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The Ductus Arteriosus -

a Fetal Vessel Coming of Age

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The Ductus Arteriosus - a Fetal Vessel Coming of Age

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Voor Daniel in het jaar waarin hij volwassen werd

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Chapter 1

General introduction

Introduction

Historical background

“Nature’s destruction of fetal structures that are superfluous in the adult seems to me something much greater than her original creation of those structures” wrote Galen when he recognized the fetal structures of the ductus arteriosus (DA) and the foramen ovale in the second century AD.¹ In countries under German influence the first description of the ductus arteriosus is erroneously attributed to the late-renaissance Italian surgeon Leonardo Botallo. Working in France he gave his name to the foramen ovale “le trou de Botal”.² Due to a mistake in the German edition of Botallo’s work his name became associated with the DA. The understanding of the functional role of the fetal arterial connection between pulmonary artery and aorta was possible after the discovery of the blood circulation by William Harvey, who in 1628 described ductus arteriosus and foramen ovale in “unripe births of mankind” in *Exercitatio Anatomica De Motu Cordis et Sanguinis in Animalibus*.³ But still it took many years until the transitional circulatory changes at birth were described and graphically demonstrated in vivo by Barclay et al.⁴ and the disturbances of this physiological process noted by Lind.⁵ Virchow is credited with being the first to note the histological differences between DA and the surrounding arteries and to point out the clinical significance of his findings for postpartum closure.⁶

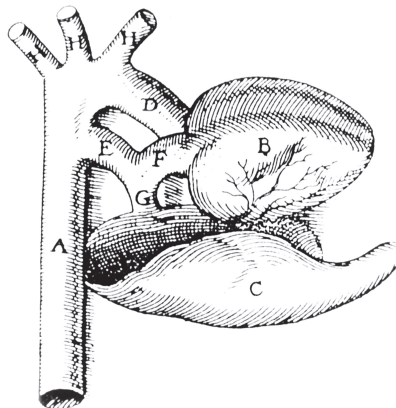


Figure 1.1 The ductus arteriosus as depicted in „de formatu fetu“ Tab VI, Fig XV, Hieronymus Fabricius ab Aquapendente (1537-1619). Source: Venice edition 1600, Bibliotheca Universitaria, Bologna: Aula-A VIII HH I 5 Legends: A: descending aorta, B: heart, C: left lung, D: ascending aorta, E: ductus arteriosus originally described as „propago magnae arteriae in venam arterialem, que in foetibus ampla apparet: in natis ut funiculus fit“ F: pulmonary trunk, G: left pulmonary artery, H: aortic arch branches.

In the second half of the last century physiological studies of fetal cardiovascular development and the transition to postnatal life by Rudolph⁷ together with biochemical studies,^{8,9} and histological studies^{10,11} enormously increased our knowledge of this unique process. From the appreciation of the two main regulators of DA dilatation and constriction (i.e. prostaglandin and oxygen) it was a small step to use synthetic prostaglandins and prostaglandin-synthesis inhibitors for the medical treatment of DA related pathology in neonates.¹²

The DA and the development of pediatric cardiac surgery and pediatric cardiology

In view of the development of pediatric cardiac surgery and pediatric cardiology the persistent DA (PDA) was ascribed the role of a pathfinder by RM Marquis.¹³ The first successful pediatric cardiac surgical procedure was the ligation of a PDA performed on an eight year old girl at Children's Hospital Boston in 1938 by Robert E. Gross.¹⁴ In 1944 the concept of Helen Taussig to create an artificial DA hereby improving pulmonary perfusion in deeply cyanosed children with a tetralogy of Fallot opened a new era in pediatric cardiology and pediatric cardiac surgery.¹⁵ The introduction of synthetic prostaglandins in 1975 was the beginning of the next new era, which enabled pediatric cardiologists to keep neonates with complex cardiac defects alive and pediatric cardiac surgeons to treat them.¹² With an open DA most critically ill ductus-dependent neonates can be stabilized before surgical treatment is offered. A better preoperative condition of the patients and the development of more sophisticated surgical techniques made that nowadays more than 15% of the cardiac defects are treated at neonatal age. Not only pediatric cardiac surgery but also pediatric cardiology was stimulated by new treatment options for the DA. In 1967 Porstmann demonstrated with the first closure of a PDA by catheter technique that congenital cardiac defects could be “corrected” in the catheterization lab.¹⁶ This took place just one year after pediatric interventional cardiology was founded by the first palliative procedure, the balloon atrial septostomy, reported by Rashkind and Miller.¹⁷ In 1979 Rashkind reported the first successful catheter interventional treatment of a PDA in an infant.¹⁸ Since then various devices have been invented for closure of small and larger PDA¹⁹⁻²⁵ and catheter treatment has become the method of choice for treatment of PDA beyond neonatal age. Later catheter techniques have been developed to palliate neonates with complex DA-dependent anomalies by DA-stenting.^{26,27} The stented DA functions as a substitute for a (modified) Blalock-Taussig shunt or guarantees the

systemic circulation in infants palliated with the staged hybrid procedure for hypoplastic left heart syndrome or its variants.²⁸⁻³⁰ After stenting the combination of physiological and stent-related remodeling of the DA puts the patient at risk for occlusion of the DA.³¹ As procedural and long-term success of DA stenting techniques depend on reliable DA patency in the first months of life it is important to increase the knowledge of the underlying molecular mechanisms of physiological DA closure in the neonate.

Patent or persistent DA – not only a matter of definition

In clinical practice the expression “patent DA” is often used synonymously with persistent DA and both are abbreviated as PDA although they differ in morphology,¹⁰ causes and clinical implication. Definition, morphology and physiological changes during process of normal DA closure are extensively reviewed in chapter 2.

As in our review we will use “patent DA” in this thesis as umbrella term for all situations in which the DA is open either physiologically or pathologically. The structurally normal DA in neonates is at risk to remain patent mostly due to immaturity³² and altered environmental conditions. An open DA in children beyond the age of three months after full gestation is defined as “persistent DA”.³³ The persistent DA is a structural anomaly, characterized by an abnormal amount of elastin in its wall and the presence of a subendothelial elastic lamina.¹⁰ In chapter 2 various causes of structural anomalies of the DA are discussed such as perinatal viral infections (i.e. rubella) and diverse genetic syndromes (i.e. Char, CHARGE, Noonan, Cri-du-chat, Holt-Oram syndrome). A structurally abnormal DA is also found in association with other congenital heart diseases (CHD), heritable disorders of connective tissue and mutations in contractile proteins^{34,35} These associations, documented in families (chapter 6), indicate that under certain circumstances a PDA is part of a more complex cardiac or systemic vascular disorder. Recently the strict distinction between functional anomalies of the immature DA and structural anomalies of the DA has been challenged by studies on the genetic influence on patency of DA in premature babies.³⁶⁻³⁸ Genetic variations in the *TFAP2B* gene, also affected by a dominant-negative mutation in patients with Char-syndrome, were identified as genetic risk factors for patency of the DA in premature infants^{36,37} suggesting a functional role for this transcription factor during normal DA closure in neonates. By comparing gene expression in the DA and aorta in fetal rats we identified the DA-dominant expression of the rat

analogue of *TFAP2B* in endothelial (EC) and smooth muscle cells SMC at the end of gestation (chapter 5).

Remodeling of the DA and similar vascular remodeling processes

In all air-breathing vertebrates the DA can be traced back to a specific segment of the 6th pharyngeal arch that matures and remodels different from all adjacent vascular structures.³⁹

As reviewed in chapter 2 the unique remodeling process of the DA starts already during the second trimester of pregnancy when intimal thickening regulated by prostaglandins develops.^{40,41} At birth contraction of SMC in response to rising oxygen partial pressure and prostaglandin withdrawal closes the DA and initiates its degeneration by apoptosis and cytolytic necrosis.^{10,32,40} Intimal thickening and loss of SMC are histological features that are comparable between DA remodeling, the arteries of children with Hutchinson-Gilford progeria syndrome (HGPS)^{42,43} and ageing individuals. This similarity and the knowledge that progerin, the truncated lamin A protein causing HGPS, is also expressed in genetically normal aged individuals, triggered us to study (chapter 4) lamin A/C and progerin expression in the neonatal DA. Because of the mutually exclusive spatiotemporal expression of lamin A/C and progerin in the neonatal DA we propose that activation of alternative splicing of the lamin A/C gene is involved in the circulatory system during neonatal DA closure. The relation between DA closure and ageing needs further clarification.

Aim and chapter outline of the thesis

In this thesis we have studied various aspects of physiological DA closure and PDA in animal models and humans. We were intrigued by the fact that the DA reacts completely different to the postnatal change in environmental conditions than the adjacent vessels. Therefore we aimed to identify genes and molecular mechanisms, which give the DA a specific genetic signature and might explain some of its characteristic morphological features. As all current therapeutic approaches to the DA have major side effects we hope that knowledge of genes that maintain fetal ductal patency and promote closure of the DA will help to develop new therapeutic strategies. These strategies might be tailored to individual genomes in order to improve the present approach targeting the prostaglandin pathway, using implantable intravascular devices or surgery. Furthermore we studied PDA-patients and their extended families with the aim to identify patients in whom the PDA is a heritable structural anomaly either of the DA or a more general vasculopathy.

In **chapter 2** we give insights into the pathogenesis and genetic background of patency of the DA. For this purpose normal DA closure, animal models of PDA and genetic syndromes with PDA are reviewed in this chapter.

In **chapter 3** we introduce a new model of a small laboratory animal with PDA. In the BN-inbred rat strain a small PDA and aortic fragility are associated and the implications of this association are discussed.

In **chapter 4** we studied progerin and lamin A/C expression in the neonatal human DA considering that activation of alternative splicing of the lamin A/C gene might be involved in human neonatal DA closure.

In **chapter 5** we analyzed DA- and aorta-specific transcriptional profiles selectively in ECs and SMCs harvested by laser-capture microdissection from the fetal DA and aorta of Wistar-rats. Using microarray and quantitative RT-PCR technique we identified genes that have not earlier been described in relation with DA-specific remodeling.

In **chapter 6** data of a retrospective patient-based study assessing the prevalence of PDA and other cardiovascular disease in families of PDA-patients are presented. These data provide evidence for a genetic association of PDA and aortic disease in these families.

