2*) also present as a syndrome of thoracic aortic aneurysms and PDA [34]. This association between abnormal contractile proteins and PDA is not surprising, given the interactions between actin and myosin required to generate contraction in muscle cells. Finally, mutations in the gene *TFAP2B* (*Tcfap2b* in the mouse) have been found to result in Char syndrome, a rare disorder characterized by facial dysmorphism, hand anomalies, and PDA [35,36]. *TFAP2B* mutations have also been reported in familial non-syndromic cases of PDA, which likely reflects phenotypic variability in Char syndrome [37,38]

Preterm infants

Two retrospective twin studies, investigating the concordance rates of PDA in monozygotic compared to dizygotic preterm twins, have suggested a familial component for PDA in preterm infants. The first study included 70 monozygotic twin pairs and 89 dizygotic twin pairs and reported a 93% heritability of PDA requiring indomethacin therapy and 48% heritability for PDA requiring surgical ligation [39]. The second study included 99 monozygotic twin pairs and 333 dizygotic twin pairs and found that genetic factors or a shared environmental factor accounted for 76% of the variance in liability to PDA, with only 12% being accounted for by genetic factors [40]. Thus, although these studies both identified a familial/heritable contribution to PDA, there was disagreement with respect to the magnitude of the genetic contribution.

In addition to twin studies that can quantify heritability (but not risk from a specific locus), candidate gene studies have identified specific gene polymorphisms associated with PDA in preterm infants. For example, the p allele of the PvuII pP polymorphism (rs2234693) in the estrogen receptor alpha gene (*ESR1*) is associated with a decreased risk of PDA [41]. A polymorphism in the interferon gamma gene (+874) (rs2430561 T allele) was also associated with a decreased risk for PDA [42]. This result may help explain the clinical finding that bacterial infection is associated with both reduced DA closure and increased reopening following initial closure [43,44]. Finally, polymorphisms in *TFAP2B* (rs987237, G allele,) and in *TRAF1* (TNF receptor-associated factor 1) (rs1056567, T allele) have been reported to be associated with the presence of a PDA. An additional analysis considering combinations of neighboring alleles identified *PTGIS* (prostaglandin I2 synthase) as a gene containing polymorphisms (rs493694, G allele and rs693649, A allele) associated with the absence of a PDA (i.e., protective). The association of sequence variants in *TFAP2B* with PDA in preterm infants supports the concept that common variants in the same genes that are responsible for syndromic forms of PDA may be responsible for isolated, non-syndromic forms of PDA given the right environmental or developmental context. *TFAP2B* genotype is also

Gene Name	Symbol	Alias	% neonatal PDA	% neonatal mortality	Reference
Prostaglandin-endoperoxide synthase 1	*Ptgs1*	Cyclooxygenase-1	0		[12]
Prostaglandin-endoperoxide synthase 2	*Ptgs2*	Cyclooxygenase-2	33	35	[12]
Prostaglandin-endoperoxide synthase 2	*Ptgs2*	Cyclooxygenase-2	57	57	[13]
Prostaglandin-endoperoxide synthase 2[a]	*Ptgs2*		0	0	[13]
Ptgs2 (-/-) and *Ptgs1* (+/-)			74	79	[12]
Ptgs2 (-/-) and *Ptgs1* (-/-)			100	100	[12,14]
Prostaglandin E synthase	*Ptges*	Microsomal prostaglandin E synthase-1	0		[62]
Prostaglandin E receptor 4	*Ptger4*	PGE receptor EP4	>95	>95	[16-18]
Solute carrier organic anion transporter family, member 2A1	*Slco2a1*	Prostaglandin transporter		100	[20]
Hydroxyprostaglandin dehydrogenase 15-(NAD)	*Hpgd*	Prostaglandin dehydrogenase	>95	>95	[21, 22]

[a]cyclooxygenase activity was inhibited without affecting peroxidase activity

Table 1: Summary of prostaglandin pathway mutant mouse models relevant to the ductus arteriosus.

associated with altered levels of mRNA encoding three ion channels that are expressed in the DA: *CACNB2* (calcium L channel beta subunit), *CACNA1* (calcium T channel) and *KCNA2* (KV1.2 potassium channel) [45]. These channels may be potential targets for therapeutic manipulation.

Gene expression profiles in the DA

Technological advances and progress in bioinformatics analysis over the past fifteen years have enabled genome-wide transcriptome analysis. Several groups have recently leveraged this by utilizing microarrays to examine DA development and function. There are four published reports that take an unbiased approach to determine the gene expression profiles of the DA [46-49]. The methodology and controls for each study differed, in part, due to the disparate questions being asked by each group of investigators. In addition to a broad survey of DA gene expression, the goals of these studies included determining the effect of maternal vitamin A exposure and the effects of oxygen and birth on DA gene expression. The results of these studies have revealed some common themes but also some differing and sometimes conflicting results.

There has only been one microarray study analyzing the human DA. Mueller and colleagues performed a comparative analysis of several vessels including the DA and pulmonary artery harvested at the time of surgery [47]. These vessels were obtained from patients ranging between 1 and 807 days of age and included vessels that were stented for different indications. Placement of a stent undoubtedly causes changes in gene expression that are unlikely to reflect aspects of normal physiologic closure in either term or preterm infants. The broad range of ages precluded the authors from being able to group the samples as biologic replicates. Together, these factors make the study's findings of little relevance to the development of the DA or function of the perinatal DA. The remaining three studies were performed using vessels isolated from the rat. In 2006, Costa and colleagues published a study examining the effects of oxygen and birth on DA gene expression at E19 and at 3 hours of life [46]. In 2007, Yokoyama and colleagues administered maternal vitamin A to increase retinoic acid levels at late gestation. They reported DA gene expression profiles at E19, E21, and at 3 to 6 hours of life [49]. In 2011, Jin and colleagues compared gene expression profiles of the DA and aorta E19 and E21 [48]. Rat gestation is approximately 21 days. Thus, E19 represents a late preterm gestational age and E21 represents term. The characteristics and platforms utilized in each study are listed in Table 2. While the methodology was different, important data can be obtained by grouping the differentially expressed genes into functional categories.

