Discerning the Role of Prostaglandins in Ductus Arteriosus Remodeling

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ABSTRACT

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Artiom Gruzdev

(Under the direction of Dr. Beverly H. Koller)

The ductus arteriosus (DA) is a fetal pulmonary bypass shunt that constricts and permanently remodels during the transition from fetal to adult circulation. Prostaglandin E_2 (PGE_2) is potent mediator of numerous physiological responses both in homeostasis and disease state. PGE_2 play a vital role in DA maturation and closure, although the exact molecular role is unclear. We attempt to discern the nature of PGE_2 involvement in DA maturation and closure. Here we generate a conditional null allele of the prostaglandin E receptor 4 (EP4), which has been previously shown to be responsible for PGE_2 signaling in the DA. Utilizing various tissue specific Cre recombinase transgenes, we have shown that EP4 expression on the neural crest derived smooth muscle cells of the DA is critical for proper DA closure. We have also shown that endothelial expression of the PGE_2 vasodilatory receptors (EP4 and EP2) is non-essential for DA closure or vascular development.

Genome wide expression profiling of the wildtype DA and EP4 deficient DA were used to assess the transcriptional consequences of PGE_2/EP4 signaling in the DA. Differentially expressed genes in the wildtype DA indicate that EP4 receptor expression leads to the up-regulation of numerous cytoskeletal genes. The relative minor increase (<2

fold) of numerous cytoskeletal genes may explain why the wildtype and EP4 deficient DA appear morphologically similar *in utero* but have antithetical fates after birth.

Here we also document the existence of a prostaglandin-independent mechanism of DA maturation and closure in mice. Selective mating generated a recombinant inbred (RI) mouse strain that undergoes DA maturation and closure without any contribution from prostaglandin signaling. Single locus inheritance of patent ductus arteriosus (PDA) in common inbred strains (CIS) seemed at odds with the complex inheritance pattern of PDA in larger animals, but the RI strain indicates that DA closure in non-CIS mice is also a complex trait. The study of both prostaglandin-dependent DA closure of CIS mice and prostaglandin-independent DA closure of RI mice provides a mouse model for understanding the complex trait of larger animals.

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BP	Base pair
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CIS	Common inbred strains
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
Cre	Cyclization recombination
cRNA	Complementary RNA
DA	Ductus arteriosus
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
eNOS	Endothelial nitric oxidase synthase
EP1-4	Prostaglandin E receptor 1-4
ES	Embryonic stem
FLP1	Flipase
FRT	Flippase recognition target
HPGD	Hydroxyprostaglandin dehydrogenase
ICF	Intimal cushion formation

IP	Prostacyclin receptor
LOD	Logarithm of odds
LoxP	Locus of X-over P1
MB	Mixed background
MYCOD	Myocardin
MYH11	Smooth muscle myosin heavy chain 11
Neo	Neomycin Resistance
NO	Nitric oxide
NSAIDs	Non-steroidal anti-inflammatory drugs
PCR	Polymerase chain reaction
PD	Pulmonary duct
PDA	Patent ductus arteriosus
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin endoperoxide
PGK	Phosphoglycerate kinase
Ptger1-4	Locus of EP1-4 receptor
QTL	Quantitative trait locus
RI	Recombinant Inbred
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SNP	Single-nucleotide polymorphism
SRF	Serum response factor
STRAP	Structure based Sequences Alignment Program

Tagln	Transgelin
Tek	Tyrosine kinase, endothelial
TFAP2β	Transcription factor AP-2 beta
Tk	Thymidine kinase
TP	Thromboxane receptor
Wnt1	Wingless-type MMTV integration site family, member 1

Chapter 1

Introduction

The mammalian cardio-pulmonary system

The work presented in this dissertation is focused on the closure of the ductus arteriosus, a critical event in the transition from fetal circulation to adult circulation. It is therefore beneficial to briefly describe the adult and fetal circulatory systems, and the transition that must occur at birth for the proper establishment of the adult circulatory system.

The mammalian cardio-pulmonary system is responsible for systemic circulation and respiration, two distinct functions that are often treated as one. Systemic circulation carries oxygenated blood from the left side of the heart to the body, and returns de-oxygenated venous blood to the right side of the heart. Pulmonary circulation is the sole source of respiration in adult mammals. Pulmonary circulation brings O_2 depleted, CO_2 rich blood to the lungs from the right side of the heart for gas exchange, and returns oxygenated blood to the left side of the heart. Separation of pulmonary circulation and systemic circulation has two main advantages over having heart, respiration, and systemic circulation in series. High respiration efficiency occurs when the pulmonary and systemic circulations are separate, as oxygenated and de-oxygenated blood cannot readily mix. Another key advantage of separating pulmonary and systemic circulation is the ability to keep the two systems at different pressure. The lungs provide significant resistance to blood flow, and greatly reduce pressure in pulmonary venous blood flow compared to the pressure of pulmonary arterial blood flow. If pulmonary circulation and systemic circulation were in series, low-pressure oxygenated blood from the lungs would have to provide O_2 to the body, which would likely result in extreme hypoxia given the great oxygen demands of the body.

A starting point for the circular flow of blood through the adult circulatory system is arbitrary, given the fact that the system, as it name implies, is circular. Therefore, the point at

which carbon dioxide rich, de-oxygenated venous blood returns to the heart through the venae cavae will be used as the starting point. In the adult circulatory system, the inferior vena cava and superior vena cava return blood to the right atrium from the lower and upper extremities, respectively. Venae cavae blood mixes in the atrium, and atrial contraction expels the blood into the right ventricle through the tricuspid valve. Right ventricular contraction propels blood, through the pulmonary valve, to the lungs where a network of capillaries will be the site of gas exchange between the oxygen rich air filled alveoli of the lungs and the carbon dioxide rich blood in the capillaries. The pulmonary veins return arterial blood to the left atrium, and contraction of the left atrium propels blood into the left ventricle through the mitral valve. Left ventricular contraction expels blood, through the aortic valve, into the aortic arch for systemic circulation. In the adult heart, the left and right sides of the heart function as two distinct pumps. The right side of the heart collects systemic venous blood flow and pumps it to the pulmonary system for respiration. The left side of the heart collects arterial blood from the lungs, and pumps it to the rest of the body for systemic circulation. All amniotic animals (birds, mammals, and reptiles) share this basic layout of circulation and respiration.

Evolution of the cardiovascular-respiratory system of vertebrata

To appreciate the mammalian system of circulation and respiration, it helps to have a frame of reference. Therefore, a brief synopsis of the cardiovascular-respiratory system found in other vertebrates is provided here to illustrate other respiratory mechanisms found in nature and the uniqueness of pulmonary respiration of amniotes.

All mammals, including human's most distant extant relative the Platypus (*Ornithorhynchus anatinus*), have a remarkably similar cardio-pulmonary system [1, 2]. In fact, the respiratory system of all amniotes, whether from a mammal, bird, or reptiles, is functionally very similar. Phylogenetic studies indicate that the ancestors of all extant amniotes shared many cardiovascular similarities with amphibians [3]. In turn, amphibians share many similarities with fish, our most distant extant vertebrate relative [3]. Like amniotes, fish and amphibians have a crucial need of O_2, but their respiratory mechanism is remarkably different from amniotes.

The development stages of fish are well documented, and there are several key stages that fish undergo from zygote to adult that involve a transition of the site of respiration [4]. The first stages of development occur before hatching, and although a heart is present in the pre-hatching embryo, O_2 and CO_2 exchange occurs through diffusion [5]. In many fish species, the transition from egg to free-feeding larvae occurs before the maturation of the gills and circulatory system, and simple diffusion accounts for most of the gas exchange of the free-feeding larvae [5]. As growth continues, the circulatory system matures and the gills become a more important respiratory organ. Diffusion will eventually become insufficient in providing gas exchange and the gills will become the sole site for respiration in most fish. The gills are well suited for water-breathing, but would collapse in air, increasing vascular resistance blocking systemic circulation, therefore terrestrial life requires an alternate respiratory organ. Gills of fish are also ill suited for respiration during period of extreme exercise and fish must either switch to air-breathing organs or suffer hypoxia during period of strenuous exercise [6, 7].

The lungfish, as their name implies, have lungs similar to that of obligate air-breathing animals. With few exceptions, the larval and juvenile lungfish develops with functional gills, and adult lungfish that maintain their neotenic gills can use gills exclusively for respiration in oxygen rich waters [8]. Lungfish destined to become obligate air-breathers initiate pulmonary respiration while the gills are still functional [8]. Lungfish possess a pulmonary bypass system consisting of an arterial duct and pulmonary artery vasomotor segment (PAVS) [9]. A closed arterial duct and open PAVS enables gill circulation, while an open arterial duct and closed PAVS enables pulmonary circulation. The arterial duct of adult lungfish is capable of channeling blood to either the gills or the pulmonary system depending on environmental conditions [9]. The free-feeding fish, from larvae to adult, can have up to three different respiratory organs (cutaneous, gills, or lung) through their life cycle, with two of the three organs functional at the same time in development.

The cardio-respiratory system of amphibians at various times in development resembles both the aquatic respiration of fish and pulmonary respiration of terrestrial animals. The amphibian larvae closely resemble fish as hatching usually occurs in an aqueous environment and the amphibian larvae relies on simple diffusion and gills for respiration. Some amphibians retain neotenic gills due to environmental pressure, but most adult amphibians do not have functional gills after metamorphosis [10]. In adult amphibians, blood returns to the right atrium oxygenated via cutaneous respiration, and pulmonary veins return oxygenated blood from the lungs to the left atrium [11]. The Plethodontidae (the lungless salamanders) and some species of frog do not have any lungs and rely exclusively on cutaneous respiration [12, 13]. Cutaneous respiration is found in virtually all amphibians and can account for 30-100% of the oxygen needs of the animal [14]. A pulmonary bypass

exists in some amphibians shunting blood from the pulmonary artery to the respiratory lamellae in the gills, and the pulmonary bypass in adults is usually not occluded [15]. In amphibians, pulmonary respiration develops while an alternate organ provides the site of gas exchange.

While amphibians undergo metamorphosis to transition to terrestrial life, it was the evolution of the amniotic egg that allows terrestrial life in all stages of development [16]. Amniotes include mammals (oviparous or viviparous), birds (oviparous), and reptiles (oviparous or oviviparous). Regardless of method parturition or hatching, the transition from fetus to neonate of all amniotes includes the initiation of pulmonary circulation without a redundant secondary site of respiration like that seen in fish and amphibians. Unlike amphibians and lungfish in which pulmonary respiration develops in free-swimming larvae, the pulmonary system of the amniotes must be functional at the time of birth. After birth or hatching there is a cessation of placental respiration or chorioallantoic respiration, respectively, and pulmonary respiration must provide all gas exchange for the amniotic neonate.

Transition from fetal to adult circulation by placental mammals

Amniotic development occurs in an aqueous environment, regardless if that environment is the uterus or the egg. Placental and metatheria mammals rely on placenta blood flow for fetal gas exchange, and avian, reptile, and monotreme fetuses rely on the gas permeability of the eggshell for gas exchange [17]. What all amniotes share is that at some point, whether it is at hatching, birth, or migration to the marsupium, the fluid-filled lungs must be cleared of liquid and the newborn must begin pulmonary respiration [18, 19]. While

there are many similarities in all amniotes, there are also significant differences between birth, hatching, and migration to the marsupium. Therefore the remainder of this chapter will focus on the cardio-pulmonary system and transition from fetal circulation to adult circulation of placental mammals [20].

In the adult circulatory system, total right cardiac output enters the pulmonary system for respiration. The fetal lungs are incompatible with the circulatory system of the adult because the fluid filled fetal lungs are resistant to blood flow and to prevent fetal pulmonary hypertension the majority of cardiac output must bypass the lungs [21-23]. The fetal circulatory system has evolved with two right-to-left shunts, the foramen ovale and the ductus arteriosus (DA), that allow blood to bypass fetal pulmonary circulation.

In utero respiration occurs in the placenta between maternal circulation and fetal circulation. The fetal heart and fetal pulmonary system do not play a role in respiration, and the fetal lungs are non-essential organs *in utero*. Oxygenated blood from the placenta enters the right atrium via the inferior vena cava and is preferentially channeled through the foramen ovale to the left atrium (See Figure 1.1 for fetal and adult cardio-pulmonary circulation). The foramen ovale is an intracardiac shunt in the atrial septum. The flap like shunt allows blood to flow down a concentration gradient from the higher-pressure right atrium to lower pressure left atrium [24, 25]. There is preferential channeling of inferior vena cava blood flow (versus superior vena cava blood flow) through the foramen ovale [26]. Approximately 60% of the right atrial blood flow is shunted through the foramen ovale. From the left atrium, blood is pumped through the mitral valve into the left ventricle. Left ventricular contraction sends blood to the aortic arch, with preferential blood flow to the upper extremities via the ascending aorta.

Blood returning to the heart from the superior vena cava, enters the right atrium and is preferentially channeled to the right ventricle (~40% of right atrial input). Right ventricular contraction expels blood into the pulmonary duct. The pulmonary duct sends as little as 10% of right ventricular output to the pulmonary arteries and fetal lungs. The remaining blood is shunted away from pulmonary circulation into the descending aorta via the DA [24, 27]. The DA is a smooth muscle lined arterial vessel that *in utero* provided right-to-left shunting of blood from the right ventricle to the descending aorta, bypassing pulmonary circulation. Blood flow through the descending aorta is directed to inferior systemic circulation and placental circulation.

At birth, the neonatal lung clears liquid from the lung mainly through transepithelial sodium reabsorption [19, 28]. The fluid free neonatal lung is capable of inflation, which decreases resistance to pulmonary blood flow. A drop in resistance to pulmonary blood flow by itself is the first step in the transition from fetal to adult circulation. Once the lungs are capable of inflation and more importantly respiration, the pulmonary bypass via the foramen ovale and the DA must be attenuated and eventually occluded to complete the transition to adult circulation [20].

Clearing of liquid from the lungs allows the lungs to inflate and deflate which decreases resistance to pulmonary blood flow [29]. Right ventricular output shifts from the DA to the pulmonary arteries as pulmonary resistance drops [30]. The increase in pulmonary blood flow decreases pressure in the DA and allows shunting of descending aorta blood flow through the DA, a reversal of fetal right-to-left shunting [31]. An obvious consequence of increased pulmonary circulation input is an increase in pulmonary circulation output, which

increases pressure in the left atrium, and causes physical closure of the foramen ovale [32]. Closure of the foramen ovale places the right atrium and ventricle in series.

The initial closure of the foramen ovale occurs because of an increase in blood flow to the left atrium from the pulmonary veins [25]. Typically, the foramen ovale will fuse with the atrial septum within the first year of life, eventually leaving only the fossa ovalis, an embryological remnant of the foramen ovale [32]. Permanent fusion of the foramen ovale with the atrial septum does not occur in almost 25% of the general population, but failure of fusion is asymptomatic in most people because of the physical closure caused by the pulmonary circulation output to the left atrium [32]. Symptomic patent foramen ovale has been linked to migraines and ischemic stroke [33, 34].

Right ventricular output is still connected to the descending aorta via a patent DA. Basic fluid dynamics can be used to describe the situation occurring in the heart with a patent DA. High-pressure blood flow in the descending aorta and high-pressure right ventricular output directly oppose each other, with only the low-pressure pulmonary arteries as a common exhaust. Descending aorta pressure is initially higher than right ventricular output pressure so there is a reversal of blood flow through the DA (left-to-right shunting) [29]. The DA typically constricts within the first 24 hours of life and remodels into a ligament (*Ligamentum arteriosum*) within the first 3 weeks of life (varies by species) [29]. Following closure of the DA, the transition from fetal cardiac blood flow to adult cardiac blood flow is completed. The left atrium, left ventricle, pulmonary system, right atrium, right ventricle, aortic arch, and systemic circulation are all in series. It is noteworthy to mention that the closure of the DA and foramen ovale is timed to occur right after birth not just for the prevention of pulmonary hypertension. *In utero*, the patent DA and foramen ovale allows all

four chambers of the heart to develop with an active workload, and heart valve development is dependent on blood flow patterns similar to the adult circulation [35, 36].

In humans, failure of DA closure within 72 hours is diagnostically considered a persistently patent DA (PDA). PDA result in an increase in pulmonary blood flow as right and left ventricular output enters pulmonary circulation through the pulmonary duct and DA, respectively. This creates inefficiency in the pulmonary system as oxygenated blood from the descending aorta is channeled through the DA (left-to-right shunting) and enters the pulmonary system for a second round of gas exchange. The internal diameter of PDA directly correlated to the severity of any symptoms. A PDA with a small diameter may be completely asymptomatic throughout an individual's life, and spontaneous closure is not uncommon [37]. However, a PDA with a large diameter typically results in two undesirable synergistic affects, pulmonary hypertension and chronic hypoxia.

If closure of a large PDA does not occur, pulmonary hypertension can result from an increase in blood flow to the lungs from the left-to-right shunting through the DA [38]. Increased pulmonary pressure can damage the delicate capillaries responsible for gas-exchange, resulting in vessel thickening and formation of scar tissue [39]. As pulmonary circulation resistance increases, right ventricular workload increases causing right ventricular hypertrophy, which further results in an increase in pulmonary hypertension [40]. As pulmonary hypertension increases, the pulmonary arterioles and capillaries thicken and remodel, decreasing gas exchange efficiency. In severe cases of chronic hypoxia, polycythemia will occur as more red blood cells are produced to counter the chronic hypoxia [41, 42]. The resulting hyper-viscosity of blood further increases workload on the right ventricle [43]. Increasing right ventricular hypertrophy and increasing resistance to

pulmonary blood flow can ultimately result in the reversal of blood flow through the DA, restoring pulmonary bypass (right-to-left shunting) in an adult. This occurs when right ventricular output minus pulmonary blood flow is at a higher pressure than the descending aorta.

Symptomatic PDA in humans can manifest itself as pulmonary hypertension caused primarily by increased resistance, pulmonary edema and hemorrhage, congestive heart failure, intraventricular hemorrhage, bronchopulmonary dysplasia, and death [38, 44]. Given the possible consequences of PDA, treatment of any diameter PDA is currently common practice. Treatment options for PDA include surgical ligation of the DA, transcatheter placement of an intravascular coil to occlude blood flow through the DA, and pharmacological inhibition of prostaglandin production in hopes of initiating normal DA closure. Surgical and transcatheter closure have become routine procedures and have a success rate of nearly 100%, but the procedures carry the same risks as other surgical or transcatheter procedures. Pharmacological treatment is currently only an option in neonates and infants, and its efficacy is highly variable, and may cause serious side effects without inducing DA closure.

Characteristics and closure of the ductus arteriosus

Giulio Cesare Aranzi first described the DA over 400 years ago, but the molecular pathways responsible for its closure are still unclear. The DA develops from the sixth aortic artery in mammals, and *in utero* morphologically resembles other great arteries of the heart. The DA, like all major arteries, can be subdivided into 4 layers: an internal endothelium, surrounded by the tunica intima (composed of internal elastic lamina and fibrocollagenous

tissue), a thick tunic media of smooth muscle cells, and an external tunica adventitia (comprised of fibrocollagenous tissue and external elastic lamina). Thickness of the layers greatly varies from species to species. In humans and other large mammals, the DA begins to differentiate itself from other great arteries of the heart *in utero* with intimal cushion formation (ICF) [45, 46]. Antenatal ICF is characterized by lifting of the endothelium and expansion of sub-endothelial space. Hyaluronic acid synthesis and elastin fiber fragmentation facilitates the migration of smooth muscle cells into the sub-endothelial space. Antenatal ICF has not been reported in mice. This may be due to ICF not being necessary in mouse DA closure, or characteristics of mouse ICF are not easily identified by microscopy. In most large animals, only the outer 1/3 of the smooth muscle media is vascularized by vasa vasorum, and the inner smooth muscle layer receives nutrients and O_2 via luminal blood flow [47]. In mice, the DA is not vascularized, and receives all its nutrients and O_2 from luminal blood flow [48]. After DA constriction, ischemia plays a role in initiating permanent remodeling of the vessel into the ligamentum arteriosum [49, 50]. Currently, the exact cause of DA constriction and initiation of remodeling are unclear. There are numerous components known to play a role in DA closure such as gestational maturity, O_2 tension, and prostaglandin levels (Table 1.1) [27, 51-53]. Many genes have been implicated in DA closure, however the exact role these genetic components play in DA maturation and closure and their possible interactions with each other remains unclear (See Table 1.2 for list of genes with known association with DA maturation/patency). Prostaglandins and their role in DA closure are briefly described in the following section.

Prostaglandins and the ductus arteriosus

Prostanoids were implicated in the transition from fetal to adult circulation after an increase in incidence of premature *in utero* DA closure was reported in infants exposed to non-steroidal anti-inflammatory drugs (NSAIDs). Indomethacin, a potent NSAIDS, was routinely used to delay premature labor, however it was discovered that following maternal indomethacin treatment, the fetal DA often underwent closure, which can potentially lead to fetal pulmonary hypertension and other serious complications [54, 55]. These serendipitous discoveries lead to the use to indomethacin (and other NSAIDs) as pharmacological agent to induce DA closure in neonates that had not undergone proper DA closure after birth.

NSAIDs inhibit cyclooxygenase (COX1 and COX2) activity, resulting in a decrease in all prostanoids. Prostanoids, a subclass of eicosanoids, are signaling molecules made by oxygenation of essential fatty acids, and include prostaglandins, prostacyclins, and thromboxanes (Figure 1.2). Prostanoids are metabolites of arachidonic acid (AA), and have numerous physiological roles including vascular tone, platelet aggregation, inflammation, and fever [56]. AA is a 20-carbon polyunsaturated fatty acid found in the phospholipid membrane of cells. COX, officially named prostaglandin-endoperoxide synthases, convert AA into prostaglandin endoperoxide (PGH_2) through a two-step catalytic reaction involving dioxygenase activity and peroxidase activity. PGH_2 is further metabolized by one of five enzymes: thromboxane synthase, prostacyclin synthase, or prostaglandin D, E, or F synthase.

Prostaglandin E_2 synthase (PTGES) generates prostaglandin E_2 (PGE_2) from PGH_2 by breaking the peroxide bond between C9 and C11. Prostaglandin D synthase and prostaglandin F synthase break the same peroxide bond, and the only principle difference between PGD, PGE, and PGF is the location of either a keto or alcohol group at C9 and C11.

PGE_2 has wide range of physiological effects including vasodilation, fever induction, uterine contraction, bone resoprtion, and developmental control. Although PGE_2 has a relative short biological half-life of about 30 seconds in circulation, PGE_2 is tightly regulated by hydroxyprostaglandin dehydrogenase (HPGD) that breaks down PGE_2 into biologically inactive 15-keto-PGE_2 [57, 58].