Finally, **chapter 7** presents concluding considerations of the topics covered by the **chapters 2 to 6**. It brings the data from basic research in the clinical context of all medical specialties that deal with problems related to the process of closure of the normal neonatal DA and the presence of a PDA.

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Chapter 2

Insights into the pathogenesis and genetic background of patency of the ductus arteriosus

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Modified after Neonatology 2010;98:6-17

Abstract

The unique differentiation program of the ductus arteriosus (DA) is essential for its specific task during foetal life and for the adapting circulation after birth. Phenotypic changes occur in the DA during the normal maturation and definitive closure. Morphological abnormalities of the vessel wall characterise the persistent DA (PDA) in older children. Here, we give an overview of the animal models of DA regulation and remodelling. Genetic research has identified the cause of syndromic forms of PDA, such as the *TFAP2B* mutations in Char syndrome. Genes that interfere with the remodelling of vascular smooth muscle cells (SMCs) of the ductal media are affected in virtually all of these anomalies. Therefore, the pivotal regulatory role of SMCs is emphasised. A better understanding of the genetic background of this developmental process may help develop new strategies to manipulate the DA in premature infants, neonates with duct-dependent anomalies, and patients with syndromic and non-syndromic PDA.

Introduction

Reflecting the increased survival of premature infants, the prevalence of patent ductus arteriosus (DA) has risen to 13.5% of all heart defects present at birth.¹ In clinical practice, the expression patent DA is often used synonymously with persistent DA. Both are abbreviated as “PDA”, although they differ in morphology^{2,3} and therapeutic implications. In this review, we will use the abbreviation PDA to refer to persistent DA.

The term “patent DA” is an umbrella term for all situations in which the DA is open either physiologically or pathologically. In healthy premature infants of 30-37 gestational weeks, spontaneous closure of the DA is documented by echocardiography in 50-58% of infants on the second and 81-87% of infants on the third day of life.⁴ In 90% of healthy term babies, the DA is functionally closed by 72 hours of life.⁵ Term infants with respiratory failure show a significant delay in ductal closure within the first 24 hours of life⁶ and sometimes suffer from a hemodynamically significant patent DA that requires treatment.⁷ Causes for the prolonged patency of the DA can be structural, as well as functional, immaturity and altered physiological conditions. Targeted deletions of genes that are essential in the physiological closing process have produced animal models of patent DA.

PDA in older children (beyond three months after full gestation) is histologically characterised by an abnormal high amount of elastin and the presence of a subendothelial elastic lamina.² Variants of PDA have also been documented in adult animals.^{8,9} PDA should be distinguished from the immature but normally developed patent DA in premature and young infants and must be considered as a primary congenital malformation of the vessel wall.² Because it usually occurs sporadically, PDA has not been regarded as a typical genetic disorder. Nonetheless, PDA occurs in 5% of siblings of PDA cases¹⁰ suggesting a genetic component to the pathogenesis, which has typically been presumed to be multifactorial. Very recently, the contribution of genetic factors to the incidence of patent DA in premature infants has also been documented.^{11,12}

Heritable PDA has been described in various animal models.^{8,9} In humans, 8-11% of cases with PDA have a chromosomal abnormality.^{13,14} The search for a functional link between the genes affected in genetic syndromes associated with PDA may elucidate the complex interactions between mutations and modifier genes as well as between environmental factors and genetic polymorphisms related to PDA and patent DA in premature infants. Serious side effects and

therapeutic failure are associated with the present pharmacological approach to the DA targeting on the prostaglandin-pathway. Knowledge of which genes maintain the balance between patency and closure of the DA may help to develop pharmacogenetic strategies tailored to individual genomes in order to improve our therapeutic protocols. In the present paper, human and animal data on DA maturation and remodelling and the genetic background of DA closure and patency are reviewed.

Animal models

Physiological ductal closure in animals

Physiological ductal closure has been studied in various vertebrate species.¹⁵ Most of the data are derived from mammals. Since 2006, there is growing amount of literature on birds.¹⁶⁻¹⁸ Birds and humans share basic mechanisms of vasoreactivity and response to oxygen at the end of the foetal period. The easy accessibility of the foetal circulation in a chicken egg makes it an attractive model for DA physiological studies.¹⁹

The DA remodels and matures in diverse ways among the different vertebrates, but its origin can be traced back to a specific segment of the sixth pharyngeal arch in all of them. In all species, the foetal DA is a large muscular artery composed of layers of vascular smooth muscle cells (SMCs) separated by layers of elastin. Figure 2.1 presents a schematic overview of the morphological appearance of the human DA during maturation and the processes involved in its functional and permanent closure. The characteristic histological features of delayed ductal closure in the premature neonate and the PDA are included in this figure. In larger animals, such as dogs⁸, lambs²⁰, and primates²¹, ductal closure is accompanied by intimal cushion formation, which starts during foetal life. Although there is some resemblance in the process of ductal closure between larger animals and humans, the relevance of intimal cushions seems to be different. Humans show the earliest onset of intimal thickening and, by far, the most extensive cushion formation.²² The DA of small animals, such as rats, rabbits, and mice, show less pronounced intimal thickening. These ducts close functionally within minutes after birth, predominantly by vasoconstriction.¹⁵ The same sequence of structural changes occurs in all species: (1) endothelial detachment from the internal elastic lamina (IEL); (2) subendothelial accumulation of the extracellular matrix; (3) migration of SMCs

through a fragmented IEL; (4) occlusion of the lumen by the thickened intima; and (5) degeneration of the DA into a fibrous remnant.¹⁵ In larger⁸ as well as smaller¹⁶ animal models, the process proceeds from the pulmonary to the aortic end of the DA. Anatomic remodelling and permanent closure of the DA require the environmental and physiological changes related to birth and air breathing. For instance, intense hypoxia within the constricted vessel wall of the DA at birth is a trigger for SMCs to express vascular endothelial cell growth factor (VEGF).^{21,23} VEGF stimulates neointima proliferation and vasa vasorum in-growth, thereby playing a role during permanent DA closure in lambs and baboons and probably also in humans.²⁴ These interactions between functional and morphological changes in the DA inspired us to include all aspects of foetal regulation and of physiological and anatomical DA closure in one figure (Figure 2.2).

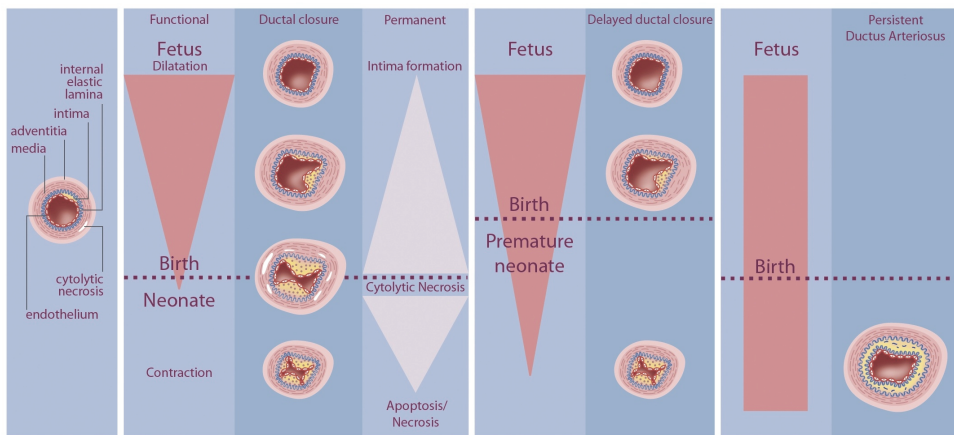


Figure 2.1 Schematic overview of the processes related to functional and anatomical closure in the human ductus arteriosus. Normal: During intrauterine life, the ductus remains in a dilated state. Functional closure after birth is achieved by immediate contraction of the ductus. Postnatal occlusion of the lumen and anatomical closure is facilitated by the development of intimal cushions, completely obliterating the lumen in the mature ductus. The ductus finally degenerates into a fibrous remnant through apoptosis and necrosis. The histological maturity stages are derived from Gittenberger et al.²² Premature: The delayed closure of the ductus arteriosus in premature neonates with poorly developed intimal cushions. PDA>3 months: Schematic representation of the PDA of older children, which is characterised by additional subendothelial elastic lamellae and less-developed, elastin-rich intimal cushions.

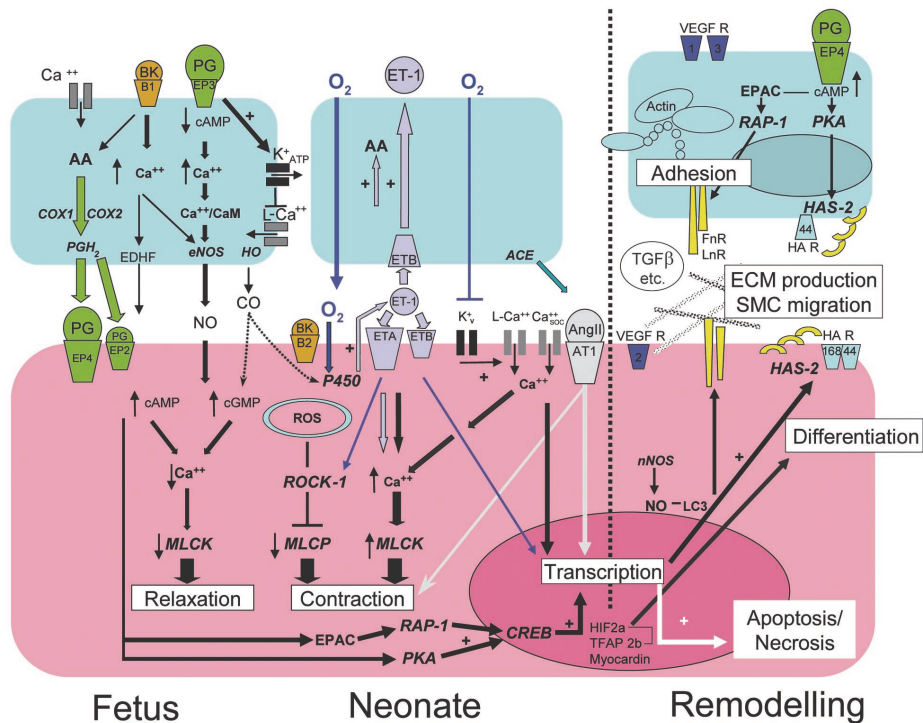


Figure 2.2 Diagram of the experimental data on intracellular and intercellular signalling related to ductus closure and remodelling that is presented in this review. Endothelial cells (ECs) are depicted in light blue. SMCs are indicated by the large pink rectangle. For the sake of clarity, not all downstream signalling steps are included. On the left side, the dominant signalling pathways in the foetus are shown. The middle section illustrates the changes related to the increase of oxygen saturation at birth. The section to the right of the vertical dotted line focuses on anatomical remodelling. Remodelling proceeds through a sequence of events including the differentiation of SMCs and ECs, extracellular matrix production, SMC migration, and finally apoptosis and necrosis.