Structural genes

Changes in cytoskeletal gene expression during late gestation may reflect a critical step in DA vascular smooth muscle differentiation in preparation for the rapid changes at the time of birth. Some of these genes include those encoding myofibrillar proteins and genes associated with muscle differentiation. Contraction of vascular smooth muscle is mediated by myosin II. Myosin II consists of both heavy chains and light chains. The heavy chain classically associated with smooth muscle is encoded by the gene *Myh11* [50]. There are two groups of light chains – essential light chains (ELCs) and regulatory light chains (RLCs). The RLCs are encoded by the *Myl2* (a.k.a. MLC2v), *Myl5*, *Myl7* (a.k.a. MLC2a), and *Myl9* genes and can be phosphorylated by myosin light chain kinase (MLCK) [51,52]. Costa and colleagues found that relative to the aorta, the late preterm E19 DA had higher expression of myofibrillar genes such as *Myl2* and *Myh7*. By 3 hours of life, under normoxic conditions, these genes were no longer differentially expressed in the DA compared with the aorta. Yokoyama and colleagues took a slightly different experimental approach. They analyzed changes in gene expression in the DA over time but did not normalize the expression to that of any other tissue or vessel. This makes it difficult to make direct comparisons, and based on the Yokoyama data, it is difficult to determine whether these myofibrillar genes are predominant in all E19 vessels. Yokoyama found that not only *Myh7* as in the Costa study, but also other myofibrillar genes such *Actc1*, *Myl7*, and *Tnnt2* were more highly expressed in the late preterm E19 DA relative to the DA at later stages (E21 and 3-6 hours after birth). Jin and colleagues found that the myofibrillar genes *Myh11*, *Myl6*, *Actg2*, *Tpm1* were highly expressed in both the E19 and E21 (term) DA relative to aortic expression. *Myl6* encodes an ELC and thus is not a MLCK target, but may have a phosphorylation independent role in the DA [52]. In contrast to the Costa study, this study showed that *Myl2* and *Myh7* were downregulated at both ages in the DA relative to the aorta along with similar downregulation of other myofibrillar genes such as *Myl7*, *Actc1*, *Tnnt2*, *Myh6*, and *Tnni3*. Although the specificity of MLCK-mediated RLC phosphorylation is known for some tissues, the RLC in the DA is unknown. These microarray studies provide a clue that although *Myl2* and *Myl7* are sometimes considered cardiac-specific RLCs, they may play a role in the DA. It is difficult to explain this discrepancy in *Myl2* and *Myh7* between the Costa and Jin studies. Coceani proposed that this may be due to rat strain differences [53]. It is plausible that genetic background may explain differences in the magnitude of fold changes but seems unlikely to explain completely contradictory findings. The exact mechanistic role of these proteins in the DA has not been described with the exception of *Myh1* [23,33].

Author, Year	Affymetrix Platform	GEO accession number	Species	Age	Vessels	Experimental conditions	Reference
Costa, 2006	U34	GSE3290	Rat	E19, 3 Hours on P0	DA, Aorta	Prenatal maternal hyperoxia	[46]
Yokoyama, 2007	U34A	GSE3420	Rat	E19, E21, 3-6 hours on P0	DA	Prenatal maternal Vitamin A	[49]
Mueller, 2009	HG_U133_Plus_2	–	Human	1-807 days after birth	DA, Pulmonary artery	Stents	[47]
Jin, 2011	U34A	GSE3422	Rat	E19, E21	DA, Aorta	–	[48]

Table 2: Summary of the characteristics of published ductus arteriosus gene expression profiles.

As discussed in more detail below, myosin light chain (MLC)-mediated contraction in vascular smooth muscle is dependent on MLC phosphorylation. This is modulated by MLCK and MLC phosphatase (MLCP). Calcium sensitization occurs when MLCP activity is inhibited thus increasing the sensitivity of MLCK to calcium. A number of signaling pathways, including the Rho-Rho kinase system, regulate MLCP activity. Rho signaling pathways, acting through Rho-associated coiled-coil containing kinases (ROCKs), decrease MLCP activity and thus result in calcium sensitization [51,54]. Costa et al. report upregulation of the Rho-Rho kinase system in the neonatal DA. The gene encoding the RhoB GTPase (*Rhob*) was upregulated in the newborn DA relative to the aorta and also upregulated compared with the fetal DA. The gene encoding the Rho downstream effector molecule, *Rock2*, was upregulated in both the neonatal DA and aorta relative to the respective fetal vessel. Neither Yokoyama nor Jin detected differential expression in Rho-Rho-kinase genes.

Ion channels

Calcium and potassium ion channels are critical mediators of DA closure (discussed below). The Costa, Yokoyama and Jin studies revealed several differentially expressed potassium channel genes. *Kcnk3* encodes the TWIK-related acid-sensitive K1 potassium channel (TASK 1) and interestingly, this potassium channel is phosphorylated in a pulmonary artery smooth muscle ET-1 signaling pathway [55]. TASK-1 controls resting membrane potential and modulates sensitivity to vasoactive factors. Costa found that *Kcnk3* was upregulated in the neonatal DA relative to the fetal DA. Yokoyama et al. identified the ATP-sensitive potassium channel component gene *Abcc9* to be upregulated in both the E21 and P0 DA relative to the E19 DA. *Abcc9* encodes the sulfonylurea receptor subunit *Sur2*. *Sur2* and the inwardly rectifying potassium channel subunit Kir6.1 (*Kcnj8*) can function as interacting subunits to form a functional ATP-sensitive potassium channel [56]. Yokoyama et al. found that *Kcnj8* was unchanged between E19 and E21 but subsequently upregulated by 3 to 6 hours after birth. Consistent with the Yokoyama data, *Kcnj8* was highly expressed in the E19 and E21 DA relative to the aorta. Yokoyama et al. also reported another inwardly rectifying potassium channel subunit, Kir1.1 (*Kcnj1*) that was present in the DA but essentially unchanged between these three ages. They also found that the potassium channel tetramerisation domain containing 12 gene (*Kctd12*) increased progressively from E19 to E21 and to P0. The functional significance of this is unclear although recent evidence shows that this protein may function as a GABAB receptor subunit [57]. This is particularly interesting as GABA receptors can regulate both inwardly rectifying potassium channels and voltage gated calcium channels.

Jin and colleagues discovered other membrane ion channels that were highly expressed in the DA relative to the aorta. The genes encoding the Na^+/K^+ ATPase pump beta subunit (*Atp1b1*) and an auxiliary α2δ subunit of the L-type voltage sensitive calcium channel (*Cacna2d1*) were highly expressed in the DA at both E19 and E21 relative to aortic expression. L-type calcium channels regulate vascular smooth muscle contraction and are essential for DA constriction [50].

Signaling molecules

During development, specification and differentiation occur as the bilaterally symmetric aortic arch arteries undergo morphological and functional changes. The left 6th aortic arch artery transforms into the DA and acquires a very different contractile potential compared to the other aortic arch artery derivatives such as the carotid arteries and portions of the aortic arch. Endothelin (ET-1) signaling has been implicated as a potential vasoconstrictive effector of oxygen in the DA and other vessels [50,58,59] (discussed below). Although Jin and colleagues found ET-1 (*Edn1*) to be highly expressed in both the E19 and E21 DA relative to aortic expression, neither Costa nor Yokoyama detected significant expression of *Edn1* in the DA. There are two transmembrane G protein-coupled endothelin receptors, endothelin A (ET_A) and endothelin B (ET_B). These two receptors are encoded by the *Ednra* and *Ednrb* genes. The ET_B receptor inhibits cell growth and functions as a scavenger, clearing ET-1, and thus inhibiting endothelin-dependent vasoconstriction [58]. Costa et al. report downregulation of *Ednrb* in the newborn DA relative to late preterm offspring. Downregulation of ET_B would be expected to result in increased levels of ET-1 and thus have a vasoconstrictive effect. In the Yokoyama study, neither *Ednra* nor *Endnb* were found to be in a dominant gene cluster at any of the tested age groups although by qPCR, ET-1 and endothelin converting enzyme (*Ece1*) were upregulated in the postnatal DA relative to fetal levels. *Gata2* may increase expression of ET-1 [60]. Costa et al. found *Gata2* was upregulated in the newborn DA compared with the late preterm E19 DA. Carboxypeptidase A3 (*Cpa3*) has been reported to catalyze the degradation of ET-1[61]. Costa et al. report that *Cpa3* was upregulated in the neonatal DA compared with late preterm DA. This seems to contradict the hypothesis that ET-1 contributes to DA constriction after birth, as one would expect higher carboxypeptidase A3 levels to result in less ET-1. This may be consistent if the relative expression of ET-1 is higher than the carboxypeptidase A3 mediated degradation.