Of all the prostanoids that are downstream of COX inhibition by NSAIDs, it is $PGE_{1/2}$ that has a physiological role in DA patency. Exogenous neonatal infusion of $PGE_{1/2}$ maintains ductal patency in neonates. Maintaining ductal patency is crucial for infants that have congenital heart defects that rely on a shunting through the DA for survival such as transposition of the great arteries, pulmonary atresia/stenosis, tricuspid atresia/stenosis, coarctation of the aorta, hypoplastic left heart syndrome, critical aortic stenosis, and interrupted aortic arch. Patency of the DA will not sustain neonates with any of these serious heart defects, and surgical intervention is ultimately necessary. In the short term, a patent DA will allow for sufficient cardio-pulmonary circulation until surgical correction.

PGE_2 exerts its biological role through four distinct seven transmembrane domain G-protein coupled receptors, the EP1-4 receptors [56]. In human DA, the EP4 receptor is expressed antenatal and neonatal by both the smooth muscle and endothelium [59]. *In utero*, the lamb and pig DA expresses EP2, EP3, and EP4 receptors, but only the EP2 receptor is present in the neonatal DA [60, 61]. The DA of fetal rabbits and rats only expresses the EP4 receptor [62, 63]. In most species the EP4 receptor has been shown to be central to DA closure, but in some species the EP2 receptor may play a role in neonatal DA patency. The EP1 receptor has not been shown to play a role in DA maturation and closure in any species. In the lamb, EP3 receptor agonists have been reported to result in DA relaxation through a

potassium-ATP channel mechanism, but in the pig this effect was shown to be at best marginal compared to saline [60, 64]. Targeted disruption of all four PGE_2 receptors in mice has confirmed that the EP4 receptor in mice, like most other species, is critical for DA maturation and closure, and the loss of EP1, EP2, or EP3 has no affect on DA closure [65-68].

The EP4 receptor

The EP4 receptor is well conserved among amniotes (Figure 1.3). The human and mouse EP4 receptors share approximately 88% amino acid identity. In most species, the EP4 locus consists of 3 exons; the first exon is often non-coding or codes for only the first few amino acids (<20 amino acids), the second exon codes for most of the seven transmembrane domains and the third exon codes for the cytosolic tail of the receptor.

The EP4 receptor has been implicated in an extremely wide range of physiological processes, and the molecular pathways activated by EP4 receptor signaling seem to be largely dependent on the cell type in which it is expressed. The EP4 receptor is Gs-protein coupled and stimulates adenylate cyclase upon ligand binding [56]. PGE_2/EP4 signaling has a catabolic function in osteoarthritis by inhibiting proteoglycan synthesis and stimulates matrix degradation [69]. In human embryonic kidney cells, PGE_2/EP4 signaling has been shown to cause transcriptional changes, through phosphatidylinositol 3-kinase, by stimulating early response growth factor expression [70]. *Ex vivo* preparations of human pulmonary veins are dilated by PGE_2 through smooth muscle EP4 receptors, but PGE_2 exerts a minimal vasodilatory affect on the pulmonary arteries, which lack smooth muscle EP4 expression [71]. *In vivo*, the smooth muscle of most arterial vessels do not widely express the

EP4 receptors, but nevertheless the EP4 receptor has a vasodepressant affect when stimulated [72]. Additionally, a role in cell differentiation and expansion was recently reported for the EP4 receptor in T-cell helper cells [73].

Fatal non-syndromic PDA in EP4 deficient mice was counter-intuitive because the loss of a dilatory receptor was not expected to result in the inability to close and remodel the arterial vessel [68]. It has been shown that EP4 receptor expression increases with gestational age, and this is consistent with the observation that the premature DA is insensitive to prostaglandin fluctuates. Selective activation of the EP4 receptors in the rat DA has also been implicated in ICF by stimulating hyaluronic synthase 2 (HAS2) [74]. However, the link between HAS2's role in ICF in the mature DA, and the fatal patency of EP4 deficient animals is unclear, and perhaps circumstantial. Similarly, EP4 signaling has also been shown to stimulate cAMP-regulated guanine nucleotide exchange factor I (EPAC1), which plays a role in smooth muscle migration in ICF [75].

In humans, ICF is crucial for successful DA closure, and does not occur in an immature DA. Visual inspection of the mouse EP4 deficient DA and wildtype DA reveals no gross morphological difference between the wildtype and EP4 deficient DA, and the presence or absence of ICF has not been reported *in utero* [68]. The lack of any apparent morphological difference between the full-term mouse DA destined to close and remodel (wildtype) and the mouse DA that is destined to remain patent (EP4 deficient) suggests that ICF is not the critical component of EP4 receptor signaling in DA maturation, and ICF is a by product of a EP4 induced DA maturation. Although the EP4 receptor is exerting a dilatory effect on the DA, as evidenced by neonatal PGE_2 infusion, the role of the EP4 receptor in DA maturation is more complex than just vasodilation.

Char syndrome may provide some additional insight into the molecular causes of PDA. Char syndrome, an autosomal dominant disorder characterized by PDA, facial dysmorphism and hand abnormalities, is caused by mutations in the transcription factor AP-2 beta (TFAP2β)[76]. An increase in PDA incidence in infants with other developmental defects is not necessarily relevant since a variety of other genetic developmental defects can cause secondary PDA in infants [77]. However, a novel frame-shift mutation in human TFAP2β, resulting in premature termination of the protein, has recently been implicated in non-syndromic PDA [78]. Expression of endothelin-1 (ET-1) in the DA is dependent on TFAP2β expression [79]. ET-1 has shown to be critical for the oxygen-induced initial constriction of the DA at birth, and ET-1 up-regulates EP4 receptor expression in other tissues [80, 81]. The relationship between the EP4 receptor and TFAP2β in the DA is circumstantial, but nevertheless the coincidence of non-syndromic PDA in humans with TFAP2β mutations and non-syndromic PDA in mice with EP4 receptor mutations should not be ignored. The loss of TFAP2β in mice does not reproduce the PDA seen in non-syndromic TFAP2β mutation in humans [82]. This discrepancy may be due to the differences between the mouse and human DA, the difference between TFAP2β null mouse locus and the TFAP2β truncated protein in humans, or TFAP2β having no relationship with DA maturation and closure.

Ultimately, the exact molecular mechanism of DA maturation and closure is unclear, as is the role of the EP4 receptor in that maturation process. The chicken-and-the-egg circular argument can be made for many of the genetics components linked to DA maturation, does an event occur in a mature DA or does that event cause DA maturation.

Research presented in this dissertation

This dissertation aims at addressing the counter-intuitive phenomenon created by the persistent PDA (dilation) seen in mice deficient in EP4 (a dilatory receptor). Chapter 2 analyzes the tissue-specific requirement of EP4 expression in the DA, and the transcriptional consequences of EP4 receptor expression in the DA. Chapter 3 examines prostaglandin-independent DA maturation and closure that may explain the complex inheritance of PDA seen in larger animals (especially humans) that can not be explained by the disruption of a single pathway as seen in common inbred mouse strains. Concluding remarks in chapter 4 summarize the work presented here and outline possible future direction to further elucidate the role of prostanoids in DA maturation and closure.

Figure 1.1. Diagram of basic **(A-D)** fetal circulation and **(E-I)** adult circulation in mammals. Foramen ovale (*fo*), ductus arteriosus (*da*), and ligamentum arteriosum (*la*) are labeled. **(A)** Inferior vena cava blood flow from the placenta is preferentially channeled through the foramen ovale into the right atrium. **(B)** Superior vena cava blood flow from the upper extremities is preferentially channeled to the right ventricle. **(C)** Right ventricular output is channeled through the ductus arteriosus to the descending aorta to the lower extremities and the placenta. **(D)** Left ventricular output provides systemic circulation to the upper extremities. **(E-F)** Venous blood flow from the inferior vena cava and superior vena cava mixes in the left atrium. **(G)** Right ventricular output through the pulmonary arteries enters the lungs for respiration. **(H)** Oxygenated blood returns from the lungs to the left side of the heart. **(I)** Left ventricular output provides systemic circulation for the entire body.

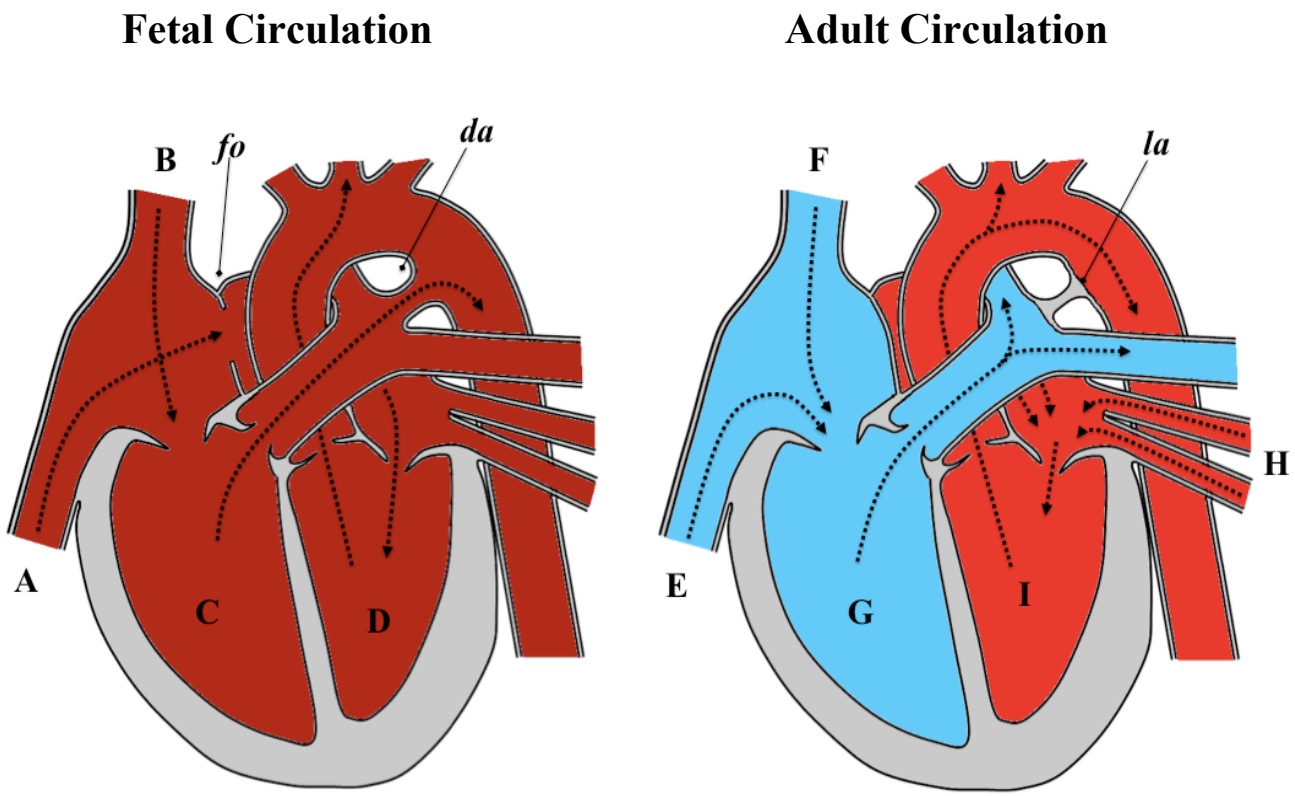

Figure 1.2. Eicosanoid metabolism pathways with specific focus on prostaglandin metabolism.

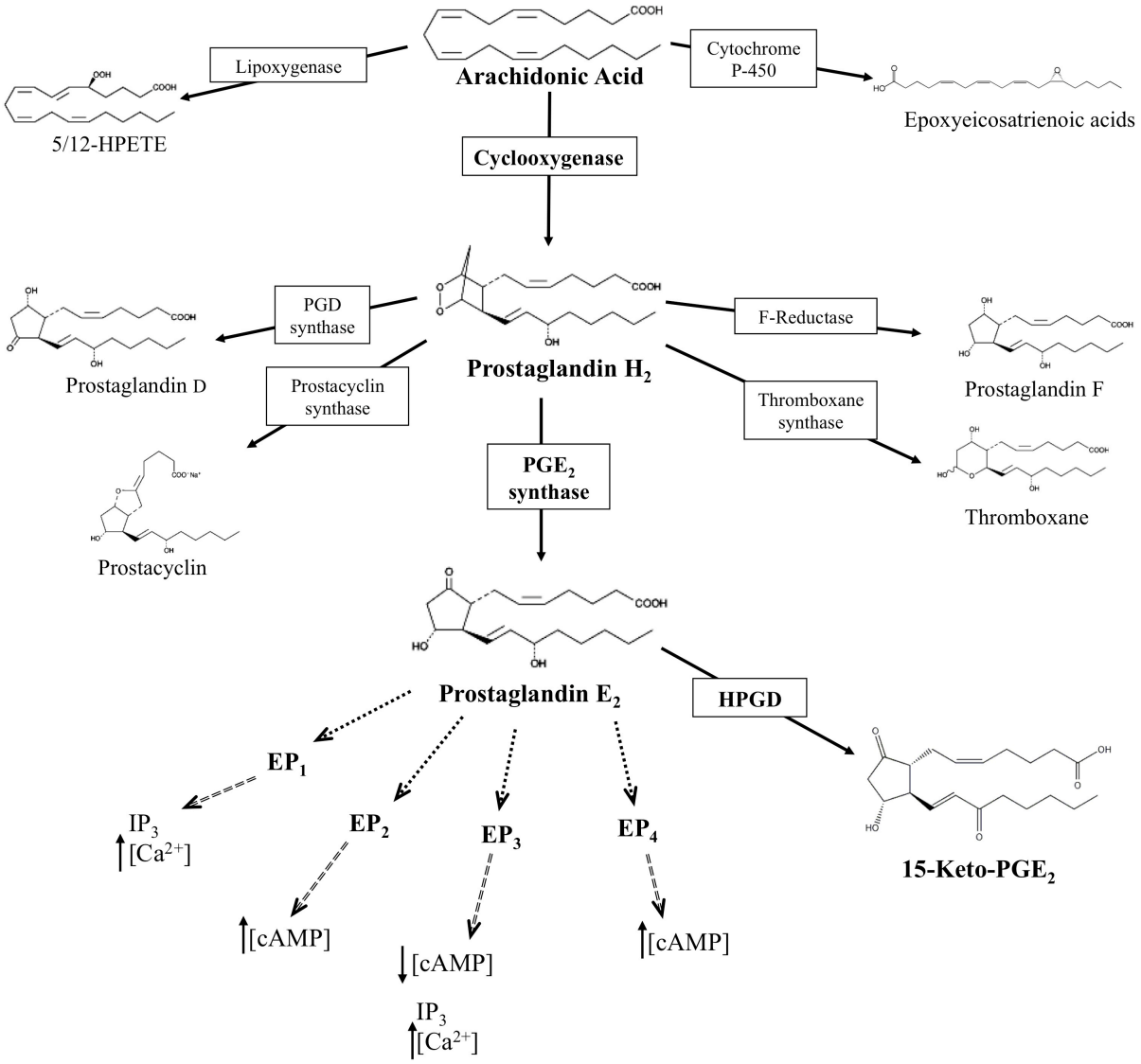

Figure 1.3. Phylogenetic tree of the EP4 receptor. Phylogenetic tree constructed using protein sequence from 10 amniotes (dog, rabbit, rat, mouse, human, chimpanzee, armadillo, cow, lizard, and chicken) and zebra fish using Structure based Sequences Alignment Program (STRAP). The mouse, rat, human, chimpanzee, rabbit, and dog EP4 receptors share >88% amino acid identity, and the mouse and chicken EP4 receptors share >70% amino acid identity.

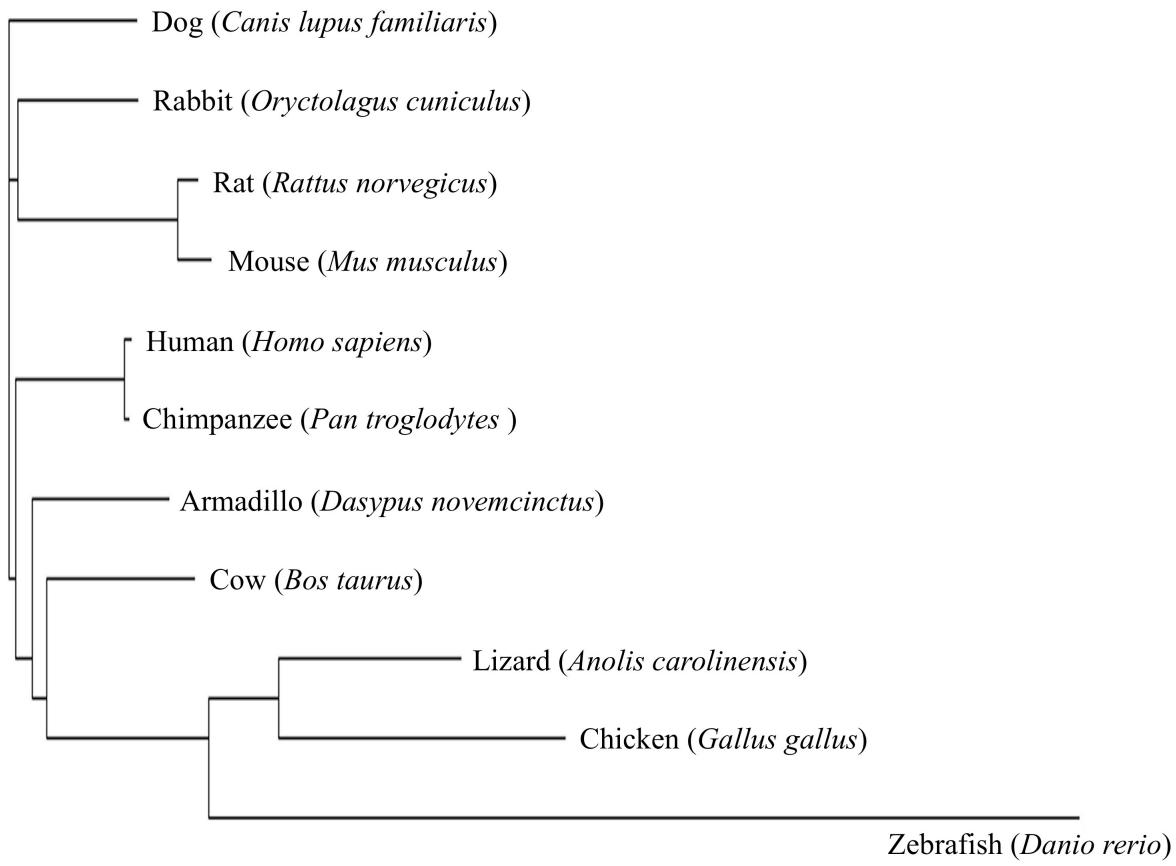

Table 1.1. Component of ductus arteriosus maturation and closure

Component	Summary	Ref.
Gestational Maturity	Gestation age inverse related to incidence of PDA	[83]
Oxygen	Initiation of pulmonary circulation results in increased plasma oxygen concentration in the DA triggering smooth muscle constriction; High altitude full-term births with increased incidence of PDA are treated by oxygen therapy	[84] [42]
Prostaglandins	Neonatal PGE infusion maintains DA patency; inhibition of PGE production triggers DA closures	[27] [85]
Bradykinin	Biphasic response to bradykinin by DA (relaxation at low levels and constriction at high levels)	[86]
Nitric Oxide	NO induced DA relaxation/dilation	[87]
Carbon Monoxide	DA relaxation/dilation with probable relationship to bradykinin and NO	[88]

Table 1.2. Genes with a known relationship to ductus arteriosus maturation and/or closure.

Genes	Summary	Ref.
PGE_2 receptors: EP2 & EP4	Common inbred EP4 null mice succumb to fatal PDA. Some species utilize the EP2 receptor instead of EP4	[68] [61]
Cyclooxygenase 1 & 2	Localized PGE_2 production in the DA plays role in DA closure, Increased incidence of PDA in COX-2 null mice	[89]
Hydroxy-prostaglandin dehydrogenase	PGE_2 main catabolizing agent. HPGD null mice succumb to fatal PDA	[90]
Myocardin	Transcription co-factor, neural crest smooth muscle loss results in fatal PDA in mice	[91]
Myosin, heavy polypeptide 11	Delayed DA closure in MYH11 null mice	[92]
TFAP2B transcription factor	Mutations in TFAP2B can result in non-syndromic PDA in humans	[78]
Rap guanine nucleotide exchange factor 3 (EPAC1)	Plays role in intimal cushion formation	[75]
IL-15	Might have an inhibitory effect on the physiologic vascular remodeling processes in closing the DA	[93]
Endothelin-1	Endothelin-1 receptor antagonism prevents DA constriction in rats	[94]

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Chapter 2

Expression of the PGE_2 EP4 receptor by smooth muscle cells is essential for closure of the ductus arteriosus at birth

ABSTRACT

Background - The ductus arteriosus (DA) is a fetal shunt that directs right ventricular outflow away from pulmonary circulation and into the aorta. In many species including humans and mice, critical roles for prostaglandin E_2 have been established in both maintaining the patency of the DA *in utero* and in its closure at birth. While studies in mice indicate that the EP4 receptor mediates these apparently incongruous actions of PGE_2 in the DA, the pathways linked to the EP4 receptor triggering these dramatic events are not known. Moreover, since EP4 receptors are expressed in the endothelial and smooth muscle compartments of the DA, the cellular mechanisms underlying these functions are also undefined.

Methods - To address these questions, we have generated mice in which loss of EP4 expression is limited to either the smooth muscle or endothelial cells and have determined the consequences of these deletions on DA closure. In addition, to define gene expression patterns linked to EP4 receptor activation in the DA, we carried out genome wide expression analysis of vessels that developed in the presence or absence of PGE_2/EP4 signaling.

Results - These studies show that EP4 receptor expression in smooth muscle is absolutely required for DA closure; pups lacking EP4 receptors limited to smooth muscle die within 48 hours of birth with a patent DA. In contrast, elimination of EP4 receptors from endothelial cells has no effect on the patency of the DA *in utero* or for closure and remodeling after birth. Analysis of DA from $EP4^{-/-}$ and wild type fetuses indicates that the PGE_2/EP4 pathway alters the gene expression signature of the DA suggesting that in addition to its role in maintaining the patency of the vessel *in utero*, the EP4 pathway may also be important for developmental changes that allow the vessel to undergo remodeling at birth.