Receptors: □, cytokine/hormone: ○, protein: —, ion-channels: ||

Abbreviations: AA=arachidonic acid, ACE=angiotensin-converting enzyme, Ang II=angiotensin II with AT1 receptor, BK=bradykinin with receptors B1 and B2, Ca⁺⁺=calcium ion and channels: Ca_{SOC}=store-operated, L-Ca=voltage-dependent; CaM=calmodulin, cAMP=cyclic adenosine monophosphate, cGMP=cyclic guanosine monophosphate, CO=carbon monoxide, COX=cyclooxygenase isoforms 1 and 2, CREB=cAMP response element-binding protein, ECM=extracellular matrix, EDHF=endothelium hyperpolarising factor, EPAC=exchange proteins activated by cAMP, ET-1=endothelin-1 with ET-A and ET-B receptors, HAS-2=hyaluronansynthase-2, FnR=fnronectin receptor, HA hyaluronic acid receptors, CD 44 and CD168=receptors for hyaluronic acid mediated motility, HIF-2α=hypoxia-inducible factor 2 alpha, HO=hemoxygenase, K⁺= potassium ion and channels: K_{ATP}=ATP-sensitive channel, Kv=voltage-gated channel; LC3=microtubule-associated protein, LnR= laminin receptor, MLCK=myosin light-chain kinase, MLCP=myosin light chain phosphatase, NO=nitric oxide, NOS=nitric oxide synthase isoforms (endothelial) eNOS and (neuronal) nNOS, PGH2=prostaglandin H2, PG=prostaglandins, predominantly PGE₂ acting via prostaglandin receptors (EP 2,3,4), PKA=protein kinase A, RAP1=small RAS-like GTPase acting downstream of EPAC, ROCK-1=Rho-associated, coiled-coil-containing protein kinase 1, ROS=reactive oxygen species, TFAP2b=transcription-factor-activating protein 2 beta, SOC=store-operated channel, VEGF=vascular endothelial growth factor.

Physiology and pharmacology of constriction and relaxation

For decades, the constrictive effect of oxygen on the DA in the neonate²⁵ as well as the relaxing effect of prostaglandin (PG) E_2 ²⁶ have been shown. Studies in various animal species and *in vitro* models revealed additional mediators for DA regulation and remodelling. Species differences in the degree, timing, and type of reaction to the stimuli have been documented. Figure 2.2 shows a compilation of these experimental *in vitro* and *in vivo* data in order to illustrate parallel actions and potential interactions of the various signalling pathways.

PGE_2 plays a major role in maintaining ductal patency in utero in smaller and larger mammals including humans.²⁷ The postnatal fall in blood levels of PGE_2 is central to the closure of the ductus. The reduction of PGE_2 is largely due to metabolism by PGDH.²⁸ Both locally released and circulating vasodilatory PGs regulate ductal closure mediated via specific receptors.²⁹ Among the four PGE_2 -receptor subtypes (EP1-4), the EP4 receptor is expressed predominantly on SMCs and is probably the most relevant mediator of prostaglandin-induced vasodilatation before birth in humans.³⁰ EP3 receptors are also found on SMCs but are dominant on endothelial cells (ECs).²⁹ They activate endothelial NO synthase (eNOS) via calcium increase and calcium/calmodulin-binding. The cyclooxygenases COX1 and COX2 catalyse PG synthesis from arachidonic acid. Although both isoforms are present in the human ductus, COX1 has a predominant role during gestation.³⁰ Exposure in utero to COX-inhibitors, such as indomethacin, can result in premature closure of the DA.³¹ Conversely, an increase of the incidence of symptomatic patent DA in infants of mothers who received indomethacin tocolysis has also been observed.³² These contradictory observations suggest a more complex role for PGs in the developmentally regulated process of DA maturation and postnatal closure. Several agents sustaining ductal patency in concert with PGs in animal studies do not act in the same way in humans.³³ Nitric oxide (NO) produced mainly via eNOS is a vasorelaxant in the DA.³⁴ NO acts as an alternative for, and synergises with, PGE_2 .³⁵ The NO mechanism is relatively more important in the preterm than in the full term ductus³⁶ and can compensate for prostaglandin-induced relaxation when PG biosynthesis is eliminated or inhibited by drugs.^{37,38} In their *in vitro* and *in vivo* studies using wild-type and gene-deleted mice, Baragatti et al.³⁵ described NO and CO as being cooperative mediators of bradykinin-induced relaxation in the absence of PGE_2 . In the absence of all three agents (PGE_2 , NO and CO), bradykinin induces a relaxant substance that behaves like endothelium-derived hyperpolarizing factor.

Although there are interspecies differences in timing, the rise in blood oxygen tension with the start of respiration is critical for early (functional) closure of the ductus.²⁵ The contraction in response to oxygen is intrinsic to SMCs of the ductus and is highly conserved. Ion channels and other vasoactive systems are involved in the acute ductal constriction immediately after birth. SMC contraction is regulated by phosphorylation/dephosphorylation of myosin light chains (MLCs). Inhibition of myosin light chain phosphatase (MLCP) and activation of the calcium/calmodulin-dependent myosin light chain kinase (MLCK) both increase phosphorylation and thereby facilitate myosin/actin interaction and contraction. Kajimoto et al.³⁹ recently determined that Rho-kinase (ROCK) activation by O₂ in the DA (but not in other foetal arteries) involves a redox-dependent activation of ROCK-1. They concluded that the DA had a relatively unique ability of increasing endogenous H₂O₂ in response to increased PO₂ and proposed a temporal sequence of O₂ constriction in three phases: (1) an early electrical phase that involves Kv channel inhibition, membrane depolarisation, and activation of the L-type, voltage-gated calcium channel; (2) a mediator phase that involves increased endothelin (ET-1) synthesis; and (3) a calcium-sensitisation phase that involves the activation of ROCK. In the neonate, the increased arterial O₂ concentration inhibits voltage-gated potassium channels (Kv) and promotes calcium entry via L-type calcium channels.^{40,41} Oxygen sensing takes place in the proximal electron transport chain in the mitochondrion and is mediated by ROS (H₂O₂).³³ The cytochrome P450 heme protein in the plasma membrane of the SMCs has been previously suggested as a receptor for oxygen-induced events.⁴²

The potent vasoconstrictor ET-1 is produced by ECs and SMCs in the DA⁴² and interacts with both endothelin receptors (ETA and ETB). *In vitro*, ET-1 is released in response to increased oxygen levels, which may be sensed by cytochrome P450.^{42,43} The role of ET-1 *in vivo* is less clear and probably varies between different species. An ETA receptor blockade in lambs does not impair ductal constriction.⁴⁴ In foetal rats⁴⁵ ET-receptor blockade prevents constriction of the DA by cyclooxygenase inhibitors. The DA in ETA receptor-deleted mice closes postnatally, but the acute vasoconstrictor response to oxygen is attenuated.⁴⁶ This may reflect the disruption of a normal reinforcing effect of ET-1 on voltage-gated potassium channels⁴⁷ or the IP₃-dependent release of intracellular calcium.³⁹ Calcium-sensitisation pathways have been documented in rabbits and humans.⁴⁸ Store-operated calcium channels act as additional components of normoxic contraction. Functional immaturity of calcium-dependent and calcium-sensitisation pathways during the hypoxic phase of remodelling might explain why the DA in

prematures tends not to stay tightly closed.⁴⁸ Gene expression data show an up-regulation of the angiotensin receptor gene (*Agtr1*) in neonatal rats.⁴⁹ Therefore, angiotensin II has also been suggested to be a vasoconstrictive effector.

PDA in animals

PDA in dogs

Most cardiac malformations known to man are also observed with a similar prevalence in dogs (5-8/1,000).⁵⁰ In 1982, Helen Taussig described that several of these malformations are heritable in specific breeds of dogs (PDA, pulmonary stenosis, and subaortic stenosis), rabbits (vestigial pulmonary artery), and rats (ventricular septal defect). The fact that these malformations occur in various animals that cannot interbreed made her conclude that the deoxyribonucleic acid (DNA) that codes for these malformations must lie in a portion of DNA that is common to all mammals and, therefore, may be genetic variants of cardiac development.⁵⁰

PDA is the most common cardiac anomaly in dogs.⁵¹ An animal model of inherited PDA has been studied in dogs by Patterson and co-workers,⁵² and the pathomorphology has been described.⁸ Histological features of the normal DA and PDA in this strain of dogs resemble those of the normal DA and PDA in humans, suggesting a similar pathogenesis in both species. Immunohistochemical data from this dog model suggest a role for prostacyclin (PGI₂) in the process of intimal thickening. The distribution of PGI₂ synthase is identical in all permanently patent vessels.⁵³ PDA, aorta, and pulmonary artery show high PGI₂ synthase expression in the endothelium and low expression in SMCs. In the normal closing DA of these dogs, however, high amounts of PGI₂ and no PGE₂ or PGDH activity were found in the SMCs of the intimal cushions.⁵³

PDA in rats

Brown-Norway rat

In a morphological study,⁹ we previously described hereditary PDA in a specific rat inbred strain – the Brown-Norway (BN) rat. The PDA in BN rats shows abundant elastic lamellae in the intima, a subendothelial elastic lamina, and failure of intimal SMC proliferation. These histological features are also hallmarks of human and

canine PDA.^{2,8} In the media of the BN ductus, the elastic lamellae are virtually absent. The aortic media shows impaired elastic lamellae,⁵⁴ with ruptures in the IEL.⁵⁵ This phenotype has been related to the increased vascular fragility that is characteristic of the BN rat.⁵⁵ In PDA, the subendothelial elastic lamina is thought to limit the access of the SMCs from the media to the intima. There is also strong evidence that intimal cells arise both during development and under pathologic conditions from delamination of ECs⁵⁶ and their circulating precursors.⁵⁷ Endothelial-mesenchymal transformation has also been described during arteriogenesis in the embryo.⁵⁸ Because ECs can also produce elastic laminae, the abundant elastic lamellae in the intima in the PDA support the idea that the intimal mesenchymal cells are also endothelially derived.