Costa and colleagues propose that angiotensin II may be a vasoconstrictive effector in the DA. They found a modest increase in the expression of the angiotensin II type 1a receptor (*Agtr1a*) in the

neonatal DA compared with late preterm DA. In follow up experiments using late preterm mouse DA explants, they showed that exposure to angiotensin II resulted in a transient dose-dependent contraction. Yokoyama and colleagues found that the angiotensin II type 2 receptor (*Agtr2*) is upregulated at E21 compared with E19 and then decreases to levels modestly higher than E19 levels by 3 to 6 hours after birth. Similarly, Jin et al. found that *Agtr2* expression in the DA increased from E19 to E21 when normalized to aortic expression but interestingly was expressed as an aortic-dominant transcript rather than a DA-dominant one. None of these groups found a difference in the angiotensin II precursor angiotensinogen (*Agt*). Waleh et al. have subsequently identified polymorphisms in the angiotensin II type 1 receptor (*Agtr1*) associated with PDA in preterm infants [45]. Surprisingly, none of the endothelin or angiotensin related genes mentioned above were highly expressed in the late preterm or neonatal DA after normalization to aortic expression, suggesting that they may be important for general vascular development and not unique to the DA.

Prostaglandins (PG) play a prominent role in maintaining DA vasodilation. Arachidonic acid is metabolized by PGH_2 synthase (a.k.a. cyclooxygenase or COX) to prostaglandin H_2. Prostaglandin H_2 can be metabolized by several enzymes but, most relevant to the biology of the DA, it is metabolized by PGE synthase to prostaglandin E_2 [62]. As discussed in more detail below, the prostaglandin receptor EP4 is the most likely PG receptor relevant to DA smooth muscle. Costa and colleagues did not report enrichment in any of the prostaglandin receptor genes or in genes encoding the enzymes involved in prostaglandin synthesis. Despite this, they did find upregulation of *Alox15* in the neonatal DA relative to the E19 DA. *Alox15* encodes arachidonate 15-lipoxygenase (previously known as 12S-lipoxygenase), an alternative metabolic pathway for PG precursor arachidonic acid. This upregulation in *Alox15* around the time of birth may not be DA-specific as this upregulation was also seen in the aorta. Neither Yokoyama nor Jin report differential expression of *Alox15*. Yokoyama and colleagues reported the EP4 prostaglandin receptor (*Ptger4*) in their term (E21) dominant cluster where it was upregulated compared with the E19 expression levels. Expression levels of *Ptger4* seemed to then modestly decrease by 3 to 6 hours after birth. Jin and colleagues reported *Ptger4* to be highly expressed in both the E19 and E21 DA relative to aortic expression. Yokoyama et al. report no apparent change in COX-1 (*Ptgs1*), but show that COX-2 (*Ptgs2*) gene expression increases between E19 and E21 and then remains fairly constant in the first few postnatal hours. Neither Costa nor Jin report differential expression of *Ptgs1* or *Ptgs2*.

The effects of prostaglandins, nitric oxide, and carbon monoxide are mediated through the second messengers, cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) in DA endothelial and smooth muscle cells (discussed below). Phosphodiesterases (PDEs) catalyze the degradation of both cGMP and cAMP. The expression of several PDEs, including *Pde1a*, *Pde1b*, *Pde1c*, *Pde3a*, *Pde3b*, *Pde4d* and *Pde5a* have been described in the sheep and baboon [45,63]. A role for *Pde5a* mediating vasoconstriction in DA smooth muscle cells has been proposed [64]. Costa et al. found that *Pde4b* was one of the most highly expressed genes in the postnatal DA relative to the aorta and that its expression increased from E19 to 3 hours after birth. This high level of expression may not be limited to the DA as they also found the same age-related pattern of *Pde4b* expression in the aorta. Yokoyama et al. found that *Pde1*, *Pde2*, *Pde3*, *Pde4b*, and *Pde5a* were expressed in the DA in at least one of the three time points they analyzed. They found both *Pde5a* and *Pde4b* in the group of genes most highly expressed at term (E21) dominant cluster. Subsequently, *Pde5a* and *Pde4b* expression decreases by 3 to 6 hours after birth. *Pde1* and *Pde2* did not change significantly in the DA at the three examined ages. *Pde3* expression progressively increases from E19 to 3 to 6 hours postnatally. Neither the E19 nor the E21 gene expression profiles reported by Jin et al. revealed any PDE genes that were highly expressed relative to aortic expression.

Other genes

Many other differentially expressed genes were reported in these three studies. Their biological significance remains to be determined. Notably absent from these studies is *Tcfap2b*. This gene is expressed in DA smooth muscle and has been implicated in human Char syndrome and in a developmental DA transcriptional network [28,65]. It is possible that *Tcfap2b* is only expressed earlier than the ages examined by these three groups. Notably, several extracellular matrix genes were also expressed. Yokoyama reported that lysyl oxidase (*Lox*), is upregulated in the E21 and postnatal DA relative to the E19 DA. Lysyl oxidase is strongly hypoxia induced and has been associated with vascular smooth muscle development [66-68]. Costa et al. did not report differential expression of *Lox*. Yokoyama et al. reported that fibronectin (*Fn1*) was the most highly expressed gene comparing term E21 to late preterm E19 expression and this high expression persisted after birth. Data from the Costa study support this finding. Fibronectin has been shown to play a role in intimal cushion formation [69,70]. Similar to *Lox*, Jin et al. found *Fn1* to be an aortic dominant gene when compared to DA expression. These findings for both *Lox* and *Fn1* may not be DA-specific as Jin et al. found both to be aortic dominant genes.

There are several important caveats to these studies. All of the rat studies used pooled samples. While pooling samples is convenient, important information is lost. If there are outlier values, pooling the mRNA may show a false positive finding of a differentially expressed gene. After pooling the samples, there is no way to identify this outlier thus masking the lack of a difference. The studies discussed above may have overcome this by pooling dozens of vessels. When assaying thousands of transcripts, statistical analysis is paramount. Statistical power is lost when samples are pooled and thus these studies likely report many false positive or false negative findings. Finally, the platform used in these studies was an early rat microarray. Not only was there incomplete probe coverage for every rat gene, but there was also incomplete probe annotation at the time of publication. The three rat studies have deposited their primary datasets (Table 2) into public databases, which will enable reanalysis of the expression data with the better annotation information available today. These microarray studies looked at a mixed cell population. In the future, a more refined approach may be to perform transcriptional profiling using specific cell populations such as smooth muscle cells, endothelial cells and fibroblasts [71] to gain insight into cell-specific mechanisms of DA development and function.