INTRODUCTION

The ductus arteriosus (DA) is fetal arterial vessel that connects the pulmonary duct to the aorta. It thus allows the majority of right ventricular output to be directed past the fluid-filled fetal lungs towards the descending aorta and placental circulatory system where oxygenation takes place. Rapid closure of the vessel occurs in most mammalian neonates within 48 hours of birth. In humans failure of the vessel to undergo closure and remodeling can lead to pulmonary hypertension later in life. Closure of the vessel is believed to occur in two steps, with the initial constriction of the vessel largely triggered by an increase in arterial oxygen tension (as ventilation of the lung is initiated) and by a precipitous drop in PGE_2 levels at birth. Remodeling of the vessels is initiated with the lifting of the endothelium from the internal elastic lamina, fragmentation of the elastin and migration of smooth muscle cells into the sub-endothelial space [1]. This is followed by dramatic structural changes that result in the eventual transformation of the vessel into the ligamentum arteriosus.

The patency of the DA, both *in utero* and immediately after birth is sensitive to NSAIDs drugs such as indomethacin which block production of all prostaglandins and thromboxane by inhibiting cyclooxygenase (COX) activity. The cyclooxygenase isozymes convert arachidonic acid into prostaglandin endoperoxide (PGH_2), a rate-limiting step in all-downstream prostaglandin, prostacyclin, and thromboxane synthesis [2]. Exposure of the fetus to indomethacin can result in DA closure *in utero* [3], and neonatal indomethacin treatment can often bring about the closure of a PDA in a neonate [4]. Furthermore, prostaglandins, including PGE_2 can relax the pre-constricted ductus *in vitro* [5] and infusion of PGE_2 maintains the patency of the vessel after birth [6]. Together these experimental and

clinical findings supported a model in which the DA was regarded as having intrinsic tone with the patency of the fetal vessel *in utero* dependent on the dilatory actions of prostaglandins; PGE_2 and perhaps prostacyclin [7]. A dramatic drop in circulating prostaglandin levels occurs at birth because of loss of the prostanoid rich placental capillary beds and because of the redirection of the right ventricular output to the pulmonary circulation where high levels of the PGE_2 catabolizing enzyme HPGD are expressed. Loss of this vasodilatory mediator allows the intrinsic tone of the vessel to close the fetal shunt. The importance of the rapid decrease in PGE_2 levels at birth in closure of the DA is supported by the finding that mice lacking HPGD die in the perinatal period with PDA and a high incidence of patent ductus arteriosus (PDA) in families carrying a mutant allele of this gene [8, 9].

This model in which PGE_2 counteracts the normal tone of this vessel is consistent with the known potent dilatory action of PGE_2 in most vascular beds. This dilatory action is endothelial-dependent and believed to be dependent largely on the ability of PGE_2 to stimulate the production of NO by eNOS present in endothelial cells. NO stimulates soluble guanylate cyclase allowing accumulation of cGMP in the smooth muscle cells. The dilatory actions of PGE_2 have been attributed primarily to two of the four PGE_2 receptors, EP2 and EP4 receptors [5]. However, when examined more carefully, the phenotype of the EP4-deficient mice suggests a more complex role for prostaglandin in the physiology of this vessel, including the possible role for this receptor on smooth muscle cells. Here we describe experiments designed to identify the cell types critical to the function of the EP4 receptor in both prenatal and perinatal physiology of this vessel and in ensuring normal closure of this vessel at birth.

METHODS

In situ hybridization

The *Ptger4* specific cDNA probe was synthesized by reverse transcription and PCR amplification using primers *Ptger4*-1F (5'-GTTTGGCTGATATAACTGGTTAAT-3') and *Ptger*4-2R (5'-ACCTGGTGCTTCATCGACTGGACC-3) and total thymus RNA as a template. The PCR fragment was cloned using a TA cloning kit (In Vitrogen) and S^{35}-labeled probes prepared using a commercially available kit (Ambion). The DA was embedded in OCT compound, transverse sections 8 to 10μm in thickness were prepared, and *in situ* hybridization carried out as described.

Generation of a loxP-flanked conditional null *Ptger4* allele

The targeting plasmid was assembled using the vector pXenaFL[2], which contains a neomycin gene flanked by loxP and Frt sites, driven by a PGK promoter and a PGK driven Tk gene that makes it possible to select against cells in which targeting plasmids have randomly integrated into the mouse genome by growth in ganciclovir. Fragments of DNA corresponding to various regions of the *Ptger4* gene were amplified by PCR from DNA prepared from the ES cell line TC1 [10]. A 4912 bp fragment of DNA corresponding to intron 2 of EP4, beginning just 94 bp from the end of exon 2, was cloned 3' of the PGKneo gene in pXenaFL2, generating pXenaFL[2]-EP4-3'. A second segment of the EP4 gene, including the last 154 bp of intron 1, exon 2 and the first 94 bp of intron 2 was amplified and cloned just 3' of the loxP site in pNEBlox. pNEBloxP was derived by insertion of a loxP sequence between the Pst1 and HindIII sites pNEB193. This EP4 fragment together with the

loxP site was then excised from pNEBloxP and cloned 5' of the neo gene in pXenaFL^2-EP4-3' generating pXenaFL^2-EP4-3'/M. A third fragment of the EP4 gene, containing approximately 5kb of 5'flanking DNA, exon 1 and the 5' portion of intron 1 was amplified by PCR and cloned 5' of the pNEBloxP derived loxP site present in pXenaFL^2-EP4-3'/M.

The Not 1 linearized targeting plasmid was electroporated into TC1 a 129S6 derived ES cell line and transfected cells selected by growth in G418 and ganciclovir. ES colonies were individually expanded, DNA prepared and subjected to Southern blot analysis, using a 751 bp probe located just 3' of the region of *Ptger4* DNA included in the targeting vector, to identify clones in which the plasmid integrated into the mouse genome by homologous recombination. These colonies were further screened by Southern analysis using a probe corresponding to exon 2, to identify those clones in which the recombination event also resulted in introduction of the loxP site into intron 1. 80 G418 ganciclovir resistant colonies were analyzed: of these 7 had integrated the plasmid by homologous recombination, and of these 3 had the loxP site present in intron 1 of *Ptger4*. These ES cell were used to generate chimeras capable of transmitting the modified *Ptger4* allele to their offspring. By breeding the chimeras with 129S6 females, the conditional null allele was maintained on the 129S6 genetic background. To remove the neomycin gene from intron 2, but leave intact exon 2 flanked by loxP sites, mice were crossed with a 129S4 line that expresses a variant of the *Saccharomyces cerevisiae FLP1* recombinase gene under the direction of the *Gt(ROSA)26Sor* promoter [11]. Mice carrying the conditional Ptger4 allele were identified by PCR.

Transgenic Mouse lines

All protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina. The generation of mice lacking EP4 and EP2 receptors is described elsewhere [12, 13]. The *Ptger4*$^{+/-}$ mice were maintained on a 129S6 background. C57BL/6 *Ptger2*$^{+/-}$ mice were crossed for 4 generations to 129S6 before use. All recombinase mouse lines were obtained from Jackson Laboratories. 129S4/SvJaeSor-Gt(ROSA)26Sortm1(FLP1)Dym/J (Stock # 003946) were maintained on a 129S6 background [11]. B6.Cg-Tg(Tek-cre)129Flv/J lines (Stock #004128), referred to here as Tek-cre mice, were crossed for seven generations to 129S6 prior to intercross with mice carrying the conditional null allele [14]. STOCK Tg(Tagln-cre)1Her/J (Stock #004746), referred to here as Tagln-cre, were crossed for nine generations to 129S6 mice prior to intercross with mice carrying the conditional null allele [15]. STOCK Tg(Wnt1-cre)11Rth Tg(Wnt1-GAL4)11Rth/J (Stock #003829), referred to here as Wnt1-cre mice, were crossed for two generations to 129S6 mice prior to intercross with mice carrying the conditional null allele [16]. B6.C-Tg(CMV-cre)1Cgn/J (Stock # 006054), referred to as CMV-cre mice in this manuscripts, was maintained on a BALB/C genetic background [17]. In all cases the presence of the Cre recombinase transgene was identified by using primers specific for cre recombinase coding sequence (upper 5'-TTACCGGTCGATGCAACGAGT-3'; lower 5'-TTCCATGAGTGAACGAACCTGG-3').

Quantitative RT-PCR

Total RNA was isolated from tissues using Qiagen RNeasy Minikit or Microkit. 4-8 ductus arteriosi of identical genotype were pooled prior to extraction of RNA. Reverse

transcription of RNA to cDNA for quantitative RT-PCR was performed using a high-capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. Primers and probes were purchased from Applied Biosystems. All reactions were performed with TaqMan PCR Universal Master Mix (Applied Biosystems) using the Applied Biosystems 7900 HT Fast Real-Time PCR System. Reactions were performed in duplicate in a 20μl final volume reaction using 10ng of cDNA. Expression levels of genes of interest were normalized to either β-2 microglobulin (B2M) or GAPDH levels. Data were analyzed using the comparative CT method as described by Applied Biosystems.

Full term DA RNA for Expression Profile

DAs were isolated from 18.5 day full-term fetuses and individually stored at -80C until the genotype of the embryos was determined. RNA, from pooled samples of 8 genetically identical vessels, was isolated using Qiagen RNeasy Micro Kit as per manufacture's tissue isolation protocol. RNA quality was accessed with Agilent Technologies' RNA 6000 Nano Chips. Samples with distinct 18s and 28s rRNA peaks and no visible degradation or genomic DNA contamination were used for Illumina Beadchip microarray analysis.

Genome Wide Expression Profile

Illumina Beadchip microarray hybridization was done as per manufacture's protocol (brief described below) by Expression Analysis (Durham, NC). The Illumina Direct Hybridization assay begins with using the Illumina® Total Prep RNA Amplification Kit for making biotin-labeled cRNA from the poly-adenylated portion of eukaryotic total RNA. The

total RNA is reverse transcribed into single-stranded cDNA. The single stranded cDNA is converted to double stranded cDNA, which is followed by column purification of the double stranded cDNA. Purified double stranded cDNA is converted to biotin-labeled cRNA during an in vitro transcription reaction. The biotin-labeled cRNA is column purified, and then quantitated using OD260 measurements. 1.5 ug biotin-labeled cRNA is mixed with a hybridization buffer and hybridized to a Sentrix Array Matrix. Gene-specific probes on the Array hybridize the biotin-labeled cRNA. After hybridization, non-hybridized material is washed away. Following the wash step, the Array is washed with a blocking solution. Next, a SA-Cy3 stain solution is washed over the Array, allowing the biotin-labeled cRNA to bind the SA-Cy3. Excess stain is then washed away and the Array is allowed to dry. The SA-Cy3 signal is then detected by the Illumina BeadArray reader.

A total of 8 microarrays were analyzed; four wildtype DA and four EP4 deficient DA. Illumiuna BeadStudio (v1.5) was used to normalize the data with cubic-spline normalization. Differential expression lists were determined based on >1.25 fold change p-value < 0.05.

Statistical Analysis

Statistical analysis of quantitative real-time RT-PCR was performed using Prism 4 (GraphPad Software). Comparisons of the distributions were made by Mann Whitney U test unless otherwise noted. Data are shown as median ± S.E.M. Differences with $p < 0.05$ were considered statistically significant and denoted with a * unless otherwise noted. Test of proportions was used to calculate statistical significance of survival data.

RESULT

Expression of the EP4 receptor in the ductus

Consistent with previous studies, analysis of transverse sections through the ductus with *Ptger4* specific probes showed high levels of expression of *Ptger4* in the vessel, with an intense signal in the layers containing the smooth muscle (Figure 2.1). *Ptger4* expression in the vasculature is generally highest in the endothelial cells. Therefore, to examine this point further longitudinal sections were prepared from the ductus. This again showed high expression of *Ptger4* in the layers containing the smooth muscle cells of the DA. However, it also reveals an abrupt change in expression at the junction between the smooth muscle of the DA and the aorta, with little to no expression in *Ptger4* in smooth muscle cells of this vessel. This was confirmed by examination of cross section of the aorta. Similarly, little expression of *Ptger4* was detected in the smooth muscle pulmonary duct. In contrast expression of *Ptger4* by endothelia cells was apparent in all vessels. Thus in the DA unlike other greater vessels *Ptger4* appears to be expressed by both the endothelial cells and the smooth muscle cells.

To quantify the difference in expression of EP4 in these muscular arteries, RNA was prepared from the DA of a full term fetus, the adult aortic arch, and adult abdominal aorta and the abundance of *Ptger4* transcripts was determined by quantitative real-time RT-PCR. *Ptger4* transcripts were six time more abundant in the RNA prepared from the DA than in samples prepared from either the abdominal aorta and aortic arch.

Generation of the mice carrying a conditional null *Ptger4* allele

To distinguish between the functions of the EP4 receptor in smooth muscle cells and endothelial cells we first generated a mouse line carrying a conditional null allele (Figure 2.2). In this allele exon2 is flanked by loxP sites and therefore is predicted to be excised in cells expressing Cre recombinase [18]. This exon was chosen because it encodes the majority of EP4 including the first six transmembrane, intra and extra cellular domains as well as a portion of the seventh transmembrane domain. Targeted ES cells were identified by Southern analysis and then subjected to further analyses to determine whether the recombination event resulted in the introduction of the loxP site into intron 1. These targeted ES cells were introduced into C57BL/6 blastocysts and the resulting chimeras bred with 129S6 mice to establish the *Ptger4*$^{\text{flox-neo}}$ mouse line. To verify that the loxP sites are functional and confirm that loss of the DNA flanked by the sites would generate a null allele these animals were mated to a transgenic line that ubiquitously expresses Cre. In these animals expression of Cre should result in recombination between loxP sites and thus the loss of exon2 and generation of a null *Ptger4* locus. As expected when these animals were intercrossed with mice heterozygous for the null *Ptger4* allele, 25% of the mice died with a PDA (data not shown).

Mice heterozygous for the modified allele were intercrossed and pups homozygous for the *Ptger4*$^{\text{flox-neo}}$ allele identified. Analysis of lines generated both by our lab and others indicate that the presence of a neomycin-resistant gene in an intron can interfere with gene expression [19]. We therefore also bred the *Ptger4*$^{\text{flox-neo/+}}$ mice to mice expressing FLP recombinase in their germline. Expression of FLP recombinase in mice carrying the *Ptger4* $^{\text{flox-neo/+}}$ is expected to result in excision of the neomycin gene from intron 2, leaving a *Ptger4* allele, *Ptger4*$^{\text{flox}}$, which differs from the wild type locus only in the presence of loxP sites and DNA linkers sequences in intron 1 and 2 (less than 400 exogenous bp). PCR and Southern

Blot confirmed the loss of the neomycin gene. Expression of *Ptger4* in mice homozygous for either the *Ptger4*^{flox} or the *Ptger4*$^{flox\text{-}neo}$ allele was then determined. RNA was prepared from a number of tissues including the DA and the abundance of *Ptger4* transcripts relative to those present in wild type animals determined by quantitative real-time RT-PCR. As can be seen in Figure 2.3 the expression of the EP4 receptor by the floxed locus did not differ significantly from that of the wild type locus in any of the arteries examined. In contrast, expression of EP4 was significantly lower in tissue isolated from animals homozygous for the *Ptger4*$^{flox\text{-}neo}$ allele indicating that the introduction of the neomycin gene had resulted in the generation of *Ptger4* hypomorph. Closure of the DA in the perinatal period was normal in the homozygous *Ptger4*^{flox} pups and also in mice homozygous for the *Ptger4*$^{flox\text{-}neo}$ allele. Given that mice heterozygous for the null allele undergo normal remodeling of the DA and that the level of expression in the hypomorphs is approximately 30-50% of that seen in the wild type animals, this is perhaps not surprising.

Endothelial cell-specific loss of EP4 and DA patency

In situ analysis (Figure 2.1) shows expression of EP4 receptor by endothelial cells, and many studies indicate that PGE_2 can modulate vascular tone through its action on this cell type. It was therefore of interest to determine whether this expression also played a role in either maintaining the patency of this vessel *in utero* or in the closure of the vessel after birth. *Ptger4*$^{flox/flox}$ mice were intercrossed with mice heterozygous for the *Ptger4* null allele that also carry a Cre transgene controlled by the endothelial specific promoter of the *Tek* gene.

A similar cross was generated using *Ptger4*$^{flox\text{-}neo/flox\text{-}neo}$ mice. We reasoned that the lower expression of the gene in the hypomorph would increase our ability to observe a physiological impact of loss of expression of the EP4 receptor by endothelial cells. No difference in the survival of embryos carrying the flox/null or flox-neo/null alleles and the cre transgene was observed (Table 2.1). Furthermore these pups survived the neonatal period and necropsy of weanlings revealed the formation of the ligamentum arteriosus. To verify that endothelial specific loss of *Ptger4* expression had occurred in these mice we examined expression of the EP4 receptor in the aortic arch and abdominal aorta (Figure 2.4), as in these vessels, *in situ* analysis indicated that expression was limited to this cell type. EP4 expression was significantly reduced in both tissues.

Endothelial cells express a second Gs-coupled PGE_2 receptor, the EP2 receptor. It is therefore possible that this receptor plays a redundant role in this cell type compensating for loss of EP4. To rule out this possibility we examined the impact of endothelial specific loss of EP4 on an EP2$^{-/-}$ genetic background. Pups lacking expression of EP2 and EP4 on endothelial cells were present in litters at expected frequency. This indicates that loss of these Gs-coupled dilatory PGE_2 receptors is not required for the maintenance of fetal blood flow. The ductus of these animals retained their dependence on PGE_2 for patency: treatment of the dams with NSAID resulted in closure of the DAs of both wild type and EP2$^{-/-}$ EP4$^{flox/-}$ Tek-cre pups.

Smooth muscle-specific loss of EP4 receptor expression

As seen in Figure 2.1, the media of the ductus expresses high levels of EP4, likely reflecting expression of this receptor by smooth muscle cells. We therefore next intercross

the mice carrying both the flox and the flox-neo *Ptger4* alleles with mice expressing Cre under the control of the Tagln(Sm22) promoter. This promoter directs Cre expression in both vascular and non-vascular smooth muscle. Expression analysis indicated that *Tagln* is expressed in the DA (data not shown). We therefore expected mice expressing the Tagln-cre and the flox alleles would lack expression of the receptor in smooth muscle cells only, allowing us to isolate the function of EP4 receptors on these cells in DA closure. As can be seen in Table 2.1, all *Ptger4*$^{flox\text{-}neo/-}$ mice expressing the Tagln-cre die in the perinatal period with a PDA indistinguishable from that observed in mice homozygous for the null allele. The loss of EP_4 in smooth muscle cells was confirmed by real-time RT-PCR analysis of RNA isolated from the DA of wild type mice, *Ptger4*$^{flox/-}$ mice, *Ptger4*$^{flox/-}$ Tagln-cre mice, and *Ptger4*$^{-/-}$ pups (Figure 2.4). As expected, no expression of the *Ptger4* was detected in the *Ptger4*$^{-/-}$ pups. Low but significant expression of the gene was detected in DA of the *Ptger4*$^{flox/-}$ Tagln-cre mice. This is consistent with the undisturbed expression of the receptor on endothelial cells in the Tagln-cre mice. However, not surprising, given muscularity of this vessel, the expression of EP4 by the Tagln-cre *Ptger4*$^{flox/-}$ mice was dramatically lower than that observed in the DA from wild type animals. To determine whether the loss of expression of the receptor on the smooth muscle altered the response of the vessel to loss of PGE_2, dams were treated with indomethacin and the patency of the vessel scored in 18.5-day fetuses. The ducti of full term fetuses lacking expression of EP4 on smooth muscle cells remained patent in the absence of PGE_2, while as expected the closure was seen in littermates with normal EP4 expression.

Loss of EP4 in Neural crest derived smooth muscle cells

Tagln is expected to drive expression of Cre in all smooth muscle cells, not only those of the DA. This raises the possibility the PDA observed in the Tagln-cre *Ptger4*$^{flox/-}$ mice reflects the combined effect of the loss of expression of this gene on multiple smooth muscle populations. To address this concern we generated mice in which loss of EP4 expression was limited to neural crest derived cell, the primary embryonic origin of the smooth muscle cells of the DA and other great arteries of the heart [20]. Mice homozygous for either the flox or flox-neo *Ptger4* allele were crossed with *Ptger4*$^{+/-}$ mice carrying the Cre transgene driven by the Wnt-1 promoter. This limits loss of EP4 expression to all smooth muscle cells of the DA and a portion of the smooth muscle cells of the other greater arteries. Neural crest-EP4 receptor deficient mice succumb to cardiovascular failure as a result of persistent patency of the DA after birth, identical to that seen in the smooth muscle EP4-deficient mice and in EP4$^{-/-}$ animals (Table 2.1).

Identification of PGE_2/EP4 dependant gene expression in the DA.

To gain a mechanistic understanding to the contribution of EP4 receptor on the smooth muscle cells to both closure of the vessel at birth and to the dilatory actions of PGE_2 *in utero* we carried out expression analysis of DA isolated from full term *Ptger4*$^{-/-}$ and *Ptger4*$^{+/+}$ fetuses. We reasoned that differences in the expression of genes in these DA would identify pathways those activities were either directly or indirectly altered by exposure of the DA to PGE_2 and activation of the EP4 pathway during embryo development. While no morphological differences are observed between the DA of wildtype and EP4-/- fetus, we were surprised to find that gene expression analysis revealed 282 transcripts with statistically

significant differential expression ($p < 0.05$, fold change > 1.25). See Table 2.2 and Table 2.3, for a list of all 282 differential expressed transcripts.

Pathway analysis on the 282 differentially expressed genes was done using the Database for Annotation, Visualization and Integrated Discovery (DAVID) at National Institute of Allergy and Infectious Diseases (NIAID), NIH [21, 22]. Gene Functional Classification clustered the genes enriched in the wildtype DA into 4 clusters (enrichment scores >1.0). A cluster of transmembrane protein (enrichment score: 1.79) and proteases (enrichment score: 1.63) without any specific relation to prostanoids or arterial vessels. However, the other two clusters enriched in the wildtype DA are troponin-myosin related (enrichment score: 3.6) and tubulin-related (enrichment score: 1.1). Gene Functional Classification clustered the genes enriched in the EP4 deficient DA into 3 clusters (enrichment score >1.0): cytochrome-related cluster (enrichment score 1.82), atapase/ion transport cluster (enrichment score: 1.09), and mast cell related cluster (enrichment score: 1.01). See Table 2.4 for list of genes in each cluster.

Additional analysis of functional clusters was done with Ingenuity Pathway Analysis (v7.5). Multiple genes in the clusters enriched in the wildtype DA are expected to be sensitive to myocardin/serum response factors. Expression of both myocardin and one of its differentially expressed targets (myosin heavy chain 11) were analyzed in the full term DA from *Ptger4*$^{+/+}$ and *Ptger4*$^{-/-}$ fetuses. Both myocardin and myosin heavy chain 11 levels were significantly decreased in the *Ptger4*$^{-/-}$ DA compared to wildtype DA (Figure 2.5). To confirm the decrease was not due to a general decrease in muscularity of the *Ptger4*$^{-/-}$ DA, myosin light chain 7 (MYL7) was used as a control. MYL7 is expressed in cardiac smooth muscle and the aorta but expression patterns of MYL7 have not been shown to be

myocardin/SRF dependent. Consistent with the hypothesis, *Myl7* transcript levels did not differ in *Ptger4*$^{+/+}$ and *Ptger4*$^{-/-}$ DA.