Linkage studies revealed different loci for elastic lamellar anomalies and PDA in BN rats. In a backcross between BN and the Louvain (LOU) reference strain, the phenotype of a ruptured IEL in the abdominal aorta and the iliac artery showed a highly significant linkage to chromosome 5 and a suggestive linkage to chromosome 10 in BN rats.⁵⁹ In the same population, Kota et al.⁵⁹ linked PDA in BN rats to two different loci on chromosomes 8 and 9.

Adult BN rats exhibit phenotypic variations in their DA morphology. Unlike other rat strains, a normal ductus ligament is only present in 14% of the rats⁵⁹ (20% in our own observations, unpublished). In the remaining 86% of BN rats, variations between grossly visible ductus ligaments and a PDA are found. These variations point to a polygenic control of normal ductal closure. Our own crossing studies in BN/Wistar rats showed one PDA (2.4%) in the F2 generation and thick ligaments in 41% and 43% of the rats in the F1 and F2 generations, respectively. In 59% of the rats in the F1 and 57% in the rats of the F2 generation, the ligament was thin, as seen in Wistar rats. These findings are comparable to the results of the crossing experiments reported by Kota⁵⁹ in BN/LOU backcross rats, in which only 3.7% of rats had a macroscopically visible ductus with a widely patent aortic and stenosed pulmonary end.

Gene-targeted mice

Gene-targeted mice have been created in order to elucidate mechanisms by which known effectors affect ductus development, prenatal patency, and postnatal ductal closure. Mice carrying deletions of components of the prostaglandin pathway, the

ET-1 receptor, and other genes interfering with SMC differentiation will be discussed in the following section.

Mouse models with deletions in the prostaglandin pathway

Targeted deletions that reduce the PGE₂ production or function paradoxically do not affect the prenatal ductal patency^{60,62} but instead may result in impaired DA closure after birth.^{61,62} Although the importance of PGE₂ signalling for postnatal DA closure has been known from *Ep4*-deleted and *Cox*-deleted animals for a long time, the explanation for this paradoxical behaviour has only recently been elucidated by Yokoyama et al.⁶³ In their study, they were able to document that PGE₂ binding to the Ep4 receptor results in intimal cushion formation via the cAMP /protein kinase A (Pka) pathway. Pka mediates the direct vasodilating effect of PGE₂ by inhibiting MLCK. Furthermore, Pka and exchange proteins activated by cAMP (EPAC1), a newly identified effector cAMP, promote hyaluronic acid production by hyaluronic acid synthase 2 (HAS2) activation. Subendothelial hyaluronic acid accumulation induces the formation of intimal cushions, which are required for DA closure (see Figure 2.2).

As cyclooxygenase–peroxidases, COX1 and COX2 catalyse the synthesis of PGH₂, which is the precursor of biologically active PGs. When looking at the *Cox*-deleted mice in detail, *Cox2* seems to be more important for DA closure than *Cox1*. Trivedi et al.⁶⁴ were the first to emphasize that *Cox2* has a vital role for ductal closure after term birth and is attenuated in preterm gestation. In addition to the *Cox2* effect, a gene-dosage dependent effect of *Cox1* has been documented.^{61,65} Two groups^{61,66} have described contrasting results concerning the impact of individual *Cox* isoform ablation on the postnatal closure of the DA in mice. Baragatti et al.⁶⁶ documented no genotype-related difference in postnatal closure of the DA in *Cox*-deleted mice born after vaginal delivery while Loftin et al.⁶¹ described mortality due to patent DA in 35% of *Cox2* (-/-) mice and 100% of mice deficient for both isoforms. The absence of the COX1 isoform did not affect DA closure in the experimental setting of Loftin et al.⁶¹ who studied the mice after a caesarean section at gestational day 19.5. The mortality (35%) and incidence of patent DA due to the absence of COX2 is, however, significantly increased (79%) when one copy of the gene encoding COX1 is also inactivated. When comparing the phenotypes of the COX2-deficient mice with a model of selective genetic COX2 inhibition that leaves its peroxidase activity intact, Yu et al.⁶⁵ surprisingly found no abnormalities in ductal remodelling after birth. These findings might imply that a

COX 1/2 heterodimer is involved in signalling in the ductus.⁶⁷ Interestingly, there is also interplay between EP4 receptor activation and Cox-2 expression. The experiments of Trivedi et al.⁶⁴ indicate that the genetic deficiency of EP4 results in attenuated Cox-2 expression.

In the late nineties Ngyuen et al.⁶² and Segi et al.⁶⁸ both generated *Ep4* receptor knockout (KO) mice and described the same phenotype. They found that the loss of the *Ep4* receptor is not lethal *in utero* but causes 95% mortality within 72 hours after delivery due to congestive heart failure. Histological examination confirmed ductal patency. In the remaining 5% of *Ep4* (-/-) mice, the ductus was either closed or severely narrowed. The animals were fertile and survived for more than a year.⁶⁸ Ngyuen et al.⁶² were able to show that selective breeding of the survivors increased the survival rate among *Ep4* (-/-) mice to 21%, suggesting that other alleles on other loci can provide an alternative to *Ep4*.

Endothelin-1 receptor KO mice

Mice lacking the ET-1 receptor ETA are described by Coceani et al.⁴⁶ These mice showed a discrepant behaviour of the ductus *in vivo* and *in vitro*. Isolated DA from *Eta* -/- mice contracted marginally to oxygen and ET-1 but responded to a thromboxane analogue. In normoxic newborn mice, reduction in the ductus lumen was equally pronounced in -/- and +/+ littermates. Conversely, no such narrowing was seen in hyperoxic -/- fetuses, although it did occur in +/+ littermates. No genotype-related difference was noted in the morphology of the ductus. These data indicated that ET-1 mediates the ductus constriction in response to oxygen but is not critical for postnatal ductus closure.

Smooth muscle myosin heavy chain (SM-Mhc) KO mice

Proper regulation of SMC contraction in response to the postnatal rise of oxygen tension is necessary for functional ductal closure. The observation of delayed (6 to 12 hours post partum) but not failed closure of the DA in *SM-Mhc* KO mice suggests that ductal SMC contractions can be generated partly (phase 2 or sustained state of contraction) by activation of the non-muscle myosin heavy chain (NM-MHC), as documented for intact bladder preparations from the same mice. The ability to recruit two distinct contractile systems, namely SM myosin for phasic contraction and non-muscle (NM) myosin during prolonged tonic contraction, can explain the unique properties of SMCs during prolonged activation.⁶⁹

Transcription factor AP2 beta (Tfap2B) KO mice

Targeted deletion of *Tfap2B*, a neural-crest-enriched transcription factor, causes apoptosis of renal epithelial cells and postnatal death, possibly related to polycystic kidney disease.⁷⁰ Ivey et al.⁷¹ did not observe morphological differences between the *Tfap2B* ^{-/-} and wild-type mice that were studied beyond mid-gestation at embryonic days (EDs) 13.5, 15.5 and 18.5. Data on postnatal ductal behaviour and morphology in *Tfap2B* ^{-/-} mice are not included in their paper. Preferential calponin expression, which characterises the advanced maturation and differentiation of ductal SMCs in wild-type mice, was lost in *Tfap2B* ^{-/-} mice by ED 18.5. These data indicate that *TfapB* disruption affects the development of SMCs within the DA. Abnormal maturation of ductal SMCs may contribute to delayed ductal closure in *Tfap2B* ^{-/-} mice and PDA in humans with Char syndrome.

Selective ablation of the myocardin gene in neural crest-derived SMCs

Myocardin regulates the expression of genes required for the contractile phenotype of neural crest-derived SMCs. Mice generated after selective myocardin ablation of cardiac neural crest-derived SMCs were symptomatic for a patent DA and died at postnatal day 3. The vascular phenotype of the ductus exhibited ultrastructural and immunohistochemic features that are generally associated with a synthetic rather than a contractile SMC phenotype.⁷² In the mutant mice, the architecture of the intima and media was markedly disorganised, showing a dramatic increase of extracellular matrix and a relative loss of SMC volume. Relatively few myofibers and a marked increase in synthetic organelles were documented by electron microscopy.

Gene transfer experiments

In vitro gene transfer experiments were carried out in order to study the maturational increase of O₂-sensitive, voltage-gated potassium (Kv) channels. Transmural Kv1.5 or Kv2.1 gene transfer "rescued" the developmental deficiency in Kv, conferring O₂ responsiveness to preterm rabbits. Targeted SMC Kv1.5 gene transfer also enhanced O₂ constriction in human DA.⁷³

Gene transfer strategies were also evaluated as a potential substitute for systemic PG administration in neonates with ductus-dependent circulations. In lambs, gene transfer is feasible and can be used to bioengineer a patent ductus. Mason et al.⁷⁴ were able to restrain SMC migration and intima formation in the ductus by inhibiting fibronectin translation. More recently, Humpl et al.⁷⁵ achieved prolonged postnatal

ductal patency by transfecting human PG synthase into the vessel wall of the ductus of newborn lambs.

PDA as part of a clinical syndrome in humans

PDA is described as part of the spectrum of cardiovascular anomalies in various genetic syndromes. Between 28-88% of PDA cases have other cardiac or non-cardiac defects, and 8-11% of cases with PDA have chromosomal abnormalities.^{13,14} In the clinical paediatric literature, the association of isolated PDA with Down's syndrome (4% of all associated congenital cardiac defects), CHARGE syndrome⁷⁶, Cri-du-chat-syndrome⁷⁷, Noonan syndrome (2.2% of all associated congenital cardiac defects)⁷⁸, and Holt-Oram syndrome⁷⁹ is well described. Insight into the cellular mechanisms underlying the phenotypes of these syndromic anomalies is increasing. Even now, a ductus-specific molecular mechanism cannot be derived from a particular syndrome. The most consistent association of PDA with mild dysmorphic features has been found in Char syndrome.