Molecular Considerations in PDA Pathobiology

Oxygen and PDA

The exquisite sensitivity of the DA to oxygen is one of its defining characteristics. Functional closure by contraction of circular and longitudinal smooth muscle cells was postulated by Virchow in 1856, but demonstration of the contractile effects of oxygen was not established until the 1940s and 1950s [72,73]. In those studies, exposure to increased oxygen tension or oxygen bubbles in the circulation caused DA constriction *in vivo* and *in vitro*. Transient oxygen exposure induced brief DA constriction that was reversible under low oxygen conditions [72,74,75]. Despite the historical nature of these

observations, decades of subsequent studies have failed to provide consensus on the mechanism for oxygen-induced DA constriction, possibly because multiple interacting pathways are involved [5,76-78].

Oxygen sensing by cytochrome P450 enzymes

Recent efforts have focused on elucidating the sequence of steps from the detection of oxygen levels to triggering a vascular response. In one well-developed scheme, a member of the cytochrome P450 (CYP) enzyme system acts as the sensor for acute changes in oxygen tension. Coceani and colleagues have extensively detailed the proposal that a monooxygenase reaction by certain CYP enzymes serves as the initial step for oxygen signaling in the DA [79]. Prevention of oxygen-induced DA constriction by carbon monoxide and various CYP inhibitors, together with localization of CYP3A in DA smooth muscle cells, support this concept. While the majority of CYP enzymes are expressed in the liver, members of the CYP2 and CYP4 family are also expressed in the cardiovascular system where they catalyze the production of epoxyeicosatrienoic (EET) and hydroxyeicosatetraenoic (HETE) acids, respectively, to modulate inflammation, angiogenesis, and vascular tone [80].

The mechanisms by which CYP enzymes might transduce oxygen levels are not yet resolved. Baragatti et al. recently examined a potential role for CYP enzymes as the source of an endothelium-derived hyperpolarizing factor (EDHF) [81] to maintain relaxation of the mouse fetal DA. A survey of CYP expression demonstrated the presence of CYP4A, CYP4B, and CYP2J (but not CYP2C) family members by conventional RT-PCR. CYP2J6 and CYP2J9 proteins were immunolocalized in the muscular media of the DA wall, with increased CYP2J6 expression in the intima and sub-endothelial region. DA explants metabolized arachidonic acid into EETs via the epoxygenase activity of CYP2J but basal concentrations were low. Although EET levels marginally increased in response to a shift in oxygen concentration from 2.5 to 30% in the culture media, this aspect of CYP function is unlikely to serve as a sensor for oxygen-induced constriction since EETs are typically associated with a vasodilatory response. An exception exists in the lung, where EETs are implicated in pulmonary vasoconstriction [80]. It is unknown whether EETs play a contractile or vasodilatory role in the DA, as this has not been directly tested. Likewise, the products of CYP4A (20-HETE and others) were either absent or did not have a definitive role in the DA. However, 12-HETE, the product of an active 12- lipoxygenase pathway or CYP4B activity, was detected in abundance. The function of 12-HETE in the DA was not determined. Although the goal of this study was to identify the CYP enzymes responsible for EDHF in the DA, insightful data were also provided on the spectrum of CYP activities that contribute to DA regulation. In a recent follow-up study, inhibition of arachidonic acid epoxygenation (presumably by CYP2J) completely blocked oxygen-induced constriction of the isolated mouse DA, lending support to the concept of CYP-mediated oxygen sensing by EETs or an unknown intermediary [82]. A similar response was noted following inhibition of the 12-lipoxygenase pathway. Together, these data show that monooxygenase and lipoxygenase metabolites of arachidonic acid that are produced in the DA wall have the ability to respond to changes in oxygen tension and influence DA tone.

Direct transduction and execution of oxygen signals by a single CYP enzyme metabolite may not be necessary. Instead, Coceani and colleagues have long proposed that oxygen-induced DA constriction occurs by a multistep process [79] that is mediated via separate mechanisms for oxygen sensing (by CYP enzymes) and effectors of the oxygen response. Endothelin-1 (ET-1) is proposed as the effector, based on its potent vasocontrictive effects, the local oxygen-stimulated production of ET-1 in the DA wall, and inhibition of its actions on the DA by receptor blockade (by BQ123 or others) or by inhibition of ET-1 synthesis (by phosphoramidon) [79,83,84]. In mice, deletion of the ET_A receptor for ET-1 results in decreased oxygen-induced DA constriction [85], further supporting its role as an effector. However, postnatal closure of the ET_A null DA occurs normally [85] and ET_A blockade in other species does not affect postnatal DA constriction [86], raising the possibility that CYP enzymes may lead to vasoconstriction by acting through other pathways. Baragatti et al. have now extended their original supposition for a CYP3A source of DA regulation [87]. In that paper, CYP3A13 was the only CYP3A family member detected in the DA and was developmentally regulated, with declining levels at term gestation. By confocal fluorescence imaging, CYP3A13 was localized in the endoplasmic reticulum and plasma membrane throughout the DA wall. Mice with targeted deletion of the *Cyp3a* gene had normal postnatal DA closure but its size was somewhat smaller than wild type, and the luminal surface lacked the usual initimal growth and endothelial undulations that are characteristic of the closing DA. Myography studies showed that the isolated DA of *Cyp3a* null mice was poorly responsive to increased oxygen tension in the bath. On closer inspection, deletion of *Cyp3a* primarily affected the tonic response to increased oxygen tension, but the periodic, phasic contractile response was maintained. This finding is in contrast to deletion of the ET_A receptor, where disruption of both tonic and phasic effects was observed [85]. This again supports the notion that CYP enzymes may modulate signaling pathways independent of ET-1. The *Cyp3a* null DA also had diminished contractile response to ET-1 compared to wild type. In both instances, the DA of animals treated with retinoic acid (used to promote maturation and oxygen sensitivity) had restoration of their contractile response to either oxygen or ET-1. Even the preterm DA on day 17 of gestation developed contractile responses that were similar to the term DA after retinoic acid exposure. Cultured smooth muscle cells from *Cyp3a* null mice had delayed intracellular calcium accumulation compared to wild type.

The findings described above support the proposed role of CYP3A as an oxygen mediator in the DA. There are other data that do not support this model, including the observation that *Cyp3a* null fetuses developed greater DA constriction in response to maternal hyperoxia and closed normally after birth. In addition, *Cyp3a* expression declined with advancing gestation – opposite to what would be expected for an oxygen sensor. However, there are at least eight CYP3A homologues in the mouse [88] and it is possible that other CYP3A family members are present in the murine DA (J.Reese; unpublished data). The identity of the hypothetical monoxygenase product that serves as messenger between the putative sensor (CYP3A13) and effector (ET-1) remains unknown, as acknowledged by the authors [82,87]. An alternative explanation might be found in the CYP epoxygenase products 11,12- and 14,15-EET, which can activate large conductance Ca^{2+}-activated potassium channels (BK_{Ca}) to stimulate vascular smooth muscle [89]. Another possibility is the interaction of ET-1 with voltage-gated potassium channels [90], although these pathways might have parallel functions in the closing DA [91,92]. Further investigation is required to determine whether links exist between these signaling systems to actively mediate oxygen-induced DA constriction.