DISCUSSION

Our studies show that the EP4 receptor is highly expressed by cells located in the media of the DA. In fact, transverse sections through the vessel indicate that the EP4 expression in this vessel serves as a marker for this great artery, distinguishing it from the adjoining pulmonary truck and aorta. Using mouse lines in which the expression of EP4 is limited to either the smooth muscle cells or endothelial cells, we provide evidence supporting both a dilatory and metabolic role for this receptor in this vessel, with both of these actions mediated through the EP4 receptor present on the smooth muscle cells.

The expression of EP4 by endothelial cells including those of the DA is consistent with a model in which activation of EP4 receptors leads to the production of dilatory mediators that act on the underlying smooth muscle cells to maintain patency of the vessel (Figure 2.6). However, while such a model accounts for the ability of PGE_2 to maintain the patency of the DA *in utero* and after birth, it is not consistent with our finding that fetal expression of the EP4 receptor by endothelial cells is not required for the normal development. Pups lacking endothelial EP4 expression are born at expected ratios, and normal closure after birth indicates that the vessel has matured and is capable of constriction and remodeling after birth. In addition, maternal exposure to indomethacin results in DA

constriction in these fetuses, despite the fact that the endothelium of the ductus of these animals would be insensitive to changes in circulating levels of PGE_2.

PGE_2 can mediate relaxation of other smooth muscle through G_s couple receptors. For example, relaxation of airway smooth muscle is mediated through the G_s coupled EP2 receptor [23]. The observation that a rapid decrease in PGE_2 levels can lead to rapid constriction of the DA, either at birth or in response to maternal indomethacin treatment, suggests that PGE_2 does act, at least in part, though similar dilatory pathways in the DA. However, if this represented the only function of smooth muscle EP4 receptor pathways, premature closure of the vessel *in utero* would be expected in the Tagln-cre *Ptger4*$^{flox/-}$ animals.

Taken together, the pharmacological and genetic studies suggest that a more complex model is required to describe the contribution of the EP4 receptor to the physiology of this vessel. PGE_2 is known to promote growth of many cell types. PGE_2 enhanced proliferation of murine aortic smooth muscle cells [24]. PGE_2 has also been shown to stimulate vascular endothelial growth factors in human airway smooth muscle cells [25]. In cardiac myocytes EP4 is linked to hypertrophy through the p42/44 MAPK pathway [26]. In one study, EP4 was reported to induce hyaluronic acid production in DA explants from rats [27]. These observations raise the possibility that, in addition to its function in maintaining the patency of the DA, PGE_2 through the EP4 receptor is essential for the maturation of the vessel, inducing the expression of genes essential for the closure and remodeling of the vessel at birth.

The demonstration here that the absence of EP4 receptors dramatically alters the expression signature of the DA supports such a model. One gene of particular interest that is differentially expressed in the *Ptger4*$^{-/-}$ and *Ptger4*$^{+/+}$ ducti is *Myh11*, smooth muscle myosin.

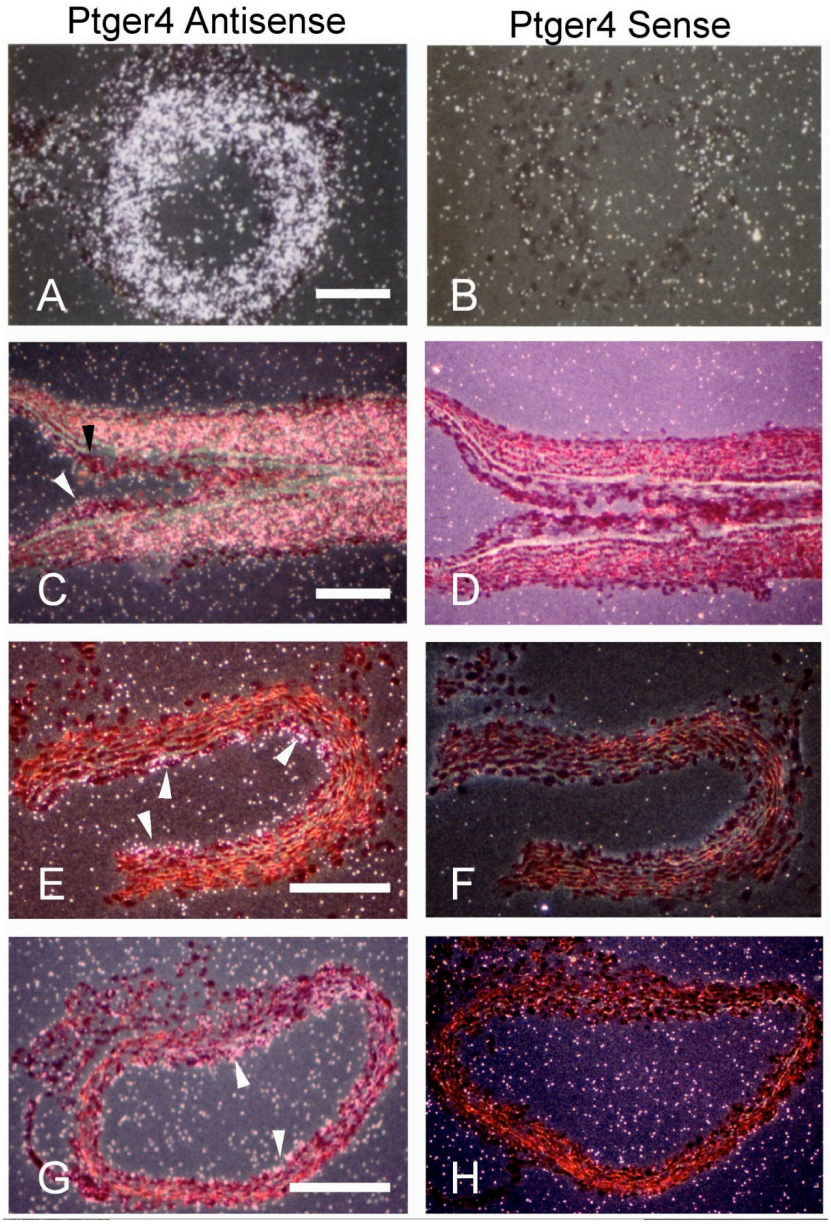
Mutations in MYH11 have been linked to hereditary PDA, and the loss of MYH11 in mice results in delayed closure of the DA [28, 29]. Expression of MYH11 and a number of other smooth muscle proteins that contribute to the contractility of the DA is regulated by myocardin. Myocardin, a coactivator for serum response factor (SRF), is a widely expressed transcription factor essential for the normal differentiation of cardiomyocytes and smooth muscle cells [30, 31]. Interestingly, although the difference in the expression of this transcription factor in *Ptger4*$^{-/-}$ and *Ptger4*$^{+/+}$ tissue did not achieve statistical significance in microarray analysis, quantitative PCR analysis indicated significantly lower levels of myocardin mRNA in the *Ptger4*$^{-/-}$ ductus. A role for this pathway in the maturation and closure of the DA is supported by previous reports of PDA in pups lacking expression of myocardin in neural crest derived smooth muscle cells [20]. This raised the possibility that the PGE_2/EP4 pathways may be required for maximal induction of myocardin and thus for the optimal development of the contractile phenotype of the DA.

The restriction of EP4 expression in the fetus to the DA smooth muscle would provide a means of limiting the growth promoting actions of PGE_2 to this vessel. Exposure of the DA to increasingly high levels of PGE_2 would trigger the synthesis of contractile proteins, together with other genes required for eventual remodeling of the vessel, increasing the tone of the vessel in late gestation. This role for PGE_2/EP4 is consistent with the PDA observed in the mice lacking EP4 receptor and with the PDA we report here in the mice lacking the expression of the EP4 receptor in the smooth muscle. The ability of EP4 to modulate the expression of genes in the DA supports a model in which this receptor carries out two function in the DA; it functions to maintain the patency of the vessel by inhibiting smooth muscle contraction and it induces the expression of genes that increase the

contractility of the vessels, and perhaps also, genes necessary for the remodeling of the vessel after birth.

The ductus is unique among the great arteries in its expression of extremely high levels of the prostaglandin receptor EP4. Our studies suggest that this expression pattern evolved to allow for rapid alteration of blood flow at birth, changes required for transition from placental to pulmonary oxygenation of blood. Expression of high levels of EP4 on DA smooth muscle cells provides a means by which the contractile properties of this vessel could be increased independent of other arteries. Importantly, by utilizing the EP4 receptor, a G_s protein coupled receptor for this task; the increase in the contractile properties of the DA could balance the concurrent increases in dilatory signaling. Furthermore, by linking the maturation and patency of the DA to EP4 a connection was established between the physiology of this vessel and the maturation of the fetus. PGE_2 levels peak late in gestation, but drop precipitously at birth, depriving the DA of the EP4 dilatory pathway and allowing the contractile properties to act unopposed contributing to closure of the fetal shunt.

Figure 2.1. Expression of *Ptger4* in the ductus arteriosus. (A, B) Transverse sections of DA from the 18.5 day mouse fetus; (C, D) longitudinal sections of DA from neonate; (E, F) transverse sections of neonatal aorta; (G, H) transverse section of neonatal pulmonary trunk; sections were hybridized with ^{35}S labeled *Ptger4* antisense probe (A,C,E,G) or *Ptger4* sense probe (B,C, F,H). Scale bar is 150μM. I) Ptger4 transcript levels in abdominal aorta, aortic arch, and DA as measured by quantitative real-time RT-PCR



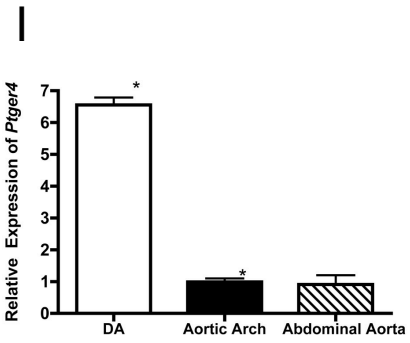


Figure 2.2. Schematic depicting the generation of a conditional null *EP4* allele. **A)** The structure of, the endogenous *EP4* locus, the targeting plasmid, and the *EP4* allele generated by homologous recombination of the plasmid with the wild type locus and the expected structures of these alleles in cells expressing either FLP or Cre recombinase, is shown. Restriction enzymes sites and probes used in identification of cells carrying a correctly targeted allele are indicated. The loxP and FRT sites are represented by filled and open triangles, respectively. EcoRI is abbreviated as RI. Filled, numbered boxes represent exons. **B and C** Identification of ES cell clones in which the targeting plasmid integrated by homologous recombination and in which this recombination event results in introduction of the loxP site into intron 1. Integration of the targeting plasmid by homologous recombination results in the introduction of an additional ScaI site generating a novel 6.6 kb Sca1 DNA fragment detectable by a probe located just 3' of the segment of the EP4 locus used to assemble the targeting construct. While only the Sca1 fragment corresponding to the endogenous EP4 locus is present in DNA shown in lane 1, the presents of a 9.8 and 6.6 kb fragment in lane 2 indicates that in this cell line one of the two *EP4* alleles has undergone homologous recombination with the targeting plasmid. Recombination between the targeting plasmid and the endogenous locus can occur at any point along regions of homology. To identify those ES cells in which recombination occurred 5' to the loxP site in intron 1, a second screen was carried out on all targeted ES cells. The presents of an EcoRI site in the linker DNA of the loxP site is expected to reduce the size of the EcoR1 fragment detected by a probe corresponding to exon 2. This probe detects an endogenous fragment of 5.5 kb and either a 7.8 kb or 3.7 kb in targeted ES cells lines, with the later fragment indicative of a crossover event 5' to the location of the loxP site in intron 1. Although the cell line

corresponding to the DNA analyzed in panel C lane 1 carries a targeted *EP4* allele, this line lacks the loxP site in intron 1. In contrast, the presence of the 3.7 kb EcoR1 fragment in lane three indicates that in this line intron 2 is flanked by loxP sites. The DNA shown in lane 2 was prepared from an untargeted ES cell line. **D.** Excision of the floxed *EP4* exon 2 in cells expressing Cre recombinase was verified by preparing DNA from mice generated by intercross of $EP4^{flox}$ mice with mice expressing Cre recombinase in their germline. The presence of a 8.9 kb Sca1 fragment detected by the 3' probe in DNA shown in lane 2 is consistent with Cre-mediated excision of exon 2 from the targeted allele. The Sca1 fragment generated from the $EP4^{floxP}$ allele is ~300bp larger than the wild type (WT) allele, due to the presence of the loxP sites and linker sequences. This small difference in size prevents resolution of these two alleles on Southern analysis with Sca1.

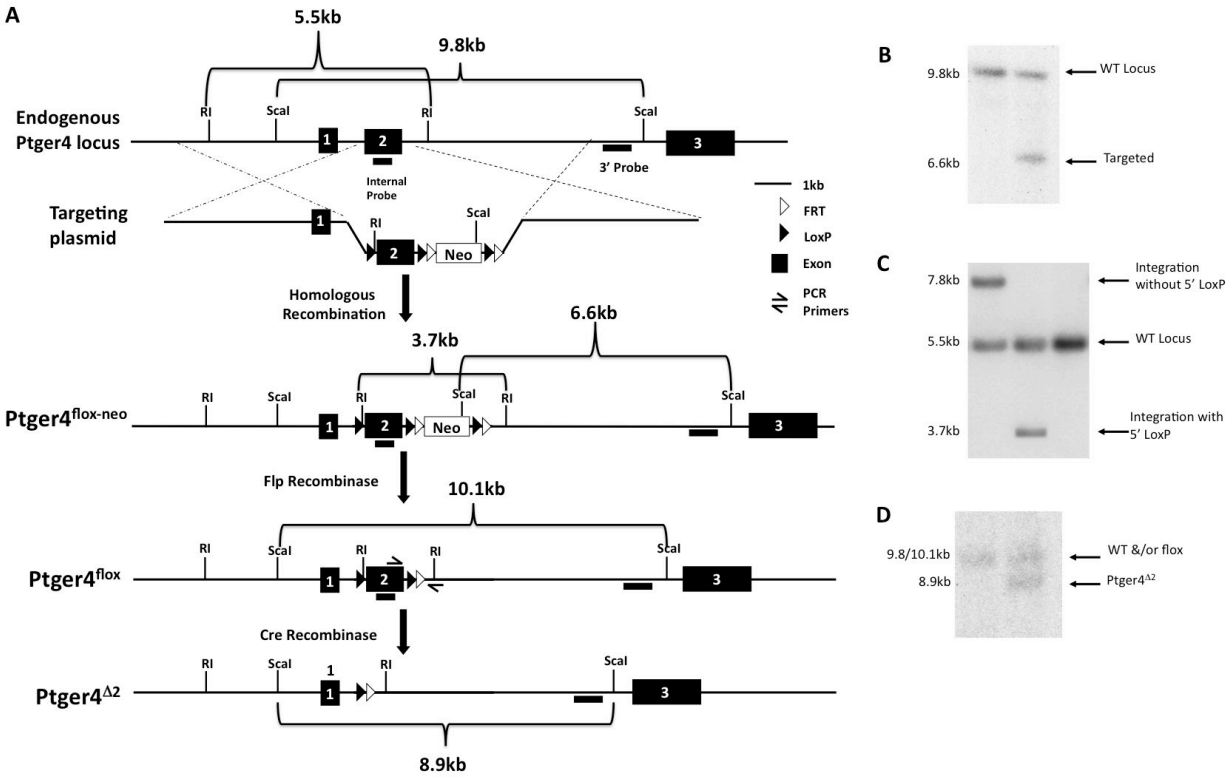


Figure 2.3. Quantitative RT-PCR demonstrating relative expression of the *Ptger4* receptor in mice homozygous for the conditional null Ptger4 alleles. RNA was prepared from the adult (A) Lung (n=5), (B) aortic arch (n=4), and (C) abdominal aorta (n=5), and from the (D) full term (18.5E) DA (n=5) of animals with the indicated genotype. Differential expression from wildtype was determined by Mann-Whitney U test: * $p < 0.05$

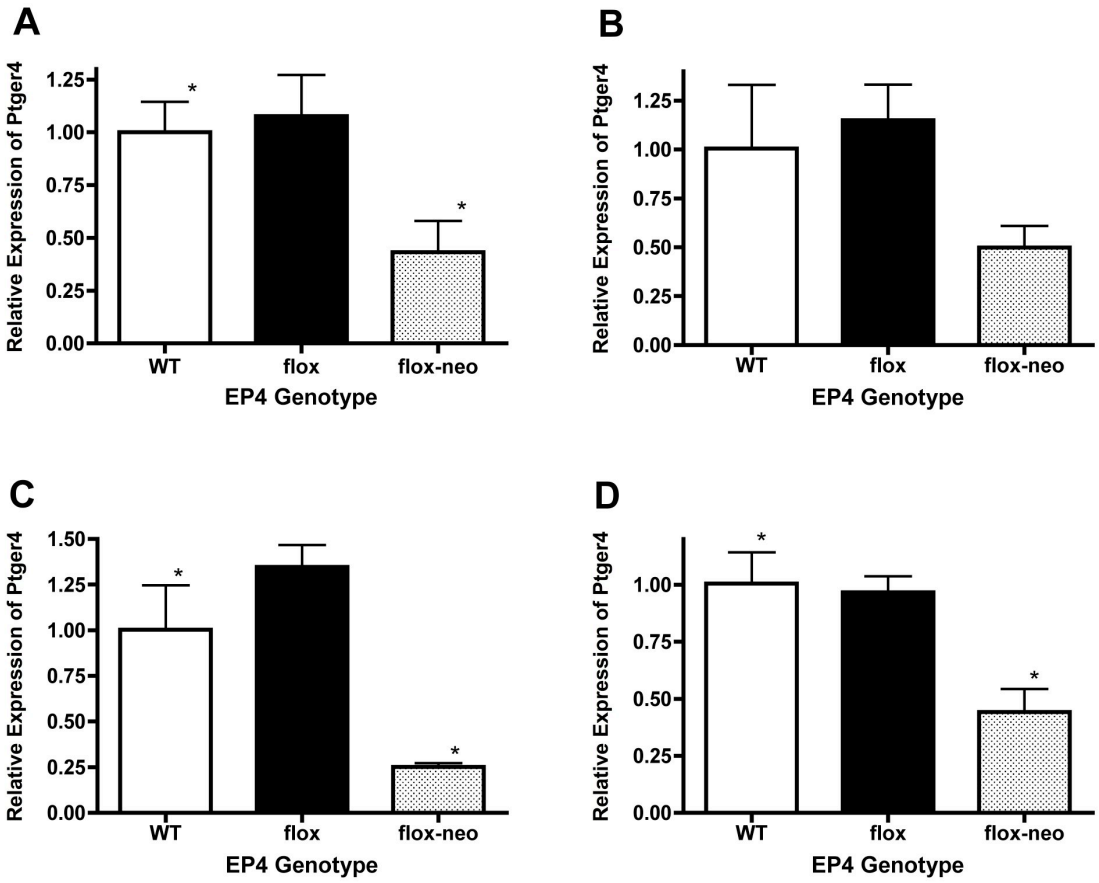


Figure 2.4. *Ptger4* receptor expression in endothelial cells and smooth muscle cells. (A) Aortic arch (n=8) and (B) abdominal aorta (n=8) RNA was examined from adult animals heterozygous for *Ptger4* null, *Ptger4*^{flox}, and genetically identical littermates expressing Tek-cre. (C) Adult kidney (n=5) from mice homozygous for the Ptger4$^{flox\text{-}neo}$ and mice heterozygous for Ptger4 null, Ptger4$^{flox\text{-}neo}$ expressing Tek-cre (D) DA (n=5 pools) and (E) fetal kidneys (n=6) RNA was examined from 18.5 day fetuses heterozygous for the Ptger4 null, Ptger4^{flox}, genetically identical embryos expressing Tagln-cre, and Ptger4 null fetuses. Differential expression determined by Mann-Whitney U test: * $p < 0.05$

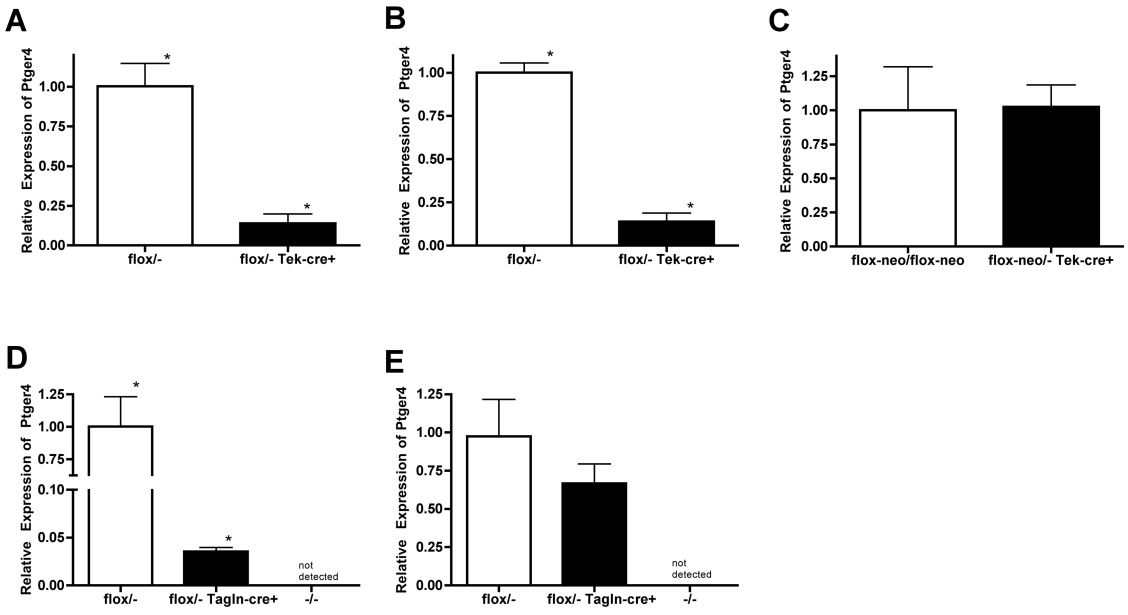


Figure 2.5. Quantitative real-time RT-PCR evaluation of *Myocd*, *Myh11, and Myl7* in the ductus arteriosus of wildtype and EP4 deficient animals. RNA was prepared from full term (18.5E) DA (n=8 pools, 4 DA/pool) of animals with the indicated genotype. * $p < 0.05$