Char syndrome is a more recently described genetic syndrome consisting of PDA in association with facial anomalies and minor skeletal anomalies and is caused by mutations in the transcription factor *TFAP2B*, which is expressed in neural crest cells.⁸⁰ Multiple dominant negative mutations have been documented. Interestingly, genotype-phenotype correlations have been documented for mutations in the basic DNA binding domain and the transactivation domain of *TFAP2B*.⁸¹ A high prevalence of PDA with very mild facial anomalies and no apparent hand anomalies are found among the carriers of the mutation in the transactivation domain. The genotype-phenotype correlation in Char families with a distinct phenotype, combining PDA with only very mild facial and no hand anomalies, suggests the existence of *TFAP2B* coactivators as a cause for ductal tissue specificity.⁸¹

Heritable disorders of connective tissue structures, such as Marfan, Loeys-Dietz, Larsen, and Ehlers-Danlos syndrome,⁸²⁻⁸⁵ and mutations in the SMC contractile proteins^{86,87} cause thoracic aortic aneurysms that lead to acute aortic dissections. Although some reports associate PDA or ductal aneurysm with these types of heritable aortic disorders, the present literature does not put particular emphasis on the DA.

Ductal aneurysm is not unanimously defined in the literature. Aneurysms of the DA are described to occur as a complication after surgical or catheter-interventional treatment of a PDA and also as an incidental finding at the aortic end of a spontaneously closed ductus in non-syndromic patients.⁸⁸ Congenital ductal aneurysm may occur during the third trimester of pregnancy⁸⁹⁻⁹¹ and is characterised by abnormal dilatation of the aortic end of the ductus with a significantly stenosed or completely occluded pulmonary end of the ductus.

New insights in the molecular pathogenesis of Marfan syndrome (MFS) have challenged the definition of this syndrome as a structural connective tissue disorder.⁹² MFS is a developmental abnormality with broad and complex effects on the morphogenesis and function of multiple organ systems. Animal data derived from fibrillin-1-deficient mice have shown that increased TGF-beta signalling contributes to the process of aortic root dilatation in MFS and that TGF-beta antagonism (achieved by angiotensin II type 1 receptor blockade) may represent a productive treatment strategy. The up-regulation of TGF-beta may be a final common pathway for aortic aneurysms in many disease states, such as Loeys-Dietz and non-syndromic thoracic aortic aneurysm/dissection.

Loeys-Dietz syndrome is a combination of altered cardiovascular, craniofacial, neurocognitive, and skeletal development caused by mutations in TGF-beta receptors 1 and 2.⁸³ The cardiovascular anomalies include aortic root aneurysm and aortic tortuosity in all patients. A PDA or ductal aneurysms were reported in 54% of affected individuals. Considering that *in vitro* and *in vivo* data⁹³⁻⁹⁶ document an important role for TGF-beta during the physiological remodelling process of the DA, it is tempting to speculate that increased TGF-beta signalling might also be the final common pathway for ductal patency or ductus aneurysms in MFS and Loeys-Dietz syndrome. The recent observation of the failure of ductal closure in mice after selective ablation of the myocardin gene in neural crest-derived SMCs⁷² also points to TGF-beta-signalling, because myocardin is able to participate in the TGF-beta 1 pathway through direct interaction with Smad3 to activate SM22-alpha, SM-myosin heavy chain, and SM-alpha actin promoters.⁹⁷

Data from Zhu et al.⁸⁶ and Guo et al.⁸⁷ highlight the importance of contractile proteins for the structural integrity of the aorta. Both studies describe a high prevalence of PDA among families with mutations in SMC contractile proteins. In 2005, Khau van Kien et al.⁹⁸ documented the association of PDA and familial thoracic aortic aneurysm and dissection in a large French family. Linkage analysis

identified 16p12.2-p13.13 as the locus responsible for both TAAD and PDA in this family. Zhu et al.⁸⁶ demonstrated that the disease is caused by mutations in the *MYH11* gene, affecting the C-terminal coiled-coil region of the SM-MHC, a specific contractile protein of SMCs.

Cyclic interaction between myosin encoded by *MYH11* and alpha-actin encoded by *ACTA2* are required for the contractile force of SMCs. Guo et al.⁸⁷ have shown that missense mutations in the *ACTA2* gene are responsible for 14% of the inherited thoracic aortic aneurysms and dissections. When reviewing the clinical characteristics and the familial segregation of *ACTA2* mutations in fourteen families, six individuals from two families with a mutation altering Arg258 had PDA. The association of PDA and thoracic aortic aneurysms and dissections in families with mutations in two components of the SMC contractile proteins suggests that unimpaired SMC contractility is, in addition to its critical role in maintaining the structural integrity of the aorta, a prerequisite for ductal remodelling, which then leads to anatomical ductal closure.

Non-syndromic PDA

The contribution of a recessive locus to non-syndromic PDA in the Iranian population has been studied by Mani et al.⁹⁹ The higher prevalence of PDA in Iran (15%) compared to that in the United States (2-7%), together with the increased rate of consanguinity (63%) among PDA cases compared to that in the general Iranian population (25%), facilitated their approach to the identification of a recessive locus. Using a genomewide linkage analysis in 21 unrelated, consanguineous PDA cases, they implicate a single locus on chromosome 12q24p. Based upon their data, they estimated that this locus contributes to the pathogenesis of one-third of the PDA cases in the Iranian population. Furthermore, they suggest a role for this locus in PDA worldwide.

Genetic predisposition to patent ductus arteriosus in preterm infants

Very recently, two studies^{11,12} dealing with the genetic contributions to ductal patency in premature babies have been published. In a quantitative heritability

study on premature twins, Bhandari et al.¹² estimated that the genetic factors or the shared environment accounted for 76.1% of the variance in liability for patent DA. In a cohort of infants less than 32 weeks of gestation, Dagle et al.¹¹ performed a single nucleotide polymorphism (SNP) analysis. Initially, they identified seven SNPs significantly associated with patent DA. A second analysis pinpointed an association between genetic variations in *TFAP2B*, *tumor necrosis factor receptor-associated factor 1*, and *prostacyclin synthase* with ductal patency in premature babies.

Conclusion

The prenatal patency of the DA and its physiological closure are obtained by highly conserved and parallel acting mechanisms. Ductal remodelling requires a specific sequence of events, which includes the differentiation of SMCs and ECs, extracellular matrix production, SMC migration, and finally apoptosis and necrosis. Based upon data generated from human genetic syndromes and experimental animals, various genes that either directly or in combination with environmental factors predispose an individual to PDA have been identified. Because genes interfering with the recruitment of medial or with the proliferation, differentiation, migration, or apoptosis of SMCs are affected in virtually all of the syndromic anomalies associated with PDA, SMCs can be considered to be a pivotal cellular structure in the pathogenesis of PDA. Because the process of recruitment and differentiation of SMCs is dependent upon reciprocal signalling between ECs and mesenchymal cells,¹⁰⁰ it is reasonable to infer that ECs in the ductus may also contain transcriptional targets or regulators necessary for the differentiation of the adjacent SMCs. Several “classical” angiogenic and vasculogenic signalling pathways are involved in ductus development and remodelling. VEGF regulates remodelling during permanent closure,²³ PDGF-BB mediates SMC proliferation,¹⁰¹ and the *Agtr1* gene is up-regulated in the neonatal ductus. In addition, angiotensin has been suggested as a novel effector of ductus closure.⁴⁹ A transcriptional network, including *Tfap2B*, endothelin-1, and hypoxia-inducible factor 1, has recently been suggested to act in ductal SMCs of developing mice.⁷¹ Our own microarray data in rats (chapter 5) show a significant up-regulation of *Tfap2B* gene-expression in ductal SMCs compared to aortic cells. A PDA in combination with only very mild dysmorphic features in patients with Char syndrome underscores the tissue specificity of *TFAP2B* mutations. Furthermore, *TFAP2B* is one of the three

genes suggested in a recent study in preterm infants to be associated with persistent patency of the DA.¹¹ In animal studies, TGF-beta signalling has also been linked to ductal remodelling.⁹²⁻⁹⁴ The role of TGF-beta signalling in the molecular regulation of the maturation and anatomical closure of the DA is not yet fully elucidated. The emerging knowledge of clinical syndromes associated with PDA or ductus aneurysms, which are related to mutations in the TGF-beta signalling cascade, draws further attention to this signalling pathway.

Technological advances in high-throughput genetic screening will facilitate the identification of pathways and genes involved in ductus development and closure. A better understanding of the genetic background of this developmental process can help develop new strategies in order to manipulate the DA in premature infants, neonates with duct-dependent cardiac anomalies, and patients with syndromic and non-syndromic PDA.