Oxygen sensing through redox state and ion channels

Kovalcik summarized contemporary concepts on oxygen-induced DA closure in the 1960s, stating, "The most obvious and most important metabolic effect of oxygen is in relation to the terminal steps

of the electron transport chain" [74]. Depolarization of the DA cell membrane by oxygen exposure was later reported in 1981 [93]. Based on observations in pulmonary arteries and other oxygen-sensitive tissues, a role for potassium channels in the early phase of oxygen-induced DA constriction was established using pharmacological inhibitors [94] and electrophysiology techniques [95]. A model emerged whereby exposure to increased oxygen tension stimulates inhibition of the voltage-gated (Kv) potassium channels that are involved in maintenance of resting membrane potential, causing subsequent membrane depolarization. This, in turn, leads to activation of voltage-dependent L-type calcium channels and entry of calcium to initiate contraction [96]. These *in vitro* findings were ultimately confirmed in whole animal studies and in human DA specimens [92]. In contrast to the multistep proposal by Coceani and colleagues, oxygen-induced DA closure might therefore be accomplished through a different series of sensors (mitochondria), mediators (peroxide), and effectors (Kv channels and L-type calcium channels) [97].

A redox reaction in smooth muscle cells may represent the earliest step in oxygen sensing by the DA [98], triggering downstream effects on redox-sensitive potassium channels [97]. Mitochondria are critical to this scheme, where the activity of specific mitochondrial enzymes (e.g., NADPH oxidase), mitochondrial energetics and the electron transport chain (ETC), and the generation of reactive oxygen species (ROS), including superoxide anion and hydrogen peroxide, determines the cellular redox status [99]. Inhibition of proximal (e.g., rotenone, for complex I), midpoint (e.g., antimycin A, for complex III), or distal (e.g., cyanide, for cytochrome oxidase) components of the mitochondrial ETC alters DA tone in humans, mammals, and birds [100,101]. Recent work by Dzialowski and colleagues confirm this concept for oxygen sensing in the DA of different avian species (chick and emu) and predict its importance in reptiles and other vertebrates [102]. In short, the overall scheme purports that oxygen stimulates mitochondrial production of ROS, and that inhibitors of different ETC complexes or mitochondrial enzymes can block this step. Peroxide (or other ROS) then inhibit redox-sensitive Kv channels in the plasma membrane, causing depolarization. This results in opening of inositol triphosphate (IP_3)-sensitive sarcoplasmic reticulum (SR) calcium stores [91], L-type calcium channels and store-operated channels (SOCs), allowing the influx of calcium. Increased intracellular calcium binds calmodulin (CaM), and the calcium-CaM complex activates myosin light-chain (MLC) kinase (MLCK). Activated MLCK then phosphorylates the MLC leading to actomyosin stimulation and muscle contraction. In opposition to this cascade, MLC phosphatase (MLCP) dephosphorylates MLC, allowing smooth muscle cell relaxation. Villamor and colleagues recently extended these findings in the chick DA to show that there is a parallel maturation of sensor, mediator, and effector functions [103]. Controversy exists regarding the nature and direction of the ROS signal that is generated during oxygen stress, along with the timing, methods, and target molecules to be assessed [78,104]. Although there is no consensus yet on the actual mechanisms by which redox changes alter vascular tone [99], this appears to be the most likely first step in oxygen signaling for DA closure.

Activation of Rho kinase, a family of small GTPases that act through ROCKs, has recently gained attention as an important pathway in DA regulation. The Rho kinase system affects vascular tone by modulating the balance between MLC kinase and phosphatase activities. In pulmonary vessels, hypoxia increases the GTP-bound (active) form of the small G-protein RhoA. This stimulates Rho kinase, which in turn, inhibits MLC phosphatase, thereby prolonging MLC phosphorylation and increasing calcium sensitization and smooth muscle contraction. Calcium sensitization yields sustained vasoconstriction independent of changes in cytosolic calcium levels and is stimulated by common vasoactive G-protein coupled receptor agonists. Inhibitors of Rho kinase lead to pulmonary vascular relaxation and prevention of pulmonary hypertension. The role of Rho kinase in hypoxic pulmonary vasoconstriction prompted interest in its potential contribution to oxygen-induced DA constriction, where it was expected to have an opposite effect. Compared to the role of RhoA/Rho kinase in the lungs, Costa and colleagues found upregulation of *RhoB* and *Rock2* expression in the DA of newborn rats [46]. Rho activation was stimulated by increased oxygen tension rather than hypoxia, as in the lung, and it was RhoB rather than RhoA that served as the initial mediator. As predicted, inhibition of Rho kinase by fasudil or similar agents blocked oxygen-induced DA constriction in rabbits [105]. A follow-up study identified RhoB and ROCK-1 as critical mediators of oxygen sensing in the rabbit and human DA [54]. RhoA, RhoB, ROCK-1, and ROCK-2 were present in the human DA and upregulated by oxygen. Approximately one-third of oxygen-mediated DA tone was attributed to Rho-mediated calcium sensitization. In the term DA, oxygen-stimulated Rho kinase effects were mimicked by the redox mediator, peroxide, and blocked by mitochondrial ETC inhibitors. Immaturity of the mitochondrial ROS system in the preterm rabbit DA was compounded by failure to upregulate Rho kinase expression in response to oxygen. Similar to hypoxic pulmonary vasoconstriction, a signaling scheme was envisioned that incorporates Rho/Rho kinase as an alternate pathway, independent of Kv and calcium channel signaling, to initiate smooth muscle constriction via its effects on MLC phosphatase [54].

Clyman et al. showed that RhoA, RhoB, and ROCK-1 are present in the fetal lamb DA, with increasing expression of RhoA in the more mature DA, but not aorta [106]. Rho kinase inhibition relaxed the isolated DA under normoxic and hypoxic conditions. Approximately 50% of normoxic tension was resistant to calcium depletion, suggesting the importance of calcium sensitizing mechanisms and the role of Rho kinases or tyrosine kinases for oxygen-induced DA constriction in sheep. More recent studies in the rat [107] and chick DA confirm the importance of Rho kinase actions on MLC phosphatase for calcium sensitization and share the view that an alternative pathway for oxygen-induced DA constriction is available and utilizes Rho signaling as an effector [100,103]. Rho kinase inhibitors may therefore provide a useful approach to maintain DA patency, although untoward side effects may limit its applicability.