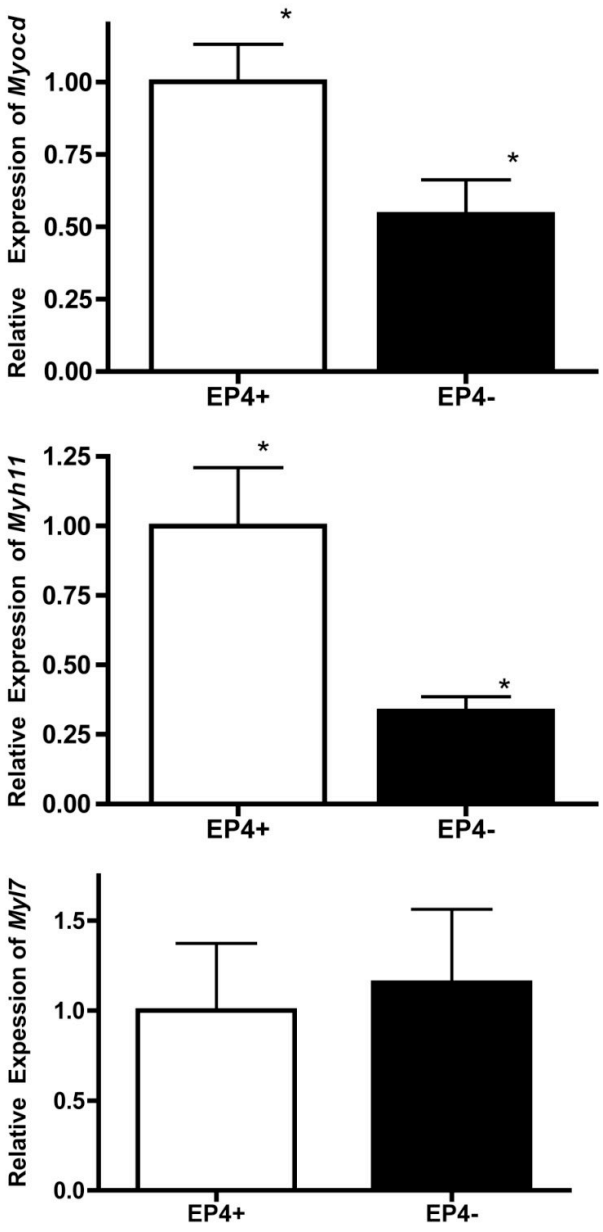


Figure 2.6. Models for the role of EP4 in the maturation and closure of the ductus arteriosus. **A)** DA has intrinsic tone that is counteracted by PGE_2 through endothelial EP4 receptors. This model predicts premature closure in EP4 deficient mice. **B)** No EP4 dependent dilatory signal is required to maintain patency. PGE_2/EP4 is responsible for the maturation of the DA, resulting in increase tone, which is required for closure at birth. However, this model is not consistent with the ability of indomethacin to rescue *HPGD*$^{-/-}$ mice. If PGE_2 had no dilatory function, the loss of PGE_2 would not be expected to cause closure. **C)** In this model, PGE_2 has two roles in the physiology of the DA. First through the smooth muscle EP4 receptors, PGE_2 provides a metabolic signal stimulating maturation. At the same time, PGE_2, through a dilatory signal via the endothelium, counteracts increasing tone of the DA. However, this model would predict premature closure in mice not expressing either EP4 or EP2 in the endothelium. **D)** In this model, PGE_2/EP4 provides two functions. In its absence, the DA fails to mature. Second, as the vessel matures, PGE_2 induced relaxation of the smooth muscle independent of the endothelium prevents premature closure. This model is consistent with the finding that mice lacking expression of EP4 in the smooth muscle die with a PDA, and mice lacking EP4 in the endothelium and EP2 in both endothelium and smooth muscle survive with no naïve phenotype.

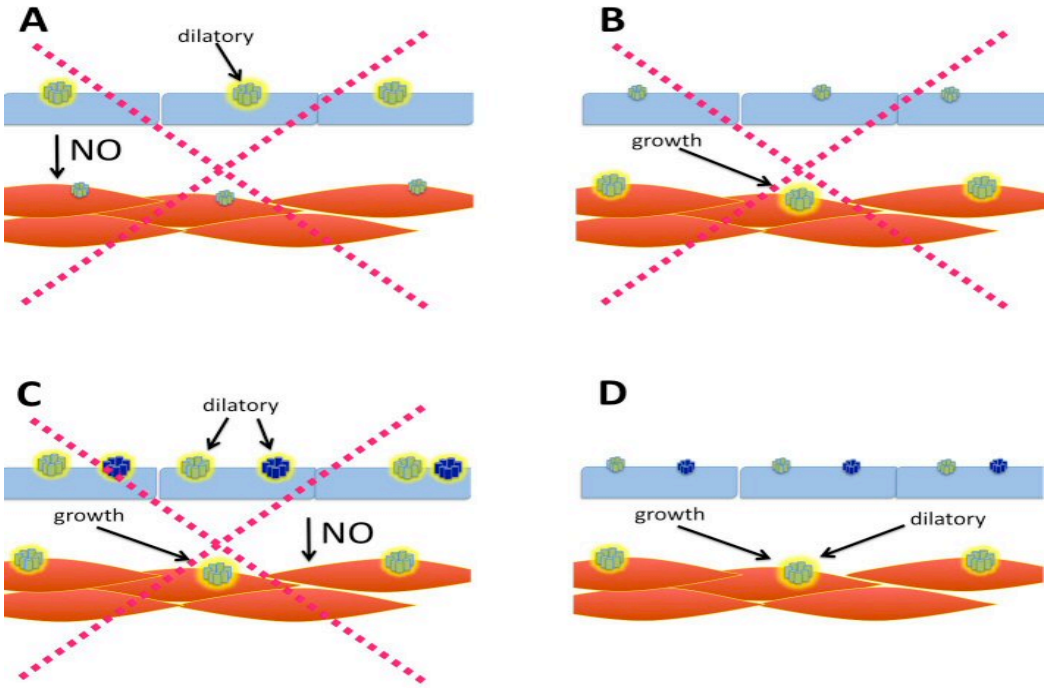

A
dilatory
NO
B
growth
C
dilatory
growth
NO
D
growth
dilatory

Table 2.1. Survival of mice with smooth muscle, neural crest, or endothelial *Ptger4* deficiency. Neonates were monitored for 72 hours after birth. Deceased pups and cyanotic pups (requiring euthanasia) are shaded. Mendelian expectation of tissue specific EP4-deficient animals is listed in brackets. Test of proportions between living and expected offspring was used to determine significance (p-value < 0.01). Test of proportions between surviving smooth muscle EP4-deficient animals (2/136) and surviving endothelial EP4-deficient animals (53/60) was significant (p-value < 0.0001).

		Living Offspring @ 72 hours										
Parental Genotype	Litters	*Ptger4*$^{+/+}$	*Ptger4*$^{+/-}$	*Ptger4*$^{flox(-neo)/+}$	*Ptger4*$^{flox(-neo)/-}$	*Ptger4*$^{+/+}$ Cre+	*Ptger4*$^{+/-}$ Cre+	*Ptger4*$^{flox(-neo)/+}$ Cre+	*Ptger4*$^{flox(-neo)/-}$ Cre+	Deceased *Ptger4*$^{flox(-neo)/-}$ Cre+	Expected *Ptger4*$^{flox(-neo)/-}$ Cre+	Significant (p-value < 0.01)
Ptger4$^{+/-}$ Tagln-Cre$^{+/0}$ x *Ptger4*$^{flox-neo/+}$	35	20	18	30	20	33	27	39	0	20	[27]	X
Ptger4$^{+/-}$ Tagln-Cre$^{+/0}$ x *Ptger4*$^{flox-neo/flox-neo}$	14	-	-	21	12	-	-	24	0	10	[19]	X
Ptger4$^{+/-}$ Tagln-Cre$^{+/+}$ x *Ptger4*$^{flox-neo/flox-neo}$	3	-	-	-	-	-	-	10	0	9	[10]	X
Ptger4$^{+/-}$ Tagln-Cre$^{+/0}$ x *Ptger4*$^{flox/flox}$	13	-	-	29	29	-	-	28	2	8	[29]	X
Ptger4$^{+/-}$ Tagln-Cre$^{+/+}$ x *Ptger4*$^{flox/flox}$	17	-	-	-	-	-	-	56	0	47	[56]	X
Ptger4$^{+/-}$ Wnt1-Cre$^{+/0}$ x *Ptger4*$^{flox-neo/flox-neo}$	17	-	-	32	35	-	-	25	0	32	[31]	X
Ptger4$^{+/-}$ Wnt1-Cre$^{+/0}$ x *Ptger4*$^{flox/flox}$	10	-	-	13	12	-	-	16	0	8	[14]	X
Ptger4$^{+/-}$ Tek-Cre$^{+/0}$ x *Ptger4*$^{flox-neo/flox-neo}$	27	-	-	52	39	-	-	55	34	7	[49]	
Ptger4$^{+/-}$ Tek-Cre$^{+/0}$ x *Ptger4*$^{flox/flox}$	11	-	-	33	22	-	-	24	19	0	[26]	

Table 2.2. mRNA down regulated in the ductus arteriosus of full-term EP4 deficient pups as determined by Illumina Beadchip Mouseref-8. Differential expression stringency: $p < 0.05$, fold change >1.25. Transcripts regulated by or with SRF/MYOCD are annotated in bold (as defined by Ingenuity Pathway Analysis). Illumina ID transcript, Accession Number, Gene Symbol, Gene Name, Fold Change, and P-value are list.

Illumina ID	Public ID	Gene Symbol	Gene Name	Fold Decrease	P-value
213948	NM_013645.2	PVALB	parvalbumin alpha	15.12	0.0130
213274	NM_011339.1	CXCL15	Chemokine (C-X-C motif) ligand 15	6.61	0.0000
222704	NM_053087.1	EPGN	Epithelial mitogen	4.68	0.0008
218569	NM_177652	RYR3	Ryanodine receptor 3	4.65	0.0253
212685	NM_027468	CPM	Carboxypeptidase M	4.44	0.0000
214286	NM_172928.1	Dcamkl3	Doublecortin and CaM kinase-like 3	3.02	0.0086
194118	NM_007739	COL8A1	Collagen, type VIII, alpha 1	2.69	0.0000
222158	NM_016754.3	MYLPF	Myosin light chain, fast skeletal muscle	2.68	0.0135
185125	NM_008728	NPR3	Natriuretic peptide receptor 3	2.50	0.0001
214631	NM_029981	ADAMTSL2	ADAMTS-like 2	2.37	0.0000
213216	NM_011824.1	GREM1	gremlin-1	2.34	0.0000
223608	NM_028472.1	BMPER	BMP-binding endothelial regulator	2.25	0.0000
210459	NM_028133.1	EGLN3	EGL nine homolog 3 (C. elegans)	2.09	0.0000
223402	NM_012028.2	SIAT7E	Sialyltransferase 7E	1.97	0.0370
214200	NM_026480.1	OOEP	Oocyte expressed protein homolog (dog)	1.97	0.0426
192308	NM_016873.1	WISP2	WNT1 inducible signaling pathway protein 2	1.97	0.0000
189372	NM_024285	BVES	Blood vessel epicardial substance	1.93	0.0000
216487	NM_011570.2	TES	Testis derived transcript, transcript variant 1,	1.89	0.0000
190311	NM_011620.1	TNNT3	Troponin T3, skeletal, fast	1.89	0.0000
211885	NM_016789.2	NPTX2	Neuronal pentraxin 2	1.86	0.0002
209643	NM_008137.2	GNA14	Guanine nucleotide binding protein, alpha 14	1.85	0.0001
211031	NM_011454.1	SERPINB6B	Serine/cysteine peptidase inhibitor, B6b	1.84	0.0000
212919	NM_010171.2	F3	Coagulation factor III	1.80	0.0000
189991	NM_009306	SYT1	Synaptotagmin I	1.78	0.0013
189308	NM_011618.1	TNNT1	Troponin T1, skeletal, slow	1.77	0.0356
186897	NM_009994	CYP1B1	Cytochrome P450, 1b1	1.75	0.0001
220885	NM_001126490	ISM1	Isthmin 1 homolog (zebrafish)	1.75	0.0000
191670	NM_009405.1	TNNI2	Troponin I, skeletal, fast 2	1.73	0.0043
188936	NM_009394.2	TNNC2	Troponin C2, fast	1.71	0.0188
209151	NM_030888.2	C1QTNF3	C1q and tumor necrosis factor related protein 3	1.70	0.0046
220036	NM_177914.1	DGKK	Diacylglycerol kinase kappa	1.68	0.0038
184867	NM_008357	IL15	Interleukin 15	1.67	0.0381
194147	NM_199473	COL8A2	Collagen, type VIII, alpha 2	1.66	0.0000
222248	NM_173767.2	INSC	Inscuteable homolog (Drosophila)	1.64	0.0024
195063	**NM_016672**	**DDC**	**Dopa decarboxylase**	**1.64**	**0.0223**
191471	NM_022322.2	TNMD	Tenomodulin	1.63	0.0034
214085	NM_013930.2	AASS	Aminoadipate-semialdehyde synthase	1.62	0.0127
219605	NM_172520.1	ARHGEF19	Rho guanine nucleotide exchange factor 19	1.62	0.0147
209457	NM_198885.2	SCX	Scleraxis	1.61	0.0000

212208	NM_181988.1	RERG	RAS-like, estrogen-regulated, growth-inhibitor	1.59	0.0005
221331	NM_007568.2	BTC	Betacellulin, epidermal growth factor family member	1.59	0.0008
195636	NM_175177	BDH	3-hydroxybutyrate dehydrogenase, type 1	1.58	0.0089
195145	NM_172496	COBL	Cordon-bleu	1.58	0.0490
184736	NM_145467	ITGBL1	Integrin, beta-like 1	1.57	0.0004
224297	NM_011448	SOX9	SRY-box containing gene 9	1.54	0.0000
196083	NM_024431	MORF4L1	Mortality factor 4 like 1	1.53	0.0233
222264	NM_008342.2	IGFBP2	Insulin-like growth factor binding protein 2	1.53	0.0000
193116	NM_011653	TUBA1	Tubulin, alpha 1A	1.53	0.0000
195245	NM_008764.2	TNFRSF11B	Osteoprotegerin	1.51	0.0004
195482	NM_013560	HSPB1	Heat shock protein 1	1.51	0.0000
222329	NM_016669.1	CRYM	Crystallin, mu	1.51	0.0013
219734	NM_008428.2	KCNJ8	Potassium inwardly-rectifying channel, J8	1.50	0.0001
208654	NM_010233	FN1	Fibronectin 1	1.49	0.0004
217063	NM_028608.1	GLIPR1	GLI pathogenesis-related 1 (glioma)	1.49	0.0000
195643	NM_026993	DDAH1	Dimethylarginine dimethylaminohydrolase 1	1.49	0.0001
189456	NM_029770	UNC5B	Unc-5 homolog B (C. elegans)	1.48	0.0000
210479	NM_178382.2	FLRT3	Fibronectin leucine rich transmembrane protein 3	1.48	0.0352
210976	NM_013507.2	EIF4G2	Eukaryotic translation initiation factor 4, gamma 2	1.47	0.0146
211631	NM_009194.1	SLC12A2	Solute carrier family 12, member 2	1.45	0.0006
216622	NM_013657.4	SEMA3C	Semaphorin 3C	1.45	0.0421
214390	NM_010317.2	GNG4	Guanine nucleotide binding protein, gamma 4 subunit	1.44	0.0223
221116	NM_172911.1	D8ERTD82E	Pragmin	1.43	0.0027
219038	NM_013519.1	FOXC2	Forkhead box C2	1.43	0.0000
211222	NM_177757.3	Kif26b	Kinesin family member 26B	1.43	0.0148
223377	NM_008176.1	CXCL1	Chemokine (C-X-C motif) ligand 1	1.42	0.0025
211787	NM_183390.1	KLHL6	Kelch-like 6 (Drosophila)	1.42	0.0005
186672	NM_178804	SLIT2	Slit homolog 2 (Drosophila)	1.42	0.0000
216680	NM_026639.1	ART4	ADP-ribosyltransferase 4	1.42	0.0001
217103	XM_147272.1	SH3BGR	SH3-binding domain glutamic acid-rich protein	1.42	0.0098
208887	NM_010158.1	KHDRBS3	KH domain containing, signal transduction associated 3	1.41	0.0181
210760	NM_010016.1	CD55	CD55 antigen	1.41	0.0036
217594	NM_009334.1	TCFAP2B	Transcription factor AP-2 beta	1.40	0.0226
221239	NM_022310.2	HSPA5	Heat shock 70kD protein 5	1.40	0.0006
220853	NM_001033301	FHDC1	FH2 domain containing 1	1.39	0.0074
221319	NM_011866.1	PDE10A	Phosphodiesterase 10A	1.38	0.0456
188577	NM_009451.3	TUBB4	Tubulin, beta 4	1.37	0.0000
194409	NM_011706.1	TRPV2	Transient receptor potential cation channel, V2	1.37	0.0020
220669	NM_152804.1	PLK2	Polo-like kinase 2 (Drosophila)	1.37	0.0000
224263	NM_008430	KCNK1	Potassium channel, subfamily K, member 1	1.36	0.0117
217240	NM_011857.2	ODZ3	Odd Oz/ten-m homolog 3 (Drosophila)	1.36	0.0006
214953	XM_147213.1	SLC15A2	Solute carrier family 15	1.36	0.0294
191087	NM_023908	SLCO3A1	Solute carrier organic anion transporter family 3a1	1.36	0.0078
214528	NM_018884.1	PDZRN3	PDZ domain containing RING finger 3	1.35	0.0367
220940	NM_172951.1	SNTG2	Syntrophin, gamma 2	1.35	0.0033
210434	NM_008549.1	MAN2A1	Mannosidase 2, alpha 1	1.35	0.0008
215425	NM_007689.2	CHAD	Chondroadherin	1.35	0.0015
209744	NM_146194	PICALM	Phosphatidylinositol binding clathrin assembly protein	1.35	0.0015
219818	NM_011375.1	SIAT9	ST3 beta-galactoside alpha-2,3-sialyltransferase 5	1.34	0.0072
222438	NM_029825	SCFD1	Sec1 family domain containing 1	1.34	0.0486
216305	NM_052973.1	STRN3	Striatin, calmodulin binding protein 3	1.34	0.0287
217595	NM_172161.2	IRAK2	Interleukin-1 receptor-associated kinase 2	1.34	0.0002

223759	NM_133897.1	LRRC8C	Leucine rich repeat containing 8 family, C	1.33	0.0364
184420	NM_153541.1	ZBTB8	Zinc finger and BTB domain containing 8b	1.33	0.0337
218073	NM_009468.1	DPYSL3	Dihydropyrimidinase-like 3	1.33	0.0006
224057	NM_008542	SMAD6	MAD homolog 6 (Drosophila)	1.33	0.0001
211657	NM_023056.2	TMEM176B	Transmembrane protein 176B	1.33	0.0180
220749	NM_010930.3	NOV	Nephroblastoma overexpressed gene	1.33	0.0172
186099	NM_153138.2	WASPIP	WAS/WASL interacting protein family, member 1	1.32	0.0097
220203	NM_172473.1	HACE1	HECT domain and ankyrin repeat containing, E3 ubiquitin protein ligase 1	1.32	0.0081
224054	NM_011846	MMP17	Matrix metallopeptidase 17	1.32	0.0000
194608	NM_178683	DEPDC1B	DEP domain containing 1B	1.32	0.0002
191350	NM_010818	CD200	CD200 antigen	1.32	0.0009
220162	NM_008760.2	OGN	Osteoglycin	1.31	0.0319
223132	NM_008516.1	LRRN1	Leucine rich repeat protein 1, neuronal	1.31	0.0425
193263	NM_011261	RELN	Reelin	1.31	0.0011
191790	NM_009416.2	TPM2	Tropomyosin 2, beta	1.31	0.0013
219312	NM_008056	FZD6	Frizzled homolog 6 (Drosophila)	1.31	0.0074
219275	NM_009386.1	TJP1	Tight junction protein 1	1.30	0.0057
210306	NM_013834.1	SFRP1	Secreted frizzled-related protein 1	1.30	0.0002
220981	NM_013846.2	ROR2	Receptor tyrosine kinase-like orphan receptor 2	1.30	0.0000
213360	NM_011851.2	NT5E	5' nucleotidase, ecto	1.30	0.0166
223065	NM_145438.1	LLGLH2	Lethal giant larvae homolog 2 (Drosophila)	1.30	0.0159
187391	NM_025356.2	UBE2D3	Ubiquitin-conjugating enzyme E2D 3	1.29	0.0251
220619	NM_009144.1	SFRP2	Secreted frizzled-related protein 2	1.29	0.0024
189442	NM_023158	CXCL16	Chemokine (C-X-C motif) ligand 16	1.29	0.0090
212800	NM_009911.2	CXCR4	Chemokine (C-X-C motif) receptor 4	1.28	0.0003
219446	NM_027052.2	SLC38A4	Solute carrier family 38, member 4	1.28	0.0203
219873	NM_007805.2	CYB561	Cytochrome b-561	1.28	0.0118
190234	NM_139300	MYLK	Myosin, light polypeptide kinase	1.27	0.0166
218918	NM_175475.2	CYP26B1	Cytochrome P450, family 26, subfamily b, polypeptide 1	1.27	0.0411
221939	NM_207142.1	OLFR1036	Olfactory receptor 1036	1.27	0.0053
210130	NM_183180.1	TSPAN18	Tetraspanin 18	1.27	0.0471
212053	NM_013607.1	MYH11	Myosin, heavy polypeptide 11, smooth muscle	1.27	0.0234
213358	NM_207663.2	DMN	Synemin, intermediate filament protein	1.27	0.0338
211655	NM_022316.1	SMOC1	SPARC related modular calcium binding 1	1.27	0.0090
210097	NM_017380.1	SEPT9	Septin 9	1.27	0.0277
221553	NM_025894.1	PSMD12	Proteasome 26S subunit, non-ATPase, 12	1.26	0.0061
216383	NM_199307	ECE1	Endothelin converting enzyme 1	1.26	0.0181
193870	NM_172294	SULF1	Sulfatase 1	1.26	0.0072
222775	NM_007876.1	DPEP1	Dipeptidase 1 (renal)	1.26	0.0012
186229	NM_027533	TSPAN2	Tetraspanin 2	1.26	0.0238
212326	NM_008057.2	FZD7	Frizzled homolog 7 (Drosophila)	1.26	0.0008
221274	NM_013470.1	ANXA3	Annexin A3	1.26	0.0000
217605	NM_009145.1	NPTN	Neuroplastin	1.26	0.0500
192016	NM_016885	EMCN	Endomucin	1.25	0.0002
209161	NM_175206.2	Fbxl22	F-box and leucine-rich repeat protein 22 (Fbxl22),	1.25	0.0210
223103	NM_007940.2	EPHX2	Epoxide hydrolase 2, cytoplasmic	1.25	0.0007
193661	NM_021273	CKB	Creatine kinase, brain	1.25	0.0000
213347	NM_010718.1	LIMK2	LIM motif-containing protein kinase 2	1.25	0.0405
213954	NM_016798.2	PDLIM3	PDZ and LIM domain 3	1.25	0.0000
224105	NM_010336	LPAR1	Lysophosphatidic acid receptor 1	1.25	0.0159

Table 2.3. mRNA up regulated in the ductus arteriosus of full-term EP4 deficient pups as determined by Illumina Beadchip Mouseref-8. Differential expression stringency: $p < 0.05$, fold change >1.25. Transcripts regulated by or with SRF/MYOCD are annotated in bold (as defined by Ingenuity Pathway Analysis). Illumina ID transcript, Accession Number, Gene Symbol, Gene Name, Fold Change, and P-value are list.