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Chapter 3

Persistent ductus arteriosus in the Brown-Norway inbred rat strain

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Abstract

Persistent ductus arteriosus (PDA) is a common cardiovascular anomaly in children caused by the pathologic persistence of the left sixth pharyngeal arch artery. The inbred Brown-Norway (BN) rat presents with increased vascular fragility due to an aortic elastin deficit resulting from decreased elastin synthesis. The strikingly high prevalence of PDA in BN rats in a pilot study led us to investigate this vascular anomaly in 12 adolescent BN rats. In all BN rats, a PDA was observed macroscopically, whereas a ligamentum arteriosum was found in adult controls. The macroscopic appearance of the PDA was tubular ($n=2$), stenotic ($n=8$), or diverticular ($n=2$). The PDA had the structure of a muscular artery with intimal thickening. In the normal closing ductus of the neonatal controls, the media consisted of layers of smooth muscle cells (SMCs) intermingled with layers of elastin. The intima was thin and poor in elastin. By contrast in the media of PDA in BN rats elastin lamellae were absent and the intima contained many elastic fibers. The abnormal distribution of elastin in the PDA of BN rats suggests that impaired elastin metabolism is related to the persistence of the ductus and implicates a genetically determined factor that may link the PDA with aortic fragility

Introduction

Patent ductus arteriosus is frequently diagnosed in children. Reflecting the increased survival of premature infants the prevalence has risen to 13.5% of all heart defects at birth.¹

Patent ductus arteriosus in premature babies is a functional anomaly, which must be distinguished from the structural anomaly of the PDA in the spectrum of congenital heart disease. To separate those two entities clinically, a PDA is defined as a ductus that has failed to close after 3 mo of age.² In the offspring of patients with PDA, the incidence of this cardiovascular anomaly is increased, suggesting a genetic background.¹ Mutations in the transcription factor AP-2B (*TFAP2B*) cause familial PDA in Char syndrome.³ Recently, a new aortic aneurysm syndrome has been described due to mutations in the genes encoding transforming growth factor (TGF- β) receptors 1 and 2 (TGF- β R1 and TGF- β R2) being associated with PDA and ductal aneurysm in 50% of the affected individuals.⁴ In human PDA, the ultrastructural changes associated with physiologic ductal closure do not occur. Intimal cushion formation is abnormal or absent and the endothelium remains attached to the internal elastic lamina or to an additional subendothelial elastic lamina; invagination of endothelium or migration of SMCs from the media are not observed.^{2,5} Until now, the only animal model of inherited PDA has been established in dogs by Patterson and coworkers.^{6,7}

The morphologic changes in canine PDA resemble those in humans. The process of physiologic ductal closure is well studied in various laboratory animals such as rats^{8,9}, rabbits⁹, and lambs.¹⁰ The BN rat differs significantly from other strains of rats with respect to thoracic and abdominal aortic elastin content and internal elastic lamina rupture.¹¹ This phenotype has been related to a genetically increased vascular fragility in the BN rat.¹² The present study describes the morphologic details of a genetically determined PDA in an inbred strain of BN rats. With our histologic study, a new model of a small laboratory animal with PDA is introduced.

Materials and methods

Animals

Twelve young adult female BN (BN/Rij) rats ranging in age between 9 and 11 wk (*i.e.* about 1–4 wk before fertility), supplied by Harlan Horst (The Netherlands) were studied. In addition to these 12 rats, 18 BN rats from the same age were studied macroscopically in our institution. Fourteen of these rats served as allogenic aortic valve donors in heterotopic aortic valve transplantation experiments published by Grauss et al.¹³ Twelve adult Wistar rats and 12 neonatal Wistar rats were used as normal controls. Animal care complied with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996). Animal experiments were approved by the local committee of animal welfare of the Leiden University Medical Center (LUMC).

Experimental protocol

Rats were killed by CO₂ inhalation followed by cervical dislocation. Neonatal rats were killed by decapitation. A median sternotomy was performed followed by en bloc removal of the lungs. The ductus arteriosus and the great vessels were inspected and documented photographically. After macroscopic examination, the great vessels and the ductus arteriosus were resected. The specimens were overnight fixed in 4% paraformaldehyde (PFA) dissolved in phosphate-buffered saline (PBS) or in 2% acetic acid in 100% ethanol overnight at 4°C. Fixed tissues were thoroughly rinsed in PBS and dehydrated in graded ethanol and xylene and embedded in paraffin. Sections of 5µm were cut and mounted onto several series of slides. Standard histologic stainings were performed with hematoxylin and eosin (HE) for a morphologic overview. Resorcin-fuchsin (RF) and sirius red (SR) were used to depict elastin and collagen, respectively. To detect SMCs, the mAb against smooth muscle -actin 1A4 (1:3000; DAKO A/S Glostrup, Denmark), horseradish peroxidase–conjugated rabbit anti–mouse antibody (1:250, DAKO) was used as second antibody. The staining procedure was performed as described by Grauss et al.¹³ Sections were briefly (5 s) counterstained with hematoxylin.

Results

Macroscopy

Controls (Wistar rats)

On analysis within 30 min after delivery, the ductus appeared as a small blood-filled vessel. Twenty-four hours after delivery, the ductus arteriosus appeared as a whitish strand of vascular tissue. There were no indications of blood inside the ductus. Both the pulmonary and aortic end of the ductus were closed. In the adult Wistar rats, the ductus was a thin ligament hardly discernible from the surrounding tissue.

BN rats

In all adolescent BN rats, a macroscopically visible ductus arteriosus was found instead of the expected thin ligament. The persistent ductus in the 14 rats that were dissected in the course of heterotopic aortic valve transplantation experiments had to be ligated to avoid hemorrhage after transplantation. There were no signs of hemodynamic consequences of the PDA. In particular, cardiomegaly and pulmonary congestion were not found during dissection of the cardiovascular system. Female rats were able to survive pregnancy and delivery without clinical signs of heart failure. The morphologic characteristics of the ductus in the 12 individual animals of the present study are summarized in Figure 3.1. The morphologic appearance varied between a longer, more tubular and a short conical structure. The largest ductus measured 25%–30% of the diameter of the ascending aorta. (Figure 3.2a). The aortic end of the ductus was open in all specimens and filled with blood at dissection. As to the morphology of the pulmonary end of the ductus arteriosus, three different types of ductus could be distinguished: 1) a tubular type with a widely patent pulmonary end in two rats (Figure 3.2a), 2) a stenotic type with a stenotic lumen at the pulmonary end filled with a small line of blood in eight rats (Figure 3.2b), and 3) a diverticular type with an occluded lumen at the pulmonary end in two rats (Figure 3.2c). Thus, the ductus was completely patent in 10 of 12 rats.

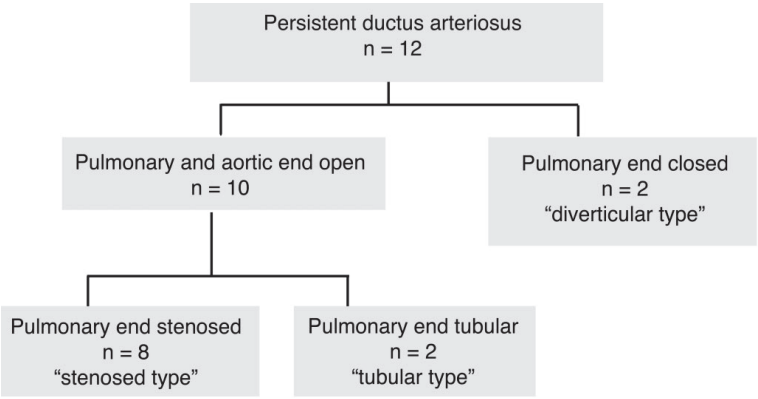


Figure 3.1 Flowchart illustrating the morphologic findings in BN rats.

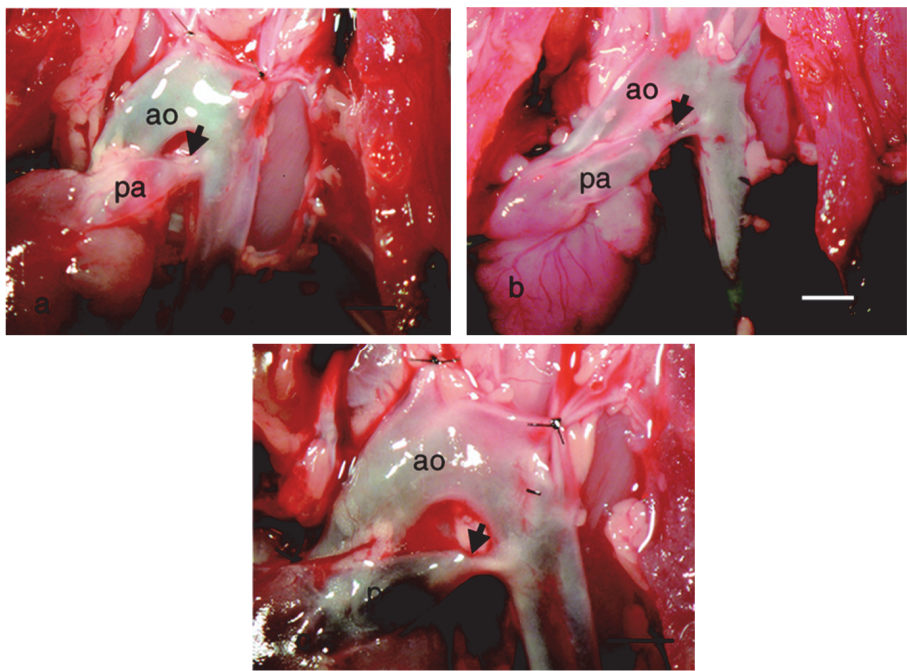


Figure 3.2 Macroscopic image of the three morphologic types of PDA in adolescent BN-rats: tubular-type PDA (a), stenosed-type PDA (b), diverticular-type PDA (c). ao, aorta ascendens; pa, pulmonary artery; the ductus arteriosus is marked by an arrow. The bar represents 2 mm.

Histology

Controls (Wistar rats)

Functional ductal closure in Wistar rats took place within the first hours after birth. The ductus of neonatal Wistar rats studied within 30 min after birth was still open. It appeared as an elastic artery with some intimal thickening inside the lumen (Figure 3.3a and 3.3b). The media consisted of 10–12 elastin lamellae (Figure 3.3b) alternating with layers of SMCs confirmed by 1A4 staining (Figure 3.3c) and layers of collagen (Figure 3.3d). The inner elastic lamina forming the border between tunica media and intima was thicker than the other lamellae. The intima consisted of three to four layers of vertically oriented cells perpendicular to the circular layers expressing SMC actin. The elastin laminae were irregular and thin. The intima was devoid of collagen layers (Figure 3.3d). The outer part of the ductus was formed by four to five layers of loose adventitia tissue containing small amounts of elastin. In all animals studied at the age of 24 h postpartum, the ductal lumen was closed. In adult Wistar rats, the ductus arteriosus was degenerated into a ligament. A vascular structure of the ductus arteriosus was not detectable. The Wistar rats showed loosely arranged short elastic fibers accompanied by some SMCs seen at the site where the ductus ligament was connected to the pulmonary artery.