Extracellular calcium entry is the principal mechanism for increased $[Ca^{2+}]_i$ and the final common pathway for DA smooth muscle constriction. L-type calcium channels are the main source for oxygen-stimulated increases in calcium. However, internal release of calcium from IP_3-sensitive SR stores has also been demonstrated and may precede calcium influx by L-type calcium channels [91]. Conversely, release of calcium from ryanodine-sensitive SR stores does not appear to be involved. Increased $[Ca^{2+}]_i$ in the DA is also the result of transient receptor potential channels (TRPCs) that are presumed to form SOCs. Inhibition of TRPC transcription or SOC function causes diminished oxygen-induced DA constriction [105,106]. Increased $[Ca^{2+}]_i$ is thus dependent on SR release and L-type channel stimulation, with calcium repletion through SOCs. Calcium entry across the plasma membrane via reverse-mode function of the Na^+/Ca^{2+} exchanger is an additional mechanism involved in DA smooth muscle response to oxygen. In sheep, pharmacological perturbation of SR replenishment or Na^+/Ca^{2+} exchange function eliminated differences in tone between the immature and mature DA under both normoxic and hypoxic

conditions, suggesting a potential therapeutic strategy. In contrast, efforts to pharmacologically manipulate L-type calcium channels were not successful in the premature DA under hypoxic conditions [106]. In a more recent paper, Thébaud et al. show that L-type calcium channels are themselves an oxygen-sensitive channel, similar to the aforementioned subset of Kv channels. In this study, stimulation of L-type channels did not have an effect on the term rabbit DA or human DA cells, but caused the preterm DA to behave like the term DA, including increased oxygen-induced constriction, increased whole-cell calcium current, and increased $[Ca^{2+}]_i$. L-type channels were expressed and physiologically capable of response in preterm tissues, but were not activated by oxygen exposure alone [108]. Previous studies showed that reduced expression and function of the oxygen-sensitive Kv1.5 and Kv2.1 channels might explain failure of the premature DA to close. Transfer of Kv channels into the preterm DA could partially restore its response, to approximately 50% of term levels [109]. In contrast, pharmacological activation of the premature DA L-type channel restored full oxygen responsiveness [108]. This intrinsic sensitivity of the L-type channel to oxygen adds another layer to the complex mechanisms for oxygen-mediated DA regulation.

A role for other calcium channels in oxygen-induced DA constriction is becoming apparent. T-type calcium channels are also members of the voltage-dependent family of calcium channels that regulate calcium influx. There was initial disagreement regarding the role of T-type calcium channels in the DA, but the presence of multiple family members has been demonstrated in the rat, where the α1G subunit was predominantly expressed [110]. In a recent study, Akaike et al. showed that the α1G subunit of the T-type channel is upregulated in rat DA cells with increased oxygenation or in neonates compared to term fetuses. Disruption of T-type channel function with a specific pharmacological agent or α1G-specific siRNAs resulted in reduced smooth muscle cell migration, decreased $[Ca^{2+}]_i$ accumulation, and impaired thickening of the intimal layers of cultured DA explants. Pharmacological inhibition also partially inhibited oxygen-induced constriction of isolated DA rings and delayed closure of the postnatal DA in newborn offspring. Overexpression of α1G promoted smooth muscle cell migration [111]. The importance of these findings was reinforced by a more recent study in humans that identified risk factors for PDA based on predisposing polymorphisms in the *TFAP2B* gene, since abnormalities in *TFAP2B* are associated with PDA in mice and humans (Char syndrome) [28,29,65]. The purpose of this study was to understand the mechanisms for failed PDA closure after treatment with prostaglandin inhibitors [45]. Genes that contribute to the increased risk of persistent PDA were identified. Here, the presence of the rs2817399 (A) allele of *TFAP2B* in human DA tissues was associated with decreased expression of specific calcium- and potassium-channel genes, including the Kv1.2 channel, the beta-2 isoform of the L-type calcium channel, and the α1G isoform of the T-type calcium channel [45]. Together, these studies implicate T-type channels as important members of the oxygen-induced events that regulate DA closure.

Prostaglandins and PDA

A role for prostaglandins in fetal DA regulation was initially suspected when DA constriction occurred *in utero* in pregnant women that were treated with salicylates or indomethacin [112,113]. Prostaglandins or their inhibitors were found to have potent effects on DA tone in animal models [114-117]. In 1976, two clinical trials subsequently established the effectiveness of indomethacin for PDA closure in premature infants [118,119]. Ibuprofen was found to have similar effects on DA closure [120,121], but clinical trials in preterm infants were not reported for another two decades [122-124].

Prostaglandins and ductus arteriosus regulation

Prostaglandins are synthesized by the cyclooxygenase enzymes COX-1 (*Ptgs1*) and COX-2 (*Ptgs2*) and are critical mediators of DA patency and closure. The COX products prostacyclin (PGI_2), and more importantly, PGE_2, have well-established roles as vasodilators of the DA [5]. However, there are inconsistencies regarding the predominance of COX-1 or COX-2 in DA regulation among different species. In mice, targeted deletion of both COX isoforms (*Ptgs1; Ptgs2* double knockout) causes uniform lethality in the immediate newborn period with a large PDA despite high levels of inspired oxygen [12,14]. Targeted deletion of COX-1 alone has little or no effect on DA closure, while COX-2 deletion makes an arguably stronger impact, resulting in PDA in 35% of offspring [12]. Trivedi et al. found increased COX-2 expression with advancing gestation and after birth, and suggested that reduced COX-2 expression in the DA of premature offspring prevented its postnatal constriction [125]. COX-2 may be coupled with downstream microsomal PGE synthases that reinforce its preferential role in prostaglandin production in the DA [62,126]. Chronic pharmacological inhibition of COX-2 mimics COX-2 deletion and also results in PDA [127,128]. Chronic inhibition of both COX isoforms results in a large PDA, similar in caliber to the COX double knockout mouse, but only if the inhibitors are given later in gestation, and not during early DA development [128].

The presence of a PDA in COX deficient or chronically COX-inhibited offspring is unexpected, since brief exposure to NSAIDs causes DA constriction. There are several competing theories that address this apparent contradiction. Some evidence suggests that NO or other vasodilatory mediators are upregulated in the absence of prostaglandins [126]. However, inhibition of NO synthesis did not rescue the PDA phenotype of knockout or chronically COX inhibited mice [128]. Alternatively, it is possible that prostaglandins play an important role in a developmental vascular program that dictates formation of the DA's contractile apparatus [14,128]. At an earlier stage in gestation, Srivastava and colleagues demonstrated the presence of a similar transcriptional program, this time under the control of TFAP2B, which modulates ET-1 and Hif2α in the DA [28], and which may be important in the human DA [45]. Based on this premise, a recent study in sheep and mice with either chronic exposure to PGE_2 or chronic COX inhibition, respectively, showed that PGE has a unique role in the development of DA contractility that is distinct from its role as a vasodilator [15]. In that study, chronic exposure of the fetal DA to PGE_2 *in vitro* increased the expression of L-type calcium channels (CACNα1c, CACNβ2) and the potassium channel genes *Kcnj8* (Kir6.1 or K_{ATP8}) and Kv1.5 (*Kcna5*) (which regulate oxygen-induced constriction), without affecting the genes that regulate Rho-kinase–mediated calcium sensitization. Conversely, chronic COX inhibition (and PGE depletion) decreased the DA's *in vitro* contractile response to stimuli that use L-type calcium channels and potassium channels, whereas the response to stimuli that act through Rho kinase-mediated pathways was not significantly affected. Chronic exposure to COX inhibitors *in utero* decreased expression of these same L-type calcium channels and K^+-channel genes, without affecting Rho kinase–associated genes [15]. Together, these observations implicate an important subset of genes that act as downstream effectors of a putative developmental program, where PGE_2 plays an important role in the expression of specific pathways that are necessary for the DA's oxygen-induced closure after delivery.