Illumina ID	Public ID	Gene Symbol	Gene Name	Fold increase	P-value
185459	NM_008797	PCX	Pyruvate carboxylase	7.45	0.0377
210568	NM_031180.1	KLB	Klotho beta	5.32	0.0064
214365	NM_001012322	SCTR	Secretin receptor	4.88	0.0280
218098	NM_178373.2	CIDEC	Cell death-inducing DFFA-like effector c	3.88	0.0387
221626	NM_009605.3	ACDC	Adiponectin	3.85	0.0133
219866	NM_153579.3	SV2B	Synaptic vesicle glycoprotein 2 b	3.69	0.0058
219899	NM_153170.1	SLC36A2	Solute carrier family 36, member 2	3.67	0.0450
220351	NM_010271.2	GPD1	Glycerol-3-phosphate dehydrogenase 1	3.28	0.0159
212767	NM_007606.2	CAR3	Carbonic anhydrase 3	3.14	0.0121
224252	NM_010779	MCPT4	Mast cell protease 4	3.07	0.0005
196155	NM_010923	NNAT	Neuronatin	2.89	0.0295
222851	NM_139134.1	CHODL	Chondrolectin	2.72	0.0002
223929	NM_133249	PPARGC1B	Peroxisome proliferative activated receptor	2.60	0.0158
220362	NM_008059.1	G0S2	G0/G1 switch gene 2	2.51	0.0170
213896	NM_009328.1	TCF15	Transcription factor 15	2.42	0.0109
218878	NM_008398.1	ITGA7	Integrin alpha 7	2.37	0.0012
213154	NM_139292.1	REEP6	Receptor accessory protein 6	2.33	0.0082
211772	NM_007498.2	ATF3	Activating transcription factor 3	2.30	0.0002
222787	NM_008573	MCPTL	Mast cell protease-like	2.26	0.0001
189441	NM_023133	RPS19	Ribosomal protein S19	2.17	0.0216
223923	NM_172119	DIO3	Deiodinase, iodothyronine type III	2.15	0.0069
218703	NM_175520.2	GPR81	G protein-coupled receptor 81	2.10	0.0021
212095	NM_007981.2	ACSL1	Acyl-CoA synthetase long-chain family member 1	2.05	0.0034
219489	NM_024495.2	CAR13	Carbonic anhydrase 13	2.01	0.0043
211789	NM_026384.2	DGAT2	Diacylglycerol O-acyltransferase 2	2.01	0.0206
219556	NM_175268.3	FAM53B	Family with sequence similarity 53	2.00	0.0238
223998	NM_013738	PLEK2	Pleckstrin 2	1.84	0.0004
216141	NM_001099635	MYH3	Myosin, heavy polypeptide 3, skeletal muscle	1.83	0.0000
219547	NM_001081106	CYTL1	Cytokine-like 1	1.79	0.0000
208882	NM_007469.2	APOC1	Apolipoprotein C-I	1.75	0.0456
223331	NM_177304.2	ENPP6	Ectonucleotide pyrophosphatase/phosphodiesterase 6	1.74	0.0035
185154	NM_020581	ANGPTL4	Angiopoietin-like 4	1.73	0.0163
215880	NM_028448	CENPV	Centromere protein V (Cenpv)	1.71	0.0240
218383	NM_008819.2	PEMT	Phosphatidylethanolamine N-methyltransferase	1.71	0.0265
212300	NM_009255.2	SERPINE2	Serine (cysteine) peptidase inhibitor, clade E, member 2	1.68	0.0001
215026	NM_013558.1	HSPA1L	Heat shock protein 1-like	1.68	0.0204
216774	NM_008940.1	PRSS19	Kallikrein related-peptidase 8	1.67	0.0000
216710	NM_146063.1	KRT79	Keratin 79	1.66	0.0359

211687	NM_178728.3	NAPEPLD	N-acyl phosphatidylethanolamine phospholipase D	1.65	0.0291
211016	NM_026730	GPIHBP1	GPI-anchored HDL-binding protein 1	1.63	0.0139
223969	NM_027391	IYD	Iodotyrosine deiodinase	1.61	0.0221
220490	NM_011157.1	SRGN	Serglycin	1.61	0.0000
221922	NM_009841.2	CD14	CD14 antigen	1.60	0.0356
211007	NM_026071.1	SLC25A19	Solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), 19	1.59	0.0368
217499	NM_182805.1	GPT1	Glutamic pyruvic transaminase 1	1.59	0.0485
213361	NM_009928.2	COL15A1	Procollagen, type XV	1.59	0.0000
213165	NM_148938.2	SLC1A3	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	1.58	0.0104
221505	NM_008002.3	FGF10	Fibroblast growth factor 10	1.58	0.0000
220489	NM_178405.2	ATP1A2	ATPase, Na+/K+ transporting, alpha 2 polypeptide	1.58	0.0020
215064	NM_177157.2	GCHFR	GTP cyclohydrolase I feedback regulator	1.57	0.0499
213671	NM_133783.1	PTGES2	Prostaglandin E synthase 2	1.57	0.0080
213534	NM_016745.2	ATP2A3	ATPase, Ca++ transporting	1.56	0.0223
222768	NM_010698.2	LDB2	LIM domain binding 2	1.55	0.0000
222379	*NM_009744.2*	*BCL6*	*B-cell leukemia/lymphoma 6*	*1.54*	*0.0125*
214170	NM_022029.1	NRGN	Neurogranin	1.52	0.0391
215279	NM_026272.2	NARF	Nuclear prelamin A recognition factor	1.51	0.0062
186307	NM_025802	PNPLA2	Patatin-like phospholipase domain containing 2	1.50	0.0383
211240	NM_007440.2	ALOX12	Arachidonate 12-lipoxygenase	1.49	0.0133
220901	NM_027857.2	ACY3	Aspartoacylase (aminoacylase) 3	1.49	0.0014
219921	NM_183263.1	RNMTL1	RNA methyltransferase like 1	1.47	0.0156
214660	NM_026516.1	TMEM178	Transmembrane protein 178	1.46	0.0330
196566	NM_145427.1	ATPAF2	ATP synthase mitochondrial F1 complex assembly factor 2	1.46	0.0009
185314	NM_028276	UTP14A	UTP14, U3 small nucleolar ribonucleoprotein, homolog A	1.45	0.0079
192989	NM_009437.2	TST	Thiosulfate sulfurtransferase	1.44	0.0208
221099	NM_148945.1	RPS6KA3	Ribosomal protein S6 kinase polypeptide 3	1.44	0.0311
215922	NM_025584.1	CD99	CD99 antigen	1.43	0.0001
215936	NM_010924.1	NNMT	Nicotinamide N-methyltransferase	1.42	0.0061
208930	NM_029020.2	ADCB8	ATP-binding cassette, sub-family B, member 8	1.42	0.0412
221447	NM_010095.2	EBF2	Early B-cell factor 2	1.42	0.0031
213067	NM_145532.2	MALL	Mal, T-cell differentiation protein-like (Mall)	1.42	0.0000
192034	NM_175106	TMRM177	Transmembrane protein 177	1.40	0.0317
220855	NM_053195.1	SLC24A3	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	1.40	0.0000
220374	NM_008787.2	PCNT2	Pericentrin	1.40	0.0061
215729	NM_007913.2	EGR1	Early growth response 1	1.40	0.0063
209344	NM_010780.1	MCPT5	Chymase 1	1.40	0.0423
208987	NM_145070.2	HIP1R	Huntingtin interacting protein 1 related	1.39	0.0000
220888	NM_207649.1	DSCR1L1	Down syndrome critical region gene 1-like 1	1.39	0.0002
215136	NM_026481.2	TPPP3	Tubulin polymerization-promoting protein family member 3	1.39	0.0005
223774	NM_183187.2	FAM107A	Family with sequence similarity 107, member A	1.39	0.0501
210091	NM_010046.2	DGAT1	Diacylglycerol O-acyltransferase 1	1.39	0.0062
216902	NM_183405.1	COXVIB2	Cytochrome c oxidase subunit VIb polypeptide 2	1.38	0.0492
192760	NM_025760	PTPLAD2	Protein tyrosine phosphatase-like A domain containing 2	1.38	0.0430
218536	NM_130884.1	IDH3B	Isocitrate dehydrogenase 3 (NAD+) beta	1.38	0.0010
213209	NM_009765.1	BRCA2	Breast cancer 2	1.37	0.0397
188466	NM_053261	IMPA2	Inositol (myo)-1(or 4)-monophosphatase 2	1.37	0.0385
214033	NM_183171.1	FEZ1	Fasciculation and elongation protein zeta 1	1.37	0.0003

224304	NM_175438	ALDH4A1	Aldehyde dehydrogenase 4 family, member A1	1.36	0.0046
218477	NM_201230	FGFR1OP	Fgfr1 oncogene partner	1.36	0.0157
214816	NM_007409.2	ADH1	Alcohol dehydrogenase 1 (class I)	1.36	0.0098
221458	NM_011338	CCL9	Chemokine (C-C motif) ligand 9	1.35	0.0413
210330	NM_013723.2	PODXL	Podocalyxin-like	1.34	0.0001
219676	NM_033569.1	CNNM2	Cyclin M2	1.34	0.0006
221353	NM_016894.1	RAMP1	Receptor (calcitonin) activity modifying protein 1	1.34	0.0001
221373	NM_029083.1	DDIT4	DNA-damage-inducible transcript 4	1.33	0.0047
213298	NM_011199.1	PTHR1	Parathyroid hormone receptor 1	1.33	0.0137
218007	NM_011304.1	RUVBL2	RuvB-like protein 2	1.33	0.0000
215828	NM_024177.2	MRPL38	Mitochondrial ribosomal protein L38	1.33	0.0003
186695	NM_178655	ANK2	Ankyrin 2	1.33	0.0279
211650	NM_010597.2	KCNAB1	Potassium voltage-gated channel, shaker-related subfamily, beta member 1	1.32	0.0112
187890	NM_026936	OXA1	Oxidase assembly 1-like	1.32	0.0199
209664	NM_007669.2	CDKN1A	Cyclin-dependent kinase inhibitor 1A (P21)	1.32	0.0001
217974	NM_010218	FJX1	Four jointed box 1	1.32	0.0344
195280	NM_145996	ARID5A	AT rich interactive domain 5A	1.31	0.0477
210469	NM_025285.1	STMN2	Stathmin-like 2	1.31	0.0225
209971	NM_010474.1	HS3ST1	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	1.31	0.0030
218963	NM_172753.2	CSGALNACT 1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	1.31	0.0085
210971	NM_028804	CCDC3	Coiled-coil domain containing 3	1.31	0.0000
212280	NM_133928.1	CHCHD4	Coiled-coil-helix-coiled-coil-helix domain containing 4	1.31	0.0181
224113	NM_173408	DCN1	Defective in cullin neddylation 1	1.31	0.0119
220989	NM_011898.2	SPRY4	Sprouty homolog 4 (Drosophila)	1.30	0.0019
215445	NM_027203.2	LENG1	Leukocyte receptor cluster (LRC) member 1	1.30	0.0311
221263	NM_197985.2	ADIPOR2	Adiponectin receptor 2	1.30	0.0137
216450	NM_010863.1	MYO1B	Myosin IB	1.30	0.0315
218564	NM_179203.1	ATAD3A	ATPase family, AAA domain containing 3A	1.30	0.0218
217105	NM_021387.1	VSTM2B	V-set and transmembrane domain containing 2B	1.29	0.0372
210635	NM_008813.2	ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	1.29	0.0291
191042	NM_177574.1	YPS37D	Vacuolar protein sorting 37D (yeast)	1.29	0.0255
223606	NM_139198.1	PLAC8	Placenta-specific 8	1.29	0.0094
219451	NM_008584.1	MEOX2	Mesenchyme homeobox 2	1.28	0.0324
219627	NM_011577.1	TGFB1	Transforming growth factor, beta 1	1.28	0.0210
195193	NM_178685	PCDH20	Protocadherin 20	1.28	0.0442
193798	NM_178114	AMIGO2	Adhesion molecule with Ig like domain 2	1.28	0.0041
220531	XM_126141.3	C1QTNF2	C1q and tumor necrosis factor related protein 2	1.28	0.0000
223570	NM_013898.1	TIMM8A	Translocase of inner mitochondrial membrane 8a1	1.28	0.0416
213944	NM_016662.2	MXD3	Max dimerization protein 3	1.28	0.0212
210167	NM_013415.2	ATP1B2	ATPase, Na+/K+ transporting, beta 2 polypeptide	1.28	0.0287
224085	NM_173749	PAMR1	Peptidase domain containing associated with muscle regeneration 1	1.28	0.0000
219811	NM_008713.2	NOS3	Nitric oxide synthase 3, endothelial cell	1.28	0.0048
212955	NM_138753.1	HEXIM1	Hexamethylene bis-acetamide inducible 1	1.27	0.0370
214901	NM_013543.1	H2-KE6	H2-K region expressed gene 6	1.27	0.0003
220032	NM_031196.1	SLC19A1	Solute carrier family 19 (sodium/hydrogen exchanger), member 1	1.27	0.0207
215908	NM_173370.3	CDS1	CDP-diacylglycerol synthase 1	1.27	0.0240
192815	NM_177683.2	VGLL4	Vestigial like 4 (Drosophila)	1.27	0.0047
216980	NM_023203.1	DCTPP1	dCTP pyrophosphatase 1 (Dctpp1)	1.27	0.0099
211069	NM_053091.1	COX4I2	Cytochrome c oxidase subunit IV isoform 2	1.26	0.0024

188390	NM_025407.2	UQCRC1	Ubiquinol-cytochrome c reductase core protein 1	1.26	0.0322
212582	NM_010925.1	RRP1	Ribosomal RNA processing 1 homolog (S. cerevisiae)	1.26	0.0342
222660	NM_025314.1	DTD1	D-tyrosyl-tRNA deacylase 1 homolog (S. cerevisiae)	1.26	0.0079
217369	NM_019695	PARD6A	Par-6 (partitioning defective 6,) homolog alpha	1.26	0.0437
219624	NM_025928.1	PMF1	Polyamine-modulated factor 1	1.26	0.0019
221027	NM_032003.1	ENPP5	Ectonucleotide pyrophosphatase/phosphodiesterase 5	1.25	0.0013

Table 2.4. Gene Functional Classification clustering by the Database for Annotation, Visualization and Integrated Discovery (DAVID) of differentially expressed genes enriched in the A) wildtype ductus arteriosus and B) the EP4 deficient ductus arteriosus.

A)

Group 1	Enrichment Score: 3.6
2481133	troponin i, skeletal, fast 2
2456978	troponin c2, fast
2636266	desmuslin
2482209	tropomyosin 2, beta
2469018	troponin t3, skeletal, fast
2460136	troponin t1, skeletal, slow

Group 2	Enrichment Score: 1.79
2734097	betacellulin, epidermal growth factor family member
1215807	gli pathogenesis-related 1 (glioma)
1259365	endomucin
1259470	riken cdna 1810009m01 gene
1246836	frizzled homolog 7 (drosophila)
1223849	cd200 antigen
1234698	tetraspanin 2
1243429	tenomodulin
2702016	riken cdna 2010011i20 gene
1227699	riken cdna 2610042g18 gene
2726030	cdna sequence ab023957
1248696	leucine rich repeat containing 8 family, member c
2774854	endothelial differentiation, lysophosphatidic acid g-protein-coupled receptor, 2
1235735	riken cdna 5830414c08 gene
1229788	coagulation factor iii
2728493	blood vessel epicardial substance

Group 3	Enrichment Score: 1.63
2754551	dipeptidase 1 (renal)
2636285	5' nucleotidase, ecto
2773800	matrix metallopeptidase 17
2629191	carboxypeptidase m

Group 4	Enrichment Score: 1.1
2602185	septin 9
1236175	tubulin, beta 4
2613740	cdna sequence bc056349
2494251	tubulin, alpha 1
2597778	guanine nucleotide binding protein, alpha 14

4B)

Group 1	Enrichment Score: 1.82
1256673	ubiquinol-cytochrome c reductase core protein 1
2612178	cytochrome c oxidase subunit iv isoform 2
2611532	solute carrier family 25 (mitochondrial deoxynucleotide carrier), member 19
1229627	cytochrome c oxidase subunit vib polypeptide 2

Group 2	Enrichment Score: 1.09
2722716	atpase, na+/k+ transporting, alpha 2 polypeptide
2638017	atpase, ca++ transporting, ubiquitous
2602902	atpase, na+/k+ transporting, beta 2 polypeptide
2590908	atp-binding cassette, sub-family b (mdr/tap), member 8
2727777	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3

Group 3	Enrichment Score: 1.03
2675232	protease, serine, 19 (neuropsin)
1240746	mast cell protease 4
1248718	chymase 2, mast cell
1227947	riken cdna e430002g05 gene
2594908	mast cell protease 5

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Chapter 3

PGE_2 Independent Maturation and Closure of the Ductus Arteriosus in a Recombinant Inbred Mouse Line

ABSTRACT

Objective - Prostaglandin E_2 (PGE_2) has a well-established role in controlling patency of the ductus arteriosus (DA). Neonatal PGE_2 infusion maintains DA patency, and pharmacological inhibition of PGE_2 production can result in DA closure. Common inbred mouse strains deficient in the prostaglandin E_2 receptor 4 (EP4) or hydroxyprostaglandin dehydrogenase (HPGD) succumb to fatal persistent patency of the DA. The genetic inheritance of PDA in humans is far more complex, indicating the existence of several modifiers or redundant pathways that contribute to DA closure.

Methods and Results - Here we report a recombinant inbred (RI) mouse strain that exhibits PGE_2 independent DA maturation and closure. The RI strain was derived from selective breeding of *EP4*$^{+/-}$ mice until EP4 deficient survival rates of 70% were achieved. The RI mice undergo DA closure during subcutaneous injection of PGE_2, the loss of HPGD, or the loss of both cyclooxygenase isozymes.

C**onclusions -** The loss of EP4, HPGD, or COX, and pharmacological inhibition of PGE_2 production indicates that the DA is capable of prostanoid-independent maturation and closure.

INTRODUCTION

Most of the blood flow through the fetal circulatory system of placental mammals bypasses the pulmonary system via the ductus arteriosus (DA) and foramen ovale. At birth, when the placenta can no longer provide the site of gas exchange, the cardio-pulmonary system of the neonate must quickly remodel from the fetal, pulmonary bypassing system, into the adult circulatory system. A primary component of this cardiovascular transition is the constriction and permanent remodeling of the DA [1]. The DA shunts blood from the right ventricle to the descending aorta, allowing nearly all the blood ejected from the right ventricle to bypass the pulmonary system. During *in utero* development, the patent DA and foramen ovale allow the four chambers of the heart to develop while allowing blood flow to bypass the pulmonary system. At birth, the air filled lungs offer less resistance to pulmonary blood flow, and increased pulmonary blood output causes an increase in left atrial pressure resulting in physical closure of the foramen ovale. An increase in oxygen tension and a decrease in plasma PGE_2 levels coincide with DA closure.

Human neonatal care has demonstrated the importance of prostaglandin E_2 (PGE_2) for DA maturation and closure. At birth, a newborn's plasma PGE_2 levels drop as pulmonary circulation catabolizes plasma PGE_2. A pharmacologically induced drop in PGE_2 via cyclooxygenase inhibition, can also trigger closure whether it is given maternally resulting in premature *in utero* DA closure, or neonatally in an attempt to initiate closure of the DA [2]. Conversely, neonatal infusion of PGE_2 maintains patency of the DA [3].

Genetic disruption of various components of prostaglandin metabolism and signaling has further refined the role of prostaglandins in DA maturation and closure. The loss of the

PGE_2 subtype 4 receptor (*Ptger4*; EP4) leads to persistent PDA in common inbred mouse strains (CIMS), resulting in congestive heart failure within the first 24 hours of life [4]. The loss of cyclooxygenase-2 (COX-2) increases incidence of PDA, and the loss of both cyclooxygenase isozymes (COX-1 and COX-2) results in 100% morbidity of pups prior to any DA remodeling [5]. Genetic disruption of hydroxyprostaglandin dehydrogenase (HPGD), the main catabolizing agent of PGE_2, results in fatal PDA in CIMS [6]. CIMS with either insufficient PGE_2 levels (COX deficiency), loss of EP4 receptor expression, or lack of quick PGE_2 catabolism (HPGD deficiency) succumb to cardiovascular failure due to a persistent PDA.

In larger mammals the inheritance of PDA is a polygenic trait and numerous molecular and environmental components may play a role in DA closure [7]. The inheritance of PDA in CIMS is simple; disruption of any part of PGE_2/EP4 signaling results in PDA. Here we present a selectively bred recombinant inbred (RI) mouse strain that exhibits PGE_2/EP4 independent DA maturation and closure that potentially represents the additional molecular components responsible for the complex genetic inheritance of PDA seen in higher mammals.

METHODS

Generation of Recombinant Inbred Strain with Prostaglandin Independent DA Closure

Disruption of the *Ptger4* locus by targeted homologous recombination in 129/Ola embryonic stem cells is described elsewhere [4]. 129 chimeras were mated to (C57BL/6 x DBA/2)F1 females, yielding a mixed genetic background (MB) carrying 129, DBA/2, and

B6 alleles. Intercrossing MB littermates generated rare *Ptger4*$^{-/-}$ mice that survived to adulthood. Surviving *Ptger4*$^{-/-}$ mice were mated to *Ptger4*$^{+/-}$ siblings. Brother-sister mating continued for >30 generations with selection for *Ptger4*$^{-/-}$ survival generated a recombinant inbred (RI) mouse line.