BN rats

The histologic findings confirmed the macroscopic diagnosis of the tubular, stenotic, and diverticular type of the PDA. The pulmonary end of the ductus was histologically patent in 10 of the 12 BN rats. Only in the two animals with the diverticular type PDA the pulmonary end of the ductus was closed. All three types of ductus arteriosus had the structure of a muscular artery with intimal thickening (Figure 3.4 and 3.5). Two types of intimal thickening were found. In the two specimens with tubular morphology of the ductus, the intima consisted of six to eight regularly organized elastin lamellae between single layers of SMCs (Figure 3.4a and b). The first intimal elastin lamella was located subendothelially (Figure 3.4a and b). In the remaining 10 ducti, belonging to the stenotic and diverticular groups (Figure 3.4d–f and 3.5), the intimal thickening was less organized than in the tubular type. Areas with elastin lamellae were found neighboring areas of intimal thickening without elastin. In eight of the 10 ducti, small vessels indicating neovascularization of the intima were present (Figure 3.5a–d). These vessels consisted of endothelial cells (ECs), elastin lamellae, and SMCs (Figure 3.5a–d).

Combinations of both types of intimal thickening were also observed. The internal elastic lamina could be identified in all specimens as a boundary between intima and media. In the media, as confirmed by 1A4 staining (Figure 3.4f and 3.5c), 10 layers of SMCs were found.

Elastin lamellae, however, were absent in the media of BN ductus (Figure 3.4a, b, e and 3.5a) in contrast to the controls (Figure 3.3b). The four to five layers of adventitial tissue contained small amounts of elastin (Figure 3.4b, d and 3.5b). Collagen was predominantly present in the adventitia as in normal controls (Figure 3.3d). Related to the internal elastic lamellae and in areas of intimal neovascularization, SR staining was more pronounced in BN rats (Figure 3.5d).

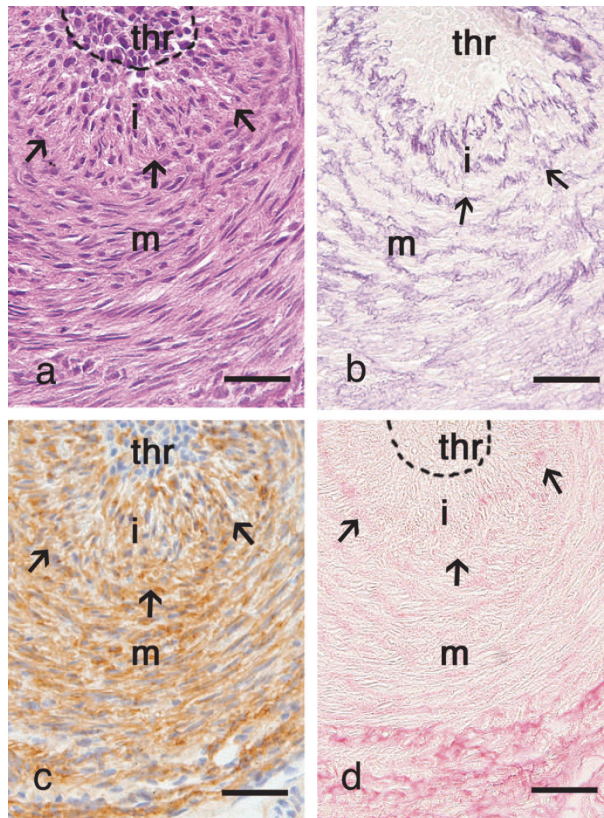


Figure 3.3 Histology of a physiologically closing ductus arteriosus of a neonatal Wistar rat 30 min post partum. Stained with HE (a), RF (b), 1A4 (c), and SR (d). Note three to four layers of intimal thickening and the media consisting of 10–12 elastin lamellae interchanged with collagen layers and layers of SMCs. The internal elastic lamina is indicated by arrows. a, adventitia; m, media; i, intima; thr, thrombus. The bar represents 100 μ m.

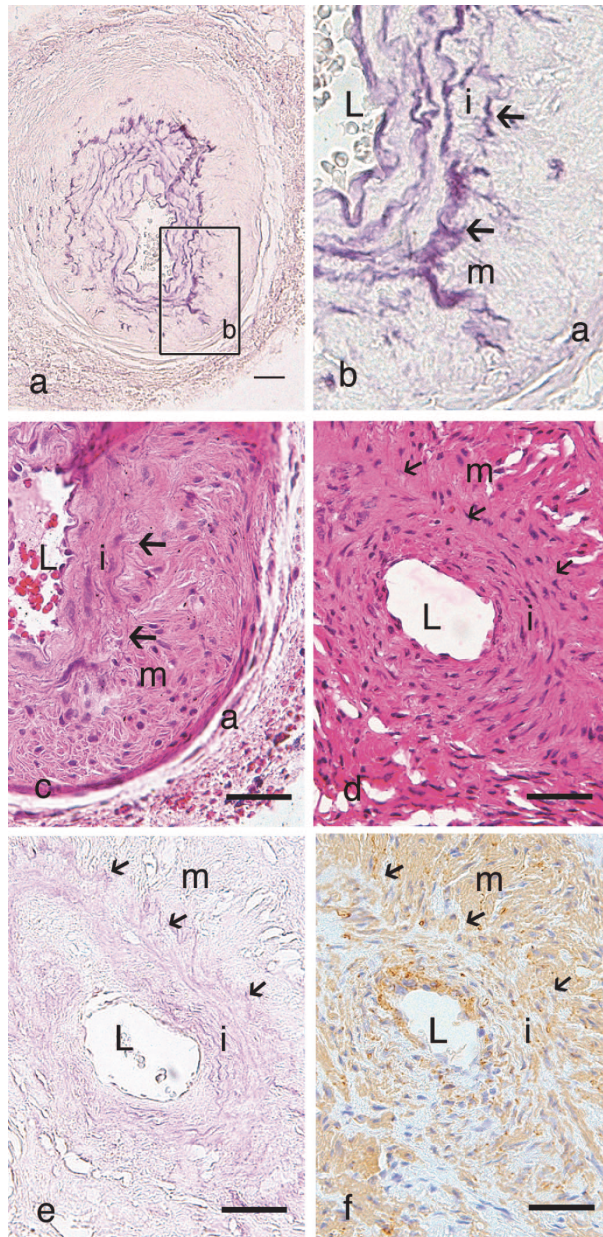


Figure 3.4 Histology of PDA of adolescent BN rats stained with RF (a, b, e), HE (c, d), and anti-smooth muscle actin 1A4 (f) (a–c) The tubular type ductus with subendothelial elastic laminae; (d–f) an example of a stenotic ductus. Here the intimal thickening consists of areas with subendothelial elastic laminae as well as areas with subendothelial accumulations of cells with less elastin. The media is devoid of elastin lamellae in both types of PDA. The internal elastic lamina is indicated by an arrow. a, adventitia; m, media; i, intima. The bar represents 100 μm .

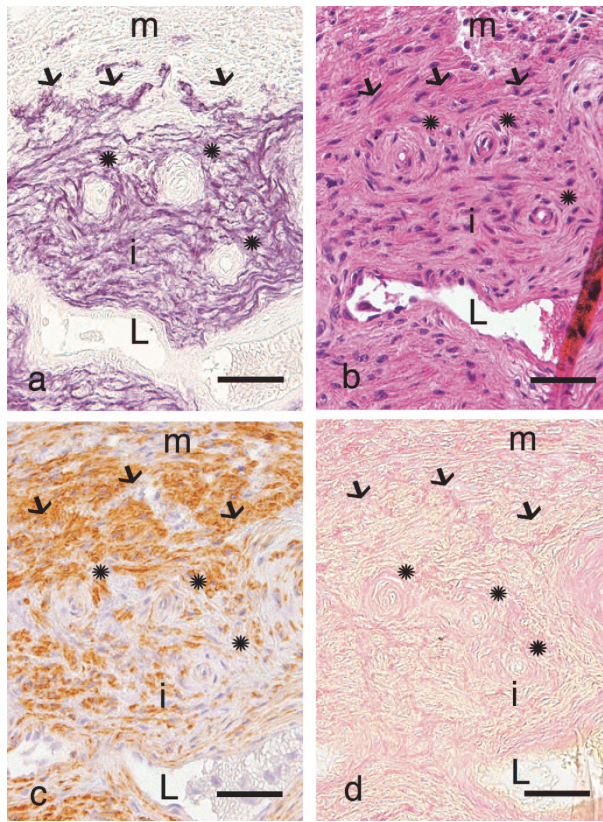


Figure 3.5 Histology of the intimal neovascularization in PDA of adolescent BN rats stained with RF (a), HE (b), anti-smooth muscle actin 1A4 (c), and SR (d). The internal elastic lamina is indicated by an arrow. As in Figure 4, the media is devoid of elastin lamellae. m, media; i, intima; asterisk indicates microvessel. The bar represents 100 μ m.

Discussion

We found a PDA in 9- to 11-week-old BN rats and failed to do so in control Wistar rats. The PDA in adolescent BN rats was histologically characterized by abundant elastin lamellae in the intima, a subendothelial elastic lamina, and the virtual absence of elastin in the media. By contrast, the normal closing ductus arteriosus in neonatal Wistar rats is an elastic vessel composed of an intima with a thin layer of endothelium and subendothelial connective tissue. The media consists of regularly organized elastin lamellae intermixed with layers of SMCs. To the best of our knowledge, this is the first report describing hereditary PDA in a specific rat