The role of COX enzymes in the human DA is less clear since

suitable tissue specimens are difficult to obtain. Nevertheless, Koehne and colleagues studied autopsy samples from fetuses of 11 - 38 weeks of gestation and found an increase in COX-1 expression with advancing maturity. COX-1 immunostaining was present in the endothelium, intima, and media, and was developmentally regulated in all three layers. COX-2 immunostaining was detected at much lower levels and was not related to maturational stage [129]. During pregnancy, the nonselective COX inhibitor indomethacin crosses the placenta and constricts the human DA in fetuses >32 weeks gestation [130,131]. The findings of Koehne, along with studies on the predominant role of COX-2 in the human uterus during parturition, suggest that selective COX-2 inhibition might be a promising approach to block uterine contractions during preterm labor without the additional risks for constriction of the fetal DA, where COX-1 would be predicted to have an important role. Unfortunately, several studies show that the fetal DA is affected by maternal COX-2 inhibition [132]. Thus, pharmacological studies suggest either that COX-2 is active in the human fetal DA or that COX-2-mediated prostaglandin synthesis in peripheral tissues (or circulating cells) is important for fetal DA patency. It will be difficult to determine whether a PGE-mediated vascular program is active in the human DA since COX inhibitors reduce vasa vasorum flow to the thick muscular media of the DA in humans and large animals, causing hypoperfusion and ischemic injury to the vessel wall [133]. This finding partially explains why infants born to some women that are treated with COX inhibitors for tocolysis have increased incidence of PDA [134,135]. The alternative possibility, that there is upregulation of other vasodilators, or disruption of a fetal vascular transcriptional program, awaits further investigation.

Prostaglandin receptors and downstream signaling

Prostaglandins exert their effects through a family of G-protein coupled receptors. There are subtle differences in the expression and function of each receptor in the DA of various species. Due to the importance of PGE in DA regulation, the EP family of receptors has been the focus of particular attention. The EP4 subtype appears to play an essential role, since mice with targeted deletion of the gene encoding EP4, *Ptger4,* die in the first few hours of life with a large PDA [16-18]. Deletion of EP4, which typically mediates a vasodilatory response, would be expected to cause DA constriction. However, the PDA phenotype in these mice may be due to vascular dysregulation, similar to COX double knockout mice, suggesting a critical ligand - receptor pathway for DA development. EP4 is also the predominant receptor in the DA of rats, rabbits, and baboons, although not in sheep [136-138]. In humans, Leonhardt et al. found significant mRNA and protein expression of the PGE_2 receptors, EP3 and EP4, along with FP, IP, and TP receptors, for PGF_2, PGI_2, and thromboxane, respectively. Of these receptors, EP4 and TP receptors were the most expressed and were primarily localized to the medial layer of the DA [139]. Rheinlaender et al. also found a predominance of EP4 protein expression in the intima and media of the human DA at the time of autopsy [129]. EP4 levels were increased in the later stages of DA maturation. More recently, Fan et al. demonstrated that the isolated preterm human DA was less responsive to oxygen *in vitro*, but that pharmacological inhibition of the EP4 receptor caused potent constriction [140]. A link between EP4 signaling and Kv channels was suggested as an underlying cause for the differential response between term and preterm human DA samples. Polymorphisms in the human EP4 gene are associated with susceptibility to aspirin-resistant asthma, Crohn's disease and other disorders [141,142], but there are no studies to date that indicate a relationship to PDA.

The downstream targets of EP4 are the subject of several ongoing investigations. Stimulation of the EP4 receptor by PGE or other agonists increases intracellular cAMP and activates cAMP dependent kinase A (PKA), resulting in relaxation of vascular smooth muscle and DA dilation. Recently, Yokoyama et al. hypothesized that PGE_2/EP4 signaling that is important for vascular remodeling in the aorta and other vessels might also play a role in promoting anatomical closure of the rat and mouse DA. In addition to the potent vasodilatory effects of PGE2, EP4 stimulation was postulated to prepare the DA *in utero* for postnatal closure by promoting subendothelial hyaluronic acid (HA) production and intimal cushion formation [19]. As in other species, EP4 was the predominantly expressed isoform. Prolonged exposure of cultured DA smooth muscle cells to a selective EP4 agonist caused cell migration that was dependent on HA synthesis; migration was inhibited by HA removal or silencing of the HAS2 enzyme for HA synthesis. Explants of immature rat DA did not respond to 48 hours of stimulation with an EP4 agonist, while the mature DA explants had increased HAS2 expression, increased HA deposition, and increased cell proliferation. Transfection of HAS2 improved lumen closure rates in immature DA explants. Moreover, the DA of *Ptger4* null mice had reduced HA deposition. HAS2 transfection also improved lumen closure in *Ptger4* null DA explants [19]. Although these detailed findings provide compelling new insights into PGE actions for DA closure, some methodological and conceptual uncertainties remain. First, it is surprising that HA accumulation was most pronounced in the adventitia and outer layers of muscular media, rather than the intima or subendothelial region of the closing DA. The proposed interaction of EP4 and HAS2 would be critical in this region. Given its proposed role in luminal closure, HA accumulation was remarkably sparse in the subendothelium - it is unclear how HA deposition in the outer wall would prepare the fetal DA for postnatal closure. Information on EP4 and HAS2 localization might also be informative. Second, there is confusion regarding the proposed role for EP4 and HA in intimal cushion formation, since rats, mice, and other small rodents do not form intimal cushions or neointimal mounds, as classically described in humans and larger species [85,143-145]. Indeed, none of the studies shown depict formation of an intimal cushion. Thus, intimal cushion formation may not be an appropriate outcome measure in these models. Although intimal thickening was also described and may be the actual difference of interest, it is difficult to distinguish intimal thickening from endothelial cell crowding that takes place as the muscular wall constricts and the lumen cross-sectional area is correspondingly reduced. Third, the contribution of intimal thickening to DA closure in the PDA of *Ptger4* null mice is particularly difficult to estimate, since the vessel wall fails to constrict and therefore does not experience the same forces that lift endothelial cells from their anchorage to the underlying internal elastic lamina. Endothelial upheaval and redundancy is regarded as part of the process that creates increased subendothelial space. While these concerns do not invalidate the hypothesis that PGE signaling is important for HA deposition, additional information is required. Mice with conditional deletion of HAS2 have an embryonic lethal phenotype [146]. However, *Prx1-Cre;Has2*$^{flox/flox}$ mice with conditional deletion of HAS2 under the control of the Prx1 transcription factor (which is expressed in the DA) survive postnatally but have severe skeletal anomalies [147]. More in-depth studies in mice with conditional HAS2 inactivation may be informative and help to resolve the interactions of PGE and HAS2 for DA closure.