Genome Wide SNP Scan

Purified genomic DNA was used for whole genome panel of 768 SNPs using the Illumina GoldenGate platform at the Mutations Mapping and Development Analysis Project (MMDAP) directed by Jennifer Moran and David Beier at Brigham and Women's Hospital, Harvard Medical School in collaboration with The Broad Institute Center for Genotyping and Analysis. Of the 768 SNPs on the panel, 694 are informative for the three founding strain (129, C57BL/6, and DBA/2) of the RI mouse line.

Histology of Ductus Arteriosus

Full-term (E18.5) females were euthanized and fetuses collected. Neonatal pups were obtained from full-term females that were observed at E19 for the initiation of natural delivery. Birth of the third pup was designated as time point zero. At 4 hours or 18 hours after birth pups were euthanized. In euthanized fetuses and euthanized pups, thoracotomy was performed to expose the DA. Whole body 10% formalin fixation was used to fix the DA *in situ*. Following fixation, the DA was removed, cryo-sectioned, and H&E stained.

Transgenic Mice and Breeding

The generation of mouse lines with targeted disruptions of the EP1 receptor, EP2 receptor, EP3 receptor, thromboxane receptor, prostacyclin receptor, HPGD, Cox-1, and Cox-2 is described elsewhere [6, 8-14]. All mice were maintained on a C57BL/6 genetic background until crossed to the RI strain. RI *Ptger4*$^{-/-}$ mice were crossed to C57BL/6 mice carrying a null allele of one the other genes of interest (EP1, EP2, EP3, prostacyclin receptor, thromboxane receptor, HPGD, COX-1, and COX-2). Offspring were backcrossed to RI mice using *Ptger4*$^{-/-}$ survival as a selection marker. Once *Ptger4*$^{-/-}$ survival rates equaled that of the parental RI *Ptger4*$^{-/-}$ mice (~4 generations of backcrossing), sibling-sibling crosses were setup to generate mice deficient in both the EP4 receptor and the other gene of interest. Survival of HPGD deficient mice was assessed in the presence and absence of the EP4 receptor. Survival of RI *Cox-1*$^{-/-}$ *Cox-2*$^{-/-}$ mice was assessed in mice that expression the EP4 receptor. Neonates were observed for the presence of a DA related phenotype for the first 96 hours after birth. The DA status of deceased pups was determined at time of death. Pups were euthanized at first instance of any pulmonary distress (cyanosis, dyspnea, etc.)

Maternal Indomethacin Treatment

Indomethacin (Sigma) was diluted in 17mM Na_2CO_3, 125mM Na_2HPO_4 (pH 7.2) to 1mg/ml. Indomethacin was intravenously administered at 1mg/g body weight to full term females. Two hours after indomethacin administration, mothers were sacrificed and fetal DA status was determined.

Neonatal PGE_2 treatment

Prostaglandin E_2 (Cayman Chemical #14010) was freshly prepared by dissolving 1mg lyophilized PGE_2 in 2ml of sterile Dulbecco's phosphate buffered saline (DPBS), yielding 0.5mg/ml PGE_2. Two hours and four hours after natural birth, a subcutaneous injection of 5µg PGE_2 or 10µl of vehicle (DPBS) was administered to neonates. At 8 hours after natural birth, neonatal DA patency was determined. In neonates that died prior to 8 hours or that required euthanasia due to pulmonary distress, DA status was determined at that time.

Quantitative RT-PCR

Total RNA was isolated from tissues using RNeasy Minikit or Microkit (Qiagen). 4-8 ductus arteriosi of identical genotype were pooled prior to extraction of RNA. Reverse transcription of RNA to cDNA for quantitative RT-PCR was performed using a high-capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. Primers and probes were purchased from Applied Biosystems. All reactions were performed with TaqMan PCR Universal Master Mix (Applied Biosystems) using the Applied Biosystems 7900 HT Fast Real-Time PCR System. Reactions were performed in duplicate in a 20µl volume using 10ng of cDNA. Expression level of genes of interest was normalized to either Beta-2 microglobulin (B2M) or GAPDH levels. Data were analyzed using the comparative CT method as described by Applied Biosystems.

Statistical Analysis

Statistical analysis was performed using Prism 4 (GraphPad Software). Comparisons of the median were made by Mann Whitney U test unless otherwise noted. Data are shown as median ± S.E.M. Differences with $p<0.05$ were considered statistically significant and

denoted with a *. Statistical significance of survival rates and response to pharmacological agents was determined by test of proportions.

RESULTS

RI Mouse Strain with Prostaglandin Independent DA closure

On the 129S6, C57BL/6, MRL, FVB, and DBA/2 genetic backgrounds, the loss of the EP4 receptor results in near complete penetrance of persistently patent DA leading to congestive heart failure within the first 24 hours of life. EP4 receptor loss in all hybrids analyzed, (129xB6)F1, (129xDBA)F1, and (B6 x DBA)F1, also resulted in fatal PDA. Over 400 CIMS and F1 litters were analyzed for EP4 deficient survivors (Table 3.1). Of the 2120 pups that survived to 5 days of age, only 12 pups were EP4 deficient (1.7% of Mendelian expectation). The few surviving EP4 deficient animals did not transmit EP4 deficient survival to their offspring.

The 129/Ola *Ptger4* chimeras were bred to (DBA/2 and C57BL/6)F1 mice. Each strain individually and pairwise results in fatal PDA in the absence of the EP4 receptor (Table 3.1). However, intercrossing of the *Ptger4* hemizygous mixed background mice resulted in a 5% survival rate of $Ptger4^{-/-}$ pups (data not shown). The mixed background EP4 deficient animals were bred to $Ptger4^{+/-}$ siblings. Further sibling-sibling mating generated a RI strain with EP4 deficient survival rates of 70%. EP4 deficient survival rates vary 40-100% from the same cohorts (data not shown). After 35 generations of selective sibling-sibling mating, survival rates have remained constant for the last 20 generations.

Genome Wide SNP Scan

Genome wide SNP genotyping of several sub-lines of the RI strain was used to determine if any of the three founding strains was preferentially selected or if heterozygosity at specific loci was crucial for EP4 deficient survival. Three sub-lines were analyzed (Figure 3.1). By F32, all 694 informative SNPs were homozygous in the RI strain (Table 3.2).

DA closure in the RI strain

A delay in DA closure was observed in EP4 deficient RI pups (Figure 3.2). The closure delay is estimated to be no more than 3-5 hours. To prevent maternal rejection, which can affect DA closure, the pups were not marked in order of birth, therefore the precise hourly age of pups is uncertain. After 24 hours, the EP4 deficient pups that have undergone closure are indistinguishable from wildtype littermates. EP4 deficient animals that had not undergone closure by 24 hours were either deceased or cyanotic from cardiovascular failure requiring euthanasia. Ultimately, the DA of RI EP4 deficient pups closes and remodels into the *ligamentum arteriosum*, indistinguishable from wildtype littermates.

Mice deficient in EP_4 and other prostanoid receptors

Three additional PGE_2 receptors are expressed in arterial vessels [15]. It was initially theorized that functional redundancy in the PGE_2 receptors was compensating for the loss of the EP4 receptor and allowing DA closure in the RI strain. *Ptger4* has the most significant differential expression in the DA compared to the aortic arch, but the other three PGE_2 receptors (*Ptger1-3*) are nevertheless expressed in the DA (Figure 3.3).

EP3 is a Gi-protein coupled receptor that causes a decrease in intercellular cAMP levels. RI *Ptger3*$^{-/-}$ *Ptger4*$^{-/-}$ animals were indistinguishable from the parental RI *Ptger4*$^{-/-}$ mice. The EP1 receptor is Gq-protein coupled, signaling the phosphatidylinositol-calcium second messenger system. RI *Ptger1*$^{-/-}$ *Ptger4*$^{-/-}$ animals are indistinguishable from the parental RI *Ptger4*$^{-/-}$ mice. The EP2 receptor was thought to be the most likely compensatory receptor because both the EP2 and EP4 receptors are Gs-protein coupled receptors, stimulate adenylate cyclases, cause smooth muscle relaxation [16]. RI *Ptger2*$^{-/-}$ *Ptger4*$^{-/-}$ animals did not have any neonatal phenotype, and underwent DA closure identical to that of the parental RI mice.

Other prostanoid receptors have been implicated in DA maturation and closure. Prostacyclin I_2 is produced by the DA, and therefore the prostacyclin receptor (IP), a Gs-protein couple receptor, was considered a possible compensatory receptor [17]. RI *IP*$^{-/-}$ *Ptger4*$^{-/-}$ mice survive to adulthood with no naïve phenotype. The thromboxane receptor (TP), a Gq-protein coupled receptor, may also play a role in DA closure, since TP receptor antagonism increase the relaxation affect of PGE_2 [18]. The RI *Ptger4*$^{-/-}$ *TP*$^{-/-}$ mice survive to adulthood with no naïve phenotype. (See Table 3.3 for survival rate of RI mice)

Mice deficient in hydroxyprostaglandin dehydrogenase

C57BL/6 mice deficient in hydroxyprostaglandin dehydrogenase (HPGD), the primary catabolizing agent of PGE_2, succumb to pulmonary distress and die with PDA within the same time frame as EP4 deficient CIMS [6]. RI *Hpgd*$^{-/-}$ mice survive to adulthood. HPGD deficient survival rates were similar in both *Ptger4*$^{+/-}$ and *Ptger4*$^{-/-}$ mice. In mice lacking the EP4 receptor, it is not surprising that the loss of ligand catabolism would have no affect.

However, RI mice with a functional EP4 receptor showed no phenotypic differences in the presence or absence of HPGD.

Pharmacological control of DA patency

To validate the hypothesis that a PGE_2-independent mechanism was responsible for DA closure in the RI strain, PGE_2 levels were controlled pharmacologically in RI full term fetuses and neonates. Indomethacin was administered to 129 and RI full term (18.5E) females (Figure 3.4). EP4 deficient fetuses had no response to maternal indomethacin treatment. Fetuses with a functional EP4 receptor underwent *in utero* closure of the DA.

Mimicking human neonatal care, exogenous PGE_2 will maintain patency of the DA in neonatal mice. To confirm the loss of PGE_2 sensitivity for maintaining patency in the RI strain, PGE_2 was subcutaneous injected into 129 wildtype, RI *Ptger4*$^{+/-}$, and RI *Ptger4*$^{-/-}$ neonates (Table 3.4). In 129 wildtype neonates, PGE_2 maintained ductal patency through out the first 12 hours of life, at which point morbidity with PDA was near 100%. In RI *Ptger4*$^{+/-}$ neonates, PGE_2 maintained DA patency for the first 10 hours of life at which point DA closure was seen in ~50% of the pups. RI *Ptger4*$^{-/-}$ neonates had no response to PGE_2 injections and underwent DA closure by 12 hours after natural birth.

Mice Deficient in Cyclooxygenase

On a mixed genetic background of C57BL/6J and 129/Ola, *Cox-1*$^{-/-}$ *Cox-2*$^{-/-}$ mice die shortly after birth prior to DA closure [5]. The Cox-1 and Cox-2 loci were independently breed to the RI strain, using EP4 deficiency survival as a selection marker. After 4 generations of back crossing, the Cox-1 and Cox-2 line were in-crossed to generate *Cox-1*$^{+/-}$

Cox-2$^{+/-}$ cohorts. From these cohorts, 1 in 16 pups are COX deficient. Ultimately, male mice heterozygous for one isozyme and deficient in the other were utilized to increase the incidence of Cox deficient pups to 1 in 8. Females were heterozygous for both isozymes due to the previously reported breeding defects observed in both Cox-*1*$^{-/-}$ and *Cox-2*$^{-/-}$ females [19]. In the RI strain, COX deficient pups undergo DA closure (Table 3.5). Life expectancy of the COX deficient mice ranges from 24 hours to 2 weeks.

DISCUSSION

The evidence presented here indicates that DA maturation and closure can occur independent of prostaglandins. This prostaglandin-independent mechanism is parallel to the PGE_2/EP4-dependent mechanism. The RI wildtype mice support this since the *in utero* DA is sensitive to indomethacin induced PGE_2 depletion in the majority of fetuses (Figure 3.4), and neonatal PGE_2 administration does not maintain DA patency in all the pups (Table 3.4). A superficial discrepancy exists between the neonatal PGE_2 induced patency seen in some of the RI wildtype pups and the DA closure of RI *Ptger4*$^{+/+}$ *Hpgd*$^{-/-}$ pups. Persistent patency following exogenous PGE_2 treatment in RI mice can be attributed to either the highly variable penetrance of prostanoid-independent DA closure, or possibly a decreased efficacy of the prostanoid-independent mechanism to rescue pups that have initiated, but aborted, the prostaglandin-dependent DA closure. The latter scenario is similar to the increased incidence of PDA in full-term neonates that were exposed to indomethacin in utero [20, 21]. Therefore, once PDA closure is initiated but is unsuccessful or aborted, a second attempt of DA closure

may be less likely to succeed. CIMS rely exclusively on the PGE_2/EP4 mechanism and succumb to fatal PDA when any component of PGE_2/EP4 signaling is dysfunctional. The RI mice utilize the PGE_2/EP4 mechanism when available, but are able to utilize a secondary mechanism of closure if the prostanoid mechanism does not initiate successful DA closure.

Survival of RI $EP4^+$ $HPGD^-$ pups is further evidence that this secondary mechanism acts constitutively and in parallel to the prostanoid dependent mechanism of DA closure. There is no known role of HPGD in DA maturation, and in CIMS deficient in HPGD pharmacological depletion of plasma PGE_2 levels is sufficient in initiating DA closure in neonates [6]. The RI *Ptger4*$^{+/+}$ *Hpgd*$^{-/-}$ mice develop *in utero* with functional PGE_2/EP4 signaling, and it is not until after birth that the loss of HPGD becomes a factor. However, the RI *Hpgd*$^{-/-}$ pups are able to undergo DA closure without indomethacin treatment. This further indicates that the initiating signal for closure in this secondary mechanism is PGE_2 independent.

Survival of cyclooxygenase deficient animals further solidifies the theory that prostanoid independent closure is occurring in RI mice. The development of the DA in COX deficient fetuses likely occurs in chronically decreased levels of PGE_2 since PGE_2 can be transported through the placental barrier from the mother, but the pup lacks the ability to produce their own PGE_2 [22, 23]. Although this results in death quickly after birth prior to DA closure in CIMS deficient in COX, the RI mice survive the perinatal period and undergo DA closure and remodeling, despite the absence of any paracrine effects of localized PGE_2 production in the DA [24].

In a murine-centric context, this secondary method of DA closure would appear to be novel. However, human and canine genetic studies have shown that DA closure is a complex

trait that is difficult to attribute to a single mechanism. There are several lines of canines that have a high incidence of PDA, but 100% penetrance of PDA has not been achieved [7]. The genetics of human PDA is limited to analysis of family pedigrees that have high incidence of PDA in full term deliveries. Several consanguineous Iranian families have a significantly increased frequency of PDA; 3 fold increase over the general Iranian population [25]. QTL mapping in these families has shown a high LOD score (>6) for a chromosomal region of approximately 34 genes, one of which is a phospholipase precursor. Phospholipases are a class of enzymes responsible for the deacylation and release from the plasma membrane of arachidonic acid, the sole substrate for cyclooxygenase enzymes, which is a rate limiting step in all downstream prostanoid production [26]. Increased incidence of PDA was seen in a human population with a recessive mutation in the HPGD gene [27]. Although the mutation is predicted to completely abolish HPGD activity, penetrance of PDA is less than 40% in individuals carrying two copies of the null allele. This is drastically different than what is observed in CIMS, in which HPGD loss causes 100% penetrance of PDA. However, the incidence of PDA in the human population does mimic the increased incidence of PDA that we observe in the RI HPGD deficient mice.

Spontaneous resolution of untreated human PDA is likely to be prostanoid-independent. Patients with untreated PDA that survive early childhood, have a 0.5% yearly rate of spontaneous closure. Spontaneous DA closure has not been linked to any specific life-event [28]. At birth, high PGE_2 levels of late gestation quickly drop, and O_2 tension in the DA increases due to left-to-right shunting through the DA of oxygen-rich left ventricular output. In adults, prostaglandins have a very active role in maintaining homeostasis and

hypoxia in the aorta is rare, therefore there is no proposed mechanism for spontaneous adult DA closure.

We have shown that there is a parallel prostanoid independent mechanism of DA closure. In the RI mice, the prostanoid independent mechanism of DA closure has been separated from the dominant prostanoid dependent mechanism. Elucidating the mechanism of this prostanoid-independent closure has the potential of being exploited as another pharmacological target to initiate DA closure, where as currently only NSAIDs that inhibit Cox activity are used. We have also shown here that there is an initiating event for DA closure that does not involve PGE_2 depletion. It is probable that the non-prostanoid mechanism was marginalized in the mouse by either evolution or by selective breeding that has yielded the commercially available CIMS. The survival of a few EP4 deficient CIMS mice on various genetic backgrounds indicates that the prostanoid independent mechanism in the RI strain is not the result of a novel mutation but rather the selective breeding to re-establish in the mouse a higher penetrance for the secondary prostanoid independent mechanism for DA closure that is seen in other mammals. Commercial CIMS have a low penetrance of prostanoid independent DA closure making them unusable in the study of the complex prostanoid independent mechanism active in higher mammals. In the RI strain, this is resolved since the mice can rely exclusively on the prostanoid independent mechanism for DA closure.

Figure 3.1. Pedigree of the recombinant inbred (RI) mouse line used for genome wide SNP genotyping. Top grey box represents the mating of a male 129/Ola chimera and a (B6/DBA/2)F1 female. EP4 deficient mice were genotyped from F8, F22, F27, F31, and F32 generations (grey and black ovals).

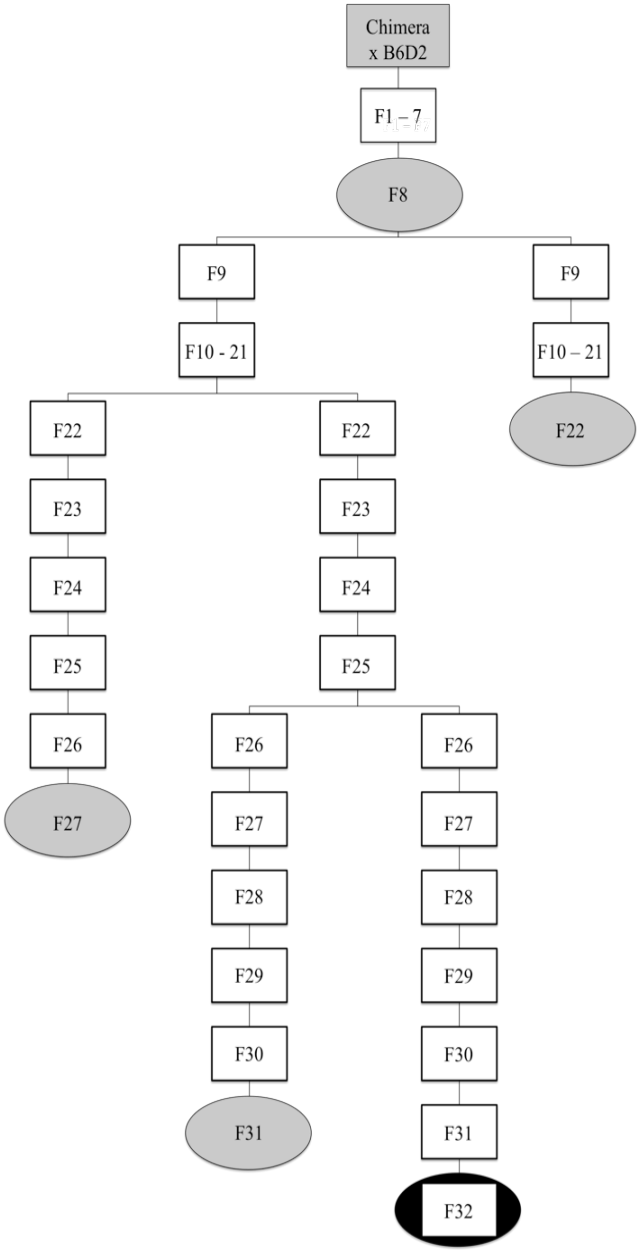

Figure 3.2. DA closure in the RI strain in the presence and absence of the EP4 receptor. The DA from full-term fetus, new born (< 4 hours old), and older neonate (12-18 hours old) in the presence and absence of the EP4 receptor were H&E stained. No gross morphological difference was observed between the EP4+ and EP4- animals. A delay of DA closure (<3 hours) was observed in the EP4 deficient animals. Delay is possibly only an artifact due of birthing process. Ultimately, the DAs of EP4 deficient animals close and form a *ligamentum arteriosum*, indistinguishable from wildtype animals.

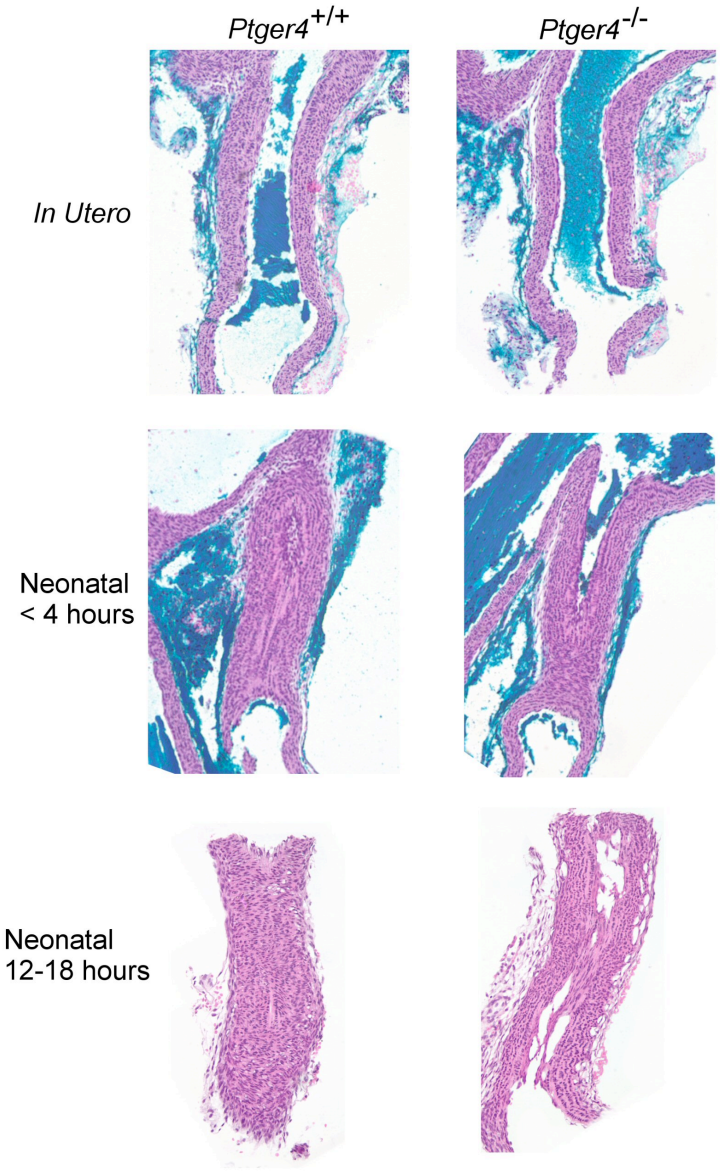

Figure 3.3. Quantitative RT-PCR demonstrating relative expression of the four PGE_2 receptors in 129 wildtype fetal ductus arteriosus and fetal aortic arch. Expression level for each receptor is individually standardized to expression level in the aortic arch. * $p < 0.05$

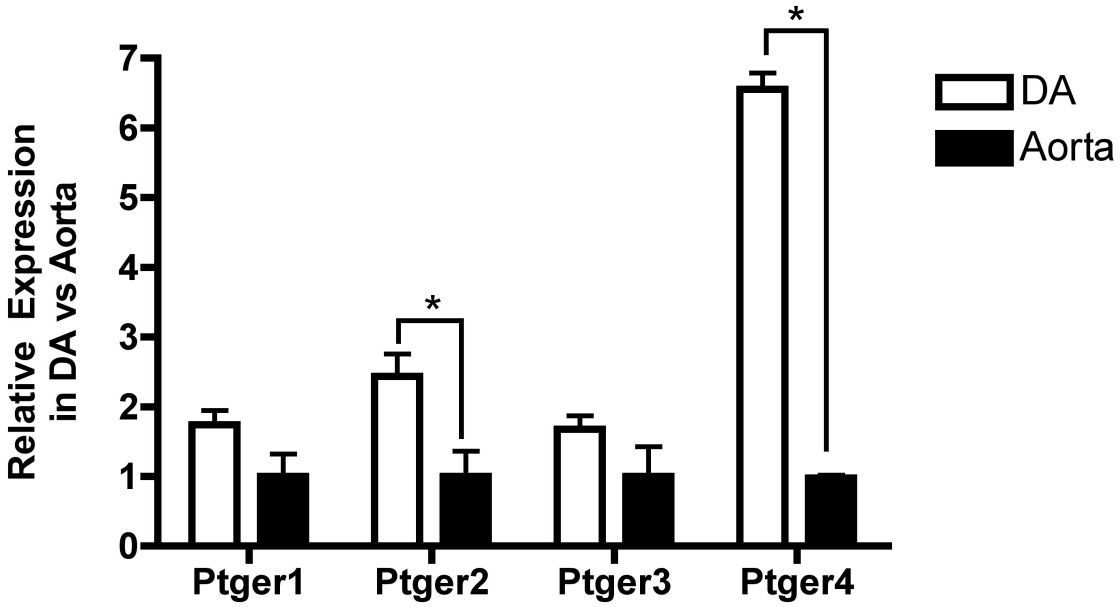

Figure 3.4. *In utero* DA closure following maternal indomethacin treatment. Indomethacin was intravenously administered at 1μg/g body weight to 18.5-day pregnant mice. Two hours later, the fetal DA was scored based on ratio of inner diameter of DA to pulmonary duct (PD) into one of three categories: Patent (DA:PD ~ 1.0), Partial constriction (DA:PD < 0.5), and Closed (DA:PD < 0.1). *In utero* constriction of the DA was seen in the majority of EP4+ fetuses (22/47 closed, 41/47 constricted). EP4 deficient fetuses had no DA response to maternal indomethacin treatment. Significance was confirmed by test of proportions of RI EP4+ (26/32 DA constricted) and RI EP4- (0/15 constricted), p-value < 0.01.

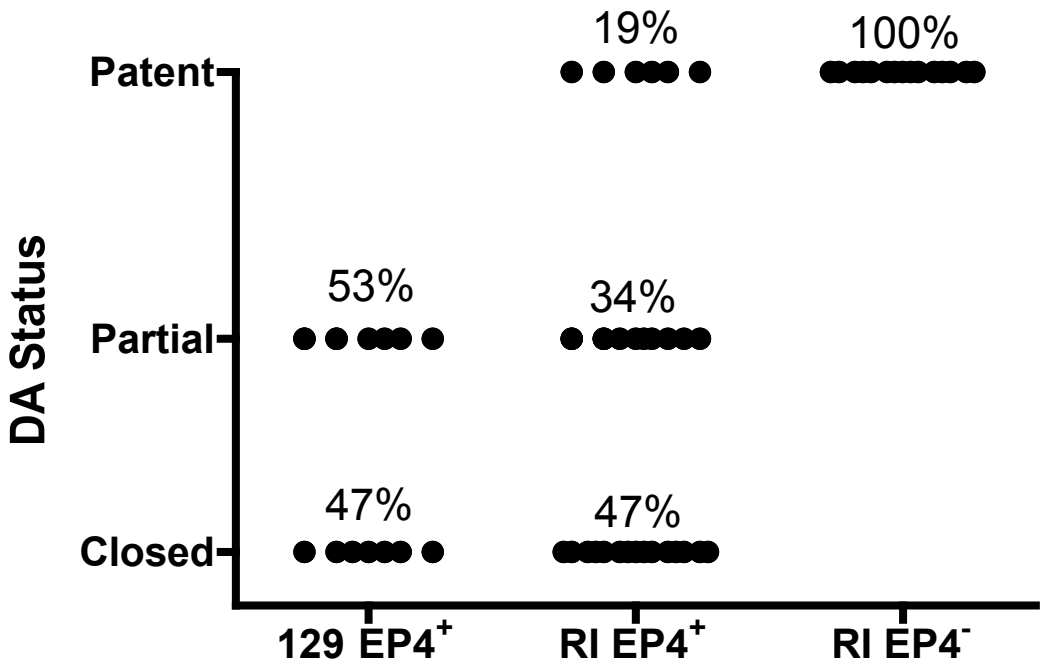

Table 3.1. EP4 deficient survival rates in on various genetic backgrounds. For CIMS and F1 litters, number of expected EP4 deficient pups were determined based on the number of EP4+/+ and EP4+/- pups surviving, which account for 75% of a litter based on Mendelian segregation of the wildtype and null locus. For the RI litters, the expected number of EP4 deficient animals is calculated from the Mendelian expectation from a combination of breeding types (+/- x +/- and -/- x +/-). Significance of observed versus expected determined by test of proportions. CIMS EP4 deficient survival (12/703) and RI EP4 deficient survival (404/577) differences were statistically significant (p-value < 0.0001)

			EP4 Deficient			Significant p-value
Strain	Litters	Pups	Observed	Expected	%	<0.01
129S6	166	724	4	240	2%	X
C57BL/6	70	344	1	114	1%	X
DBA/2	42	178	1	59	2%	X
MRL	13	72	1	24	4%	X
FVB	13	72	0	24	0%	X
129 x B6	51	393	4	130	3%	X
129 x DBA	32	166	0	55	0%	X
B6 x DBA	30	171	1	57	2%	X
CIMS	**417**	**2120**	**12**	**703**	**2%**	**X**
RI	**174**	**1189**	**404**	**577**	**70%**	**X**

Table 3.2. SNP genotyping of RI mice from 5 generations of three sub-lines. The mouse from generation F8 was a sibling of the single common ancestors of all RI sub-lines. Percent identity between the five generations genotyped is displayed. Percent homozygousity across genome within the genotyped animal is indicated in shaded squares.

	F8	**F22**	**F27**	**F31**	**F32**
F8	65.6%	50.4%	49.6%	50.6%	51.0%
F22		90.2%	58.8%	61.0%	61.1%
F27			99.1%	95.5%	96.0%
F31				99.4%	98.7%
F31					100%

Table 3.3. Survival of (A) RI mice deficient in the EP4 receptor and one other prostanoid receptors, (B) and survival of HPGD deficient RI mice. Test of proportions used to calculate significance of observed versus expected number of pups surviving > 5 days.

				Mice surviving >5 days			Observed vs Expected
	Pup Genotype	Litters	Pups	Observed	Expected	%	p-value
A)	*Ptger4*$^{-/-}$ *IP*$^{-/-}$	42	255	59	63	94%	0.376
	Ptger4$^{-/-}$ *Ptger1*$^{-/-}$	8	56	11	15	73%	0.244
	Ptger4$^{-/-}$ *Ptger2*$^{-/-}$	305	1462	312	348	90%	0.108
	Ptger4$^{-/-}$ *Ptger3*$^{-/-}$	40	184	51	57	89%	0.313
	Ptger4$^{-/-}$ *TP*$^{-/-}$	3	21	3	4	75%	0.364
B)	*Ptger4*$^{-/-}$ *Hpgd*$^{-/-}$	9	63	14	16	88%	0.374
	Ptger4$^{+/?}$ *Hpgd*$^{-/-}$	11	78	17	21	81%	0.375

Table 3.4. Exogenous PGE_2 treatment of pups within the first 2 hours after natural birth to maintain DA patency. Each neonate received a series of two injections; at 2 hours after natural birth, and a second injection two hours later. Littermates were given vehicle control. Four hours later, patency of the DA was determined. Pups deceased prior to 8 hours, had DA patency status assessed at time of death. DA patency in 129 mice (7/8) was statistically different (p-value < 0.01) from DA patency in RI mice (5/20). Vehicle was not statistically significant; 0/7 versus 3/11, p-value = 0.13.

			DA Status @ 8 hours		
Strain	EP4	Treatment	Patent	Constricted	Morbidity
129S6	+	PGE_2	7	1	4/8
129S6	+	Vehicle	0	7	1/7
RI	+	PGE_2	2	5	2/7
RI	+	Vehicle	1	2	0/3
RI	-	PGE_2	3	10	2/12
RI	-	Vehicle	2	6	1/8

Table 3.5. 24-hour survival rate and DA closure in Cox-$1^{-/-}$, Cox-$2^{-/-}$, and Cox-$1^{-/-}$ Cox-$2^{-/-}$ RI mice. Survival of Cox deficient animals (7/117) is not statistically significant from expected (15/125), p-value = 0.104.

Parental Genotype: (±/- ±/-) x (+/- +/-)		Total Pups @ 24 hours			
			Deficient Pups		
Pup Genotype	Litters	Total Pups	w/ DA closed	Expected	Dead w/ PDA or ?
Cox-$1^{-/-}$	21	183	52	37	1
Cox-$2^{-/-}$	18	131	34	32	3
Cox-$1^{-/-}$ Cox-$2^{-/-}$	15	117	7	15	5

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Chapter 4

Conclusion

The closure of the ductus arteriosus (DA) is an essential component in the transition from fetal circulation to adult circulation. A better understanding of DA maturation and closure can lead to the development of additional treatments for PDA. Currently, the use of non-steroidal anti-inflammatory drugs (NSAIDs) to inhibit cyclooxygenase (COX) activity is the only non-invasive treatment for PDA. Neonatal NSAIDs treatment carries several potential side affects, some of which can be life threatening in premature infants.

The use of prostaglandins to control DA patency has become common practice in neonatal care. PGE_2 infusion to maintain DA patency is critical for the survival of infants with severe congenital heart disease that is dependent on shunting through the DA. Indomethacin (and recently other NSAIDs) have good efficacy in initiating DA closure. However, despite the routine use of prostaglandin to control the DA, the mechanisms responsible for normal maturation and closure of the DA are still unknown.

In the work presented here, we generated a conditional null allele of the EP4 receptor, which is critical for DA closure in most mammals. Animals with tissue specific-loss of the EP4 receptor were generated with the use of various Cre recombinase transgenes. The loss of EP4 receptor expression on smooth muscles resulted in cardiovascular failure due to a persistently patent DA, similar to EP4 deficient animals. Given the unique and abundant expression of EP4 by the smooth muscle of the DA, it was not surprising that smooth muscle loss would lead to a DA-related phenotype.

Prior to the generation of endothelial EP4 deficient animals, it was difficult to predict the phenotypic consequences of EP4 receptor loss in all endothelial cells of the body. EP4 is expressed by the endothelium of all arterial vessels, including the DA. The lack of any neonatal phenotype in endothelial EP4 deficient mice was surprising. Although, we did not

predict that fatal PDA would necessarily occur in these mice, I assumed that the endothelium played some role in DA maturation and closure. However, maternal indomethacin treatment resulted in identical *in utero* DA closure in wildtype and endothelial EP4 deficient fetuses.

Although the EP4 receptor expression in smooth muscle is unique and critical for its closure, the EP2 receptor has been shown to have an overlapping physiological role in the adult animal [1]. To eliminate receptor redundancy in the endothelium we generated endothelial EP4 deficient animals on an EP2 deficient genetic background. Somewhat surprisingly, mice lacking EP4 and EP2 expression in the endothelium have no naïve phenotype. The loss of both known PGE_2 dilatory receptors, EP2 and EP4, in the endothelium indicates that their expression is non-essential in development or in the unchallenged adult. Future use of the $EP2^{-/-}$ $EP4^{flox/-}$ Tek-cre mice will include the measurement of the vasodilatory affects of exogenous PGE_2. If PGE_2-induced dilation is abolished or greatly attenuated, it will indicate that vasodilatory affects of PGE_2 occur via the endothelium of arterial vessels. However, if PGE_2-induced dilation is unaffected, it will show that PGE_2's vasodepressant role occurs outside the arterial vessel network, likely in the kidney.

We confirmed that PGE_2 signaling via the endothelium of the DA or smooth muscle EP2 receptors has no consequences on DA closure. Gene expression profiling of EP4 deficient and wildtype DA was used to assess the transcriptional consequences of EP4 receptor expression. Since the smooth muscle media of the murine DA accounts for most of the physical mass of the DA, the gene expression profile largely represents the transcriptional consequences of EP4 receptor signaling in the smooth muscle. Lower noise levels may be achieved in microarray analysis from neural crest EP4 deficient DAs, perhaps even on a EP2

null genetic background. These mice still succumb to fatal PDA, but PGE_2 signaling, or lack there of, would be limited to the smooth muscle layer of the DA.

Genes differentially up-regulated in the EP4 null DA showed no consistent pattern, whether through visual inspection of the gene list or pathway analysis through programs such as Database for Annotation, Visualization and Integrated Discovery (DAVID). The working theory had been that EP4 expression is exerting a positive transcriptional affect in the DA, and therefore activating the machinery necessary for DA maturation and closure. If this premise is assumed accurate, then the lack of pattern in genes up regulated in the EP4 null DA is not surprising. However, accepting half a premise is not an option, therefore, we must also assume that genes up regulated in the wildtype DA will reveal a consistent pattern.

Pathway analysis of genes up regulated in the wildtype DA revealed enrichment for cytoskeletal-related genes. Discovering that a smooth muscle arterial vessel has cytoskeletal gene expression is not itself a breakthrough. However, when the original EP4 null locus was generated by our lab over 10 years ago, close comparison of the wildtype and EP4 null DA revealed no apparent morphological difference [2]. EM analysis did not reveal any gross difference in morphology between the DA that was destined to close (wildtype), and the DA destined to stay patent (EP4 null). The lack of morphological differences is not in itself surprising if one takes into account that most cytoskeletal genes were up regulated by less than 50% in the wildtype. Expression of cytoskeletal genes by other great arteries is substantial, therefore an increased expression due to EP4 receptor expression in the DA may be difficult to detect, especially if subtle.

Further corroboration of the microarray data is necessary before victory can be declared in the war of discerning the role of the EP4 receptor in the DA. Validation via

realtime RT-PCR can easily be accomplished, but would still rely on transcript levels rather than protein levels. Semi-quantitative immunofluorescence or immunohistrochemistry may yield protein-based validation for the microarray data, but it is difficult to predict the likelihood of success. Semi-quantitative measurements in immunostaining techniques are readily performed when expression of a particular protein is either abolished or greatly increased compared to reference sample. However, the gene expression profile of the DA indicates that most of the cytoskeletal genes, at the transcriptional level, are not greatly increased in the wildtype DA.

One option is to utilize gene transfer in cultured aortic smooth muscle cells [3]. The aorta and DA share a neural crest lineage and morphologically the vessels appear similar. However, at birth the EP4 expressing DA constricts while the non-expressing aorta has no discernable reaction to the drop in plasma PGE_2 levels and an increase in O_2 tension. Adenovirus facilitated expression of the EP4 receptor in aortic smooth muscle would potentially mimic DA smooth muscle. If the forced expression of the EP4 receptor in cultured aortic smooth muscle results in an increase in cytoskeletal proteins, the role of PGE_2 signaling in the DA would be clear. Alternatively, the neural crest specific Wnt-1 promoter can be used to generate a neural crest specific EP4 transgene that would force artificial expression of the EP4 receptor in the smooth muscle of most great arteries of the heart. If the EP4 receptor expression is the primary molecular component responsible for differentiating the DA from the other great arteries of the heart, then forced expression of the EP4 receptor in the smooth muscle of the common carotid artery, brachiocephalic artery, and ascending aorta may have extremely deleterious effects on the vessels following birth as the vessels attempt to constrict and close similar to the DA. Physiologically, the contractility of the

wildtype and EP4 deficient murine DA can also be tested. The model proposed here would predict that *in utero* the full-term wildtype DA would have a stronger contraction force than the EP4 deficient DA when constricted by KCl [4]. Physiological manipulation of the DA could confirm if the wildtype DA has an increased contractility due to EP4 signaling. However, this would still leave the possibility that the EP4 receptor is allowing maturation of the DA, and increased contractility is an indirect by-product of the EP4 receptor induced maturation.

Corroboration of cytoskeletal gene up-regulation in the EP4-expressing smooth muscle of the DA would strengthen the model that EP4 receptor signaling is responsible for two distinct roles in the DA. PGE_2-induced dilation of the neonatal DA can maintain patency and most studies have shown that this is facilitated through the EP4 receptor. The second role of the EP4 receptor is increasing expression of contractile proteins during late gestation. After birth, when endogenous PGE_2 levels are depleted, the EP4 receptor has no role in closure and becomes a passive participant, since it provides neither dilatory signal nor metabolic signal in the absence of ligand. However, the up-regulated cytoskeletal protein with the absence of PGE_2 induced dilation can constrict the vessel. Unique expression of the EP4 receptor in the smooth muscle DA is an ingenious system for linking DA maturation to gestation, and DA closure to birth. In late gestation, plasma PGE_2 levels are at their highest, coinciding with the maturation of the DA. Relying on the same receptor for both metabolic growth and dilation allows for the coupling of these functions. As metabolic signaling increase contractility of the DA, dilatory signals increase maintaining patency. At birth, the DA is hopefully mature and metabolic signals via the EP4 receptor are unnecessary, and the loss of dilatory signals via the EP4 receptor allows for smooth muscle constriction.

If the consequence of PGE_2/EP4 signaling in the smooth muscle DA is an increase in cytoskeletal proteins, and possibly increased contractility of the vessel, how does prostaglandin-independent closure occur? The recombinant inbred (RI) mouse strain described here undergoes DA maturation and closure independent of the EP4 receptor. The loss of either COX isozymes of HPGD does not prevent DA closure, either of which is fatal on isogenic mouse backgrounds. Preliminary data also indicates that DA closure occurs even in RI mice deficient in all four PGE_2 receptors. If the survival of EP1-4 deficient animals is validated, this would indicate that PGE_2 is non-essential in mouse development. The RI strain has indicated that PGE_2 depletion is not the initiating event in DA closure, as *in utero* indomethacin exposure has no affect on the fetal DA. Given the fact that the mechanism of DA closure in isogenic mice is still unclear, determining a second mechanism will be a challenge.

It has been recently shown that the loss of myocardin in the neural crest results in non-symptomatic fatal PDA in mice [5]. Expression of myocardin induces the transcription of numerous cytoskeletal genes, similar to the enrichment seen in mircroarray analysis of the wildtype DA. The disruption of myocardin in the neural crest of the RI strain could possibly indicate where in the pathway of DA maturation and closure the prostanoid-independent mechanism play a role. If it is confirmed that EP4 receptor expression in the DA stimulates cytoskeletal genes, and the RI mice succumb to fatal PDA following neural crest myocardin loss, then a prostanoid independent mechanism of closure is likely to be very similar to the prostanoid-dependent mechanism. However, if neural crest myocardin loss does not restore fatal PDA in RI mice, then the prostanoid-independent mechanism is truly distinct from the prostanoid-dependent mechanism.

Gene expression profiling of the DA from RI fetuses in the presence and absence of the EP4 receptor would shed more light on the nature of DA closure in the RI mice. If the expression profile of the RI EP4 deficient DA is similar to the 129 wildtype DA, at least in terms of cytoskeletal genes, then that would support the theory that the RI mice are simply inducing expression of the same genes but independent of the EP4 receptor.

Another option for determining the nature of the prostanoid-independent mechanism of DA closure in the RI strain would be quantitative trait locus analysis. Crossing the RI strain to common inbred mouse line (in which EP4 loss is fatal), and then backcrossing that F1 to the parental RI line would generate EP4 deficient animals on a mixed genetic background. From previous studies, most of the N2 EP4 deficient animals succumb to fatal PDA. However, N2 EP4 deficient survivors do occur. Genome wide SNP analysis of the N2 EP4 deficient animals that survive versus those that succumb to PDA could be used to determine the loci that may play a role in the inheritable prostanoid-independent DA closure in the RI strain.

A caveat should be mentioned for both QTL and gene expression profiling in the RI strain. Low-resolution genome wide SNP genotyping has shown that the RI strain is homozygous at all loci by F32, and EP4 survival rates have remained constant for over 20 generations. However, the prostanoid-independent mechanism of DA closure has highly variable efficacy, even in litters from the same cohorts. This variable survival creates noise, which would necessitate an increase in the number of replicates in QTL or expression analysis to eliminate that noise.

The noise is best illustrated through an example. If a microarray analysis is done on the pooled total RNA from 4 EP4 deficient *in utero* ductii, but 25% of those pups would have

died after birth due to fatal PDA, then potentially only 3 of the 4 ductii will be expressing the genes necessary for EP4-independent DA closure. A similar situation is created in QTL analysis. If a comparison is made between EP4 deficient survivors and non-survivors, then the population of non-survivors includes individual animals that had inherited the loci necessary for DA closure but succumbed to PDA due to the variable efficacy of prostanoid-independent closure.

In summary, the work we have presented here has shown that there is a dual role for PGE_2/EP4 signaling in the smooth muscle, a dilatory role and a metabolic role. We have also shown that this signaling is non-essential in murine DA closure, and the initiation of closure is not exclusively dependent on catabolism of PGE_2 by the pulmonary system. An alternative signal initiating DA closure independent of plasma PGE_2 depletion can potentially be exploited as an alternate pharmacological target for the treatment of PDA in neonates. If PGE_2 signaling does have a metabolic role in the DA, then extra-uterine maturation of the DA of premature infant may be possible by exogenous PGE_2, which will hopefully result in DA constrict without the need of surgical or NSAIDs intervention.

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