A recent follow-up study by Yokoyama et al. examined whether a newly defined target of cAMP, exchange protein activated by cAMP

(Epac), is an important downstream effector of PGE_2-EP4 cAMP signaling during postnatal DA constriction [148]. *Epac1* and *Epac2* mRNA expression was upregulated at term gestation and after birth, with immunolocalization in the media and endothelium of the closed rat DA. EP4 stimulation activated both the cAMP-PKA and cAMP-Epac pathways. A selective agonist of the cAMP-Epac pathway stimulated DA smooth muscle cell migration, whereas cAMP-PKA stimulation was inhibitory. Adenoviral-mediated overexpression or siRNA-mediated inhibition of each isoform suggested the importance of Epac1 over Epac2 for cell migration. The selective agonist of the cAMP-Epac pathway inhibited cell proliferation and did not upregulate hyaluronin synthesis, while stimulation of the cAMP-PKA pathway successfully stimulated HA accumulation, as seen in their previous publication [19]. Explants of immature rat DA had increased intimal thickening after transfection with *Epac1*, but not *Epac2* [148]. Reservations regarding the formation of intimal cushions as an outcome measure also exist for this paper. It is unclear whether the difference between acute (PKA) and chronic (Epac) activation of EP4 occurs *in vivo*. However, the overall data demonstrate a unique, second pathway for the downstream mechanisms of PGE_2-EP4-cAMP actions. In contrast to PKA, Epac-promoted DA closure was independent of cell proliferation and HA synthesis. Further study is required to determine whether these mechanisms are active in the human DA and could be exploited therapeutically.

Platelets and PDA

A relationship between circulating platelet counts and closure of the DA in preterm infants has recently become the focus of considerable research. Echtler et al. first reported this relationship in 2010 [149]. The authors demonstrated that activated platelets accumulated in and adhered to the lumen of the constricted DA within minutes after birth in newborn mice. Mice with defective platelet adhesion or biogenesis had high rates of persistent PDA, even after treatment with indomethacin. Echtler then performed a retrospective evaluation of the relationship between thrombocytopenia, defined as platelet count ≤150,000/μl on the first day of life, and DA closure, demonstrated by echocardiogram on day of life 3-5, in a group of 123 human infants born at 24-30 weeks gestation [149]. Seventy-one percent of infants had a PDA on day 3-5. In a logistic regression model, low platelet count was identified as an independent predictor of hemodynamically significant PDA (OR 13.1, p=0.0001). The authors did not present a similar model for all (both hemodynamically significant and asymptomatic) PDAs. Based on these findings, it was concluded that formation of a platelet plug is a critical step in closure of the DA, linking the initial reversible constriction and final anatomic remodeling.

Since the publication of Echtler's mouse and human studies, several additional studies in human subjects have been performed. In a similar retrospective cohort, Fujioka reported that a platelet count ≤150,000/μl on the first day of life was not related to the rate of DA closure in 142 Japanese infants 24-30 weeks gestational age [150]. Median platelet counts in the two studies were similar, but overall rate of PDA was significantly lower in the Japanese study. Interestingly, the thrombocytopenic Japanese infants were overall smaller and younger than those with higher platelet counts, so would have been expected to have higher rates of PDA. A study presented at the 2011 meeting of the Pediatric Academic Societies evaluated the relationship between platelet counts during the first 3 days of life and DA closure in 148 extremely low birth weight infants. Rates of both spontaneous and indomethacin-induced DA closure were lower in extremely low birth weight infants with platelet counts ≤150,000/μl [151]. However, this result is confounded by higher rates of small for gestational age and maternal preeclampsia and lower average birth weight in the thrombocytopenic infants.

The largest published study evaluating the relationship between platelets and DA patency in human subjects included 497 infants <28 weeks of gestation [152]. The cohort was managed in a single center with an aggressive protocol including prophylactic indomethacin, echocardiographic screening, and the availability of additional indomethacin treatment. Unlike previous studies, which only evaluated platelet counts in the 1-3 days of life, this study examined platelet counts over the first week of life, the time period in which initial constriction is most likely to occur in human infants. Persistence of a PDA was not related to platelet count at any time in the first week of life. Rather, high platelet counts were found to promote initial DA constriction. Neither high nor low counts influenced rates of final, permanent closure. This finding conflicts with the Echtler model, which suggests that platelets accumulate in and contribute to obliteration of the already constricted DA lumen, promoting permanent anatomic closure. Unfortunately, the true influence of platelet count on the duct in the absence of indomethacin cannot be determined in this study because all infants received prophylactic indomethacin.

Older studies, performed before the Echtler publication, are equally inconclusive. A prospective cohort study from Singapore demonstrated that, when controlling for other factors, platelet count was of borderline significance for predicting failed closure of PDA with indomethacin (OR 0.987, p=0.045) [153]. On the other hand, in a randomized trial of transfusions to keep the platelet count >150,000/μl in thrombocytopenic preterm infants, rates of PDA were nearly identical in the two groups [154].

These conflicting studies have led Sallmon and colleagues to suggest an alternative hypothesis: immature platelet function, not platelet count, plays a role in persistent patency of the DA in the preterm human infant [155]. The rationale for this theory includes evidence for impaired platelet function in preterm infants compared to term infants and adults and the important observation that term infants with severe alloimmune or autoimmune thrombocytopenia do not have higher than expected rates of PDA [156,157]. However, no definitive data in humans or animals yet exists to support the theory that developmental differences in platelet function contribute to persistent PDA in preterm infants.

Thus, despite multiple studies, the platelet-PDA relationship remains unclear. Echtler presented compelling and novel murine data about the role of platelets in successful DA closure. The few small, single center studies that have been performed in preterm human subjects are contradictory, but do not suggest that thrombocytopenia in the first days of life consistently results in failure of DA closure. Physiological differences in the mechanism of DA closure between mice and humans or population differences between cohorts in the human studies may account for these conflicting results. As Echtler and others have suggested, it is likely that the role of platelets in DA closure cannot be elucidated without large controlled clinical studies.

Conclusions

While the DA is a vessel rarely dwelled upon after patients leave the neonatal intensive care unit, it is critical for both fetal well-being and the transition to newborn life. Recent research has provided a window into the important molecular pathways regulating the development and function of the DA. However, there continues to be a tremendous

amount that is unknown. Many of the animal models have focused on the late preterm or term DA, but there is a distinct possibility that the DA of the ELBW is functionally dissimilar. Further analysis of these pathways earlier in gestation is necessary. The extension of rodent and non-human primate studies to human basic science and clinical studies will likely reveal conserved pathways with potential therapeutic targets. As detailed in this review, these targets may include modulation of hematopoietic cells, specific ion channels, prostaglandins or signaling pathways including angiotensin and endothelin. In the future, improved mechanisms by which clinicians can modulate the patency and closure of the human ductus arteriosus will undoubtedly improve the lives of countless infants.

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