strain. The morphologic characteristics of PDA in BN rats are comparable to the histologic findings both in human and canine PDA.^{6,7} The functional relevance of PDA in the adolescent BN rats is comparable to a small PDA, which is amenable to catheter interventional treatment in humans. Therefore, this rat strain may serve as a model for human PDA. BN rats differ from other rat strains with respect to the elastin content¹¹ and the rupture of the internal elastic lamina in the thoracic and abdominal aorta.¹² This phenotype has been related to increased vascular fragility characteristic of the BN rat.¹² Therefore, alterations in elastin regulation may link PDA and the aortic fragility characteristic of BN rats. By analogy, Khau van Kien *et al.*¹⁴ described the association of PDA and familial thoracic aortic aneurysm and dissection (TAA/AD) in a large French family. Recently, mutations in the *MHY11* gene were identified as cause for both TAA/AD and PDA in this family.¹⁵ Elastin in the vascular wall is a polymer of the tropoelastin protein secreted by ECs, SMCs, and fibroblasts.¹⁶ Elastin is an autocrine regulator of SMC proliferation.¹⁷ In the normal closing ductus, impaired elastogenesis coincides with increased SMC migration and proliferation causing physiologic occlusion.¹⁸ By contrast, abundance of elastin lamellae in the intima, a subendothelial elastic lamina, and failure of intimal SMC proliferation are hallmarks of PDA in humans and canine pups.¹⁹⁻²¹ PDA in BN rats is also characterized by elastin lamellae in the intima, a subendothelial elastic lamina, and the virtual absence of elastin in the media. The media of the normal closing ductus in rodents is built up by SMCs derived from the neural crest²² producing elastin lamellae. The absence of elastin in the media of a PDA in BN rats fits into a concept of an overall down-regulation of the aortic elastin production in this particular strain.¹¹ In the normal closing ductus, SMCs from the media migrate through the fragmented internal elastic lamina into the intima and cause intimal proliferation leading to closure of the ductus.⁷ In PDA, the subendothelial elastic lamina is thought to limit the access of the SMCs from the media to the intima. There is also strong evidence that intima cells arise during development and under pathologic conditions from delamination of ECs²³ and circulating precursors.²⁴ The capability of endothelial-mesenchymal transformation has also been described during arteriogenesis in the embryo.²⁵ As ECs can also produce elastic laminae, the elastic intima in the PDA supports the idea that the mesenchymal cells are derived from the endothelium. Hence, endothelium-derived SMCs in the intima exhibiting a predominantly secretory phenotype might lead to the excess local elastin production and inhibition of SMC proliferation and closure of the ductus observed in BN rats. The elastin poor media of PDA may be built up by SMCs of a different origin (probably neural crest cells) lacking secretory

properties. Genetic changes in the physiologic cell differentiation process of the ductus arteriosus might also contribute to the functional heterogeneity of SMCs. During the neonatal period, dedifferentiation of SMCs takes place in the inner intima of the ductus and has been related to intimal thickening and physiologic ductal closure in humans.²⁶ Therefore, a spatiotemporal heterogeneity in differentiation with a lack of neonatal dedifferentiation in the intima of the PDA in BN rats might also be relevant for the development of the phenotypic characteristics of PDA.

Elastin synthesis is modulated by various factors. In the rat aorta and in the caudal artery, it has been shown that TGF-beta1 increases elastin mRNA levels in SMCs and ECs.²⁷ The role of TGF-beta during ductal closure was established in lambs years ago.²⁸⁻³⁰ In the present study TGF-beta signaling was not analyzed because of the fact that the PDA in the adolescent BN rat represents the endpoint of a defective developmental process. Several lines of evidence suggest a role of TGF-beta signaling in congenital cardiovascular anomalies. Recently, a syndrome of altered cardiovascular, craniofacial, neurocognitive, and skeletal development caused by mutations in TGF-beta receptors 1 and 2 has been described.⁴ The cardiovascular anomalies included aortic root aneurysm and aortic tortuosity in all patients. A PDA or ductal aneurysms were reported in 54% of the affected individuals. Histologically, the aortic anomaly was characterized by disorganization of the elastin matrix and loss of elastic fiber architecture. Studies on the expression of collagen, connective tissue growth factor, and Smad2 in the aortic media implicated perturbations in TGF-beta signaling as underlying pathogenetic mechanism. Recent data on Marfan syndrome also focus on mutations of the TGF-beta receptor. Marfan syndrome, an extracellular matrix disorder with the cardinal cardiovascular manifestations of aortic root dilatation and aneurysm formation, has been associated with defects in the gene encoding for fibrillin at 15q21.1.³¹ A second type of Marfan syndrome (MFS II) has been related to locus 3p25-p24.2 recently, which has been shown to be caused by heterozygous mutations in the *TGFB2* gene.^{32,33}

A diverticular phenotype of the ductus was observed in the present study in two of 12 adolescent BN rats. Although strictly speaking, the diverticular ductus is not a patent ductus, it seemed justified to include the diverticular type ductus in the study group. The persistence of the aortic end of the ductus as a vascular structure has not been found in rats from other strains *i.e.* Wistar rats serving as controls. The diverticular type is considered as farthest end of a continuum of PDA. A similar phenotype has been described earlier in dogs with hereditary PDA⁷ and is referred

to as mixed-type ductus arteriosus. The mixed phenotype consists of stenosis and intimal thickening at the pulmonary end of the ductus and the lack of intimal thickening at the aortic end. In humans, a ductal aneurysm is known as a rare lesion developing in the third trimester of pregnancy.³⁴ This anomaly is observed in connective tissue disorders such as Marfan and Ehlers-Danlos syndromes and other syndromal lesions in about 25% of the patients³⁵ and is characterized by deficient or absent intima cushions and/or disorganized elastin lamellae in the ductal media.³⁴ As intima cushion formation²⁷⁻³⁰ and elastic fiber organization are both regulated by TGF-beta signaling, the phenotype of congenital ductal aneurysm might be also the result of localized or generalized perturbations of TGF-beta signaling.

In eight of 12 BN rats, we observed neovascularization of the intima at the pulmonary end of the ductus. Similar findings have been described in dogs with a hereditary form of PDA. In these genetically defective dogs, the normal process of intimal thickening is impaired. Endothelial strands are observed in the subendothelial region suggestive of capillary formation.⁷ An important factor leading to abnormal intimal neovascularization is tissue hypoxia. In atherosclerotic systemic arteries, neovascularization develops in response to hypoxia in deep parts of atherosclerotic lesions.³⁵ During physiologic ductal closure, tissue hypoxia appears to be responsible for anatomical closure³⁶ and has been related to the death of SMCs in the inner media and consecutive vascular remodeling.^{26,36} The role of tissue hypoxia in ductal closure has extensively been studied in lambs.^{37,38} In lambs, hypoxic cell death in the media has been attributed primarily to changes in vasa vasorum flow and medial thickness, which can occur before luminal blood flow had ceased.³⁸

In small animals such as rats, mice, and rabbits, intramural vasa vasorum are lacking.³⁹ It has been suggested that, in this situation, the complete obliteration of the ductal lumen is necessary to initiate hypoxia-induced anatomic remodeling of the ductus. The exact mechanisms modulating functional and anatomic closure of the ductus are still not fully elucidated. *In vitro* data demonstrate a fundamental role for developmentally regulated O₂-sensitive K-channels in ductal vascular SMCs during the initial postnatal vasoconstriction of the ductus arteriosus.⁴⁰ Recent evidence suggests a role for endothelin as oxygen messenger in the ductus during the acute postnatal increase in oxygen tension.⁴¹

Conclusion

The BN inbred rat strain has been characterized as a novel animal model of PDA. PDA in the BN rat may reflect the well-known elastin defect in this particular strain. The BN rat may serve as a model for defective vascular remodeling found in human PDA as well as in human thoracic aortic aneurysm and dissection.

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Chapter 4

Differential temporal and spatial progerin expression during closure of the ductus arteriosus in neonates

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Abstract

Closure of the ductus arteriosus (DA) at birth is essential for the transition from fetal to postnatal life. Before birth the DA bypasses the uninflated lungs by shunting blood from the pulmonary trunk into the systemic circulation. The molecular mechanism underlying DA closure and degeneration has not been fully elucidated, but is associated with apoptosis and cytolytic necrosis in the inner media and intima. We detected features of histology during DA degeneration that are comparable to Hutchinson-Gilford Progeria syndrome and ageing. Immunohistochemistry on human fetal and neonatal DA, and aorta showed that lamin A/C was expressed in all layers of the vessel wall. As a novel finding we report that progerin, a splicing variant of lamin A/C was expressed almost selectively in the normal closing neonatal DA, from which we hypothesized that progerin is involved in DA closure. Progerin was detected in $16.2\% \pm 7.2$ cells of the DA. Progerin-expressing cells were predominantly located in intima and inner media where cytolytic necrosis accompanied by apoptosis will develop. Concomitantly we found loss of α -smooth muscle actin as well as reduced lamin A/C expression compared to the fetal and non-closing DA. In cells of the adjacent aorta, that remains patent, progerin expression was only sporadically detected in $2.5\% \pm 1.5$ of the cells. Data were substantiated by the detection of mRNA of progerin in the neonatal DA but not in the aorta, by PCR and sequencing analysis. The fetal DA and the non-closing persistent DA did not present with progerin expressing cells. Our analysis revealed that the spatiotemporal expression of lamin A/C and progerin in the neonatal DA was mutually exclusive. We suggest that activation of *LMNA* alternative splicing is involved in vascular remodeling in the circulatory system during normal neonatal DA closure.

Introduction

The fetal ductus arteriosus (DA) is a muscular artery connecting the aortic arch and pulmonary trunk (Figure 4.1a). The DA shunts deoxygenated blood from the fetal right ventricle into the umbilico-placental circulation, where gaseous exchange takes place. Prenatally, the DA is indispensable. After birth, as blood becomes oxygenated in the lungs, the DA rapidly contracts followed by anatomical definitive closure (Figure 4.1a, b, f). The histological features of the normal closing muscular DA as well as the elastic structure of the aorta are depicted schematically (Figure 4.1d, e). DA closure is incomplete in about 10% of neonates¹ causing a patent or persistent DA (PDA). This is the third most common congenital heart defect.² Normal closure of the DA, which can take place in both healthy neonates as well as in neonates with congenital heart malformations, is mediated by two processes, vascular smooth muscle cell (SMC) contraction, and anatomical closure on the basis of tissue degeneration.^{1,3-5} Contraction of SMCs in the DA results from the rapid rise in oxygen tension after delivery.⁶ Anatomical closure in humans starts already in the second trimester fetus by the initiation of intimal thickening which is regulated by the prostaglandin (PGE) receptor, EP4⁷ via the protein kinase A and Epac regulatory pathways.⁸ After birth following contraction there are major changes in the inner media characterized by extracellular matrix deposition followed by apoptosis and cytolytic necrosis (CN) (Figure 4.1d). Eventually a DA ligament (Figure 4.1f) is formed.^{3,5-9} The molecular regulation of DA regression in the neonate is still incompletely understood.

The anatomical process of postnatal DA closure and regression is common in mammals but the timing of the processes regulating DA closure varies between species. In humans DA maturation already starts in the prenatal period but without strict correlation between gestational age, postnatal age and the degree of maturation.¹ For medical purposes, it is crucial to study the spatiotemporal regulation of DA regression in the prenatal and postnatal period.³⁻⁵ In neonates with ductus-dependent heart anomalies, systemic administration of PGE can prevent constriction of the DA.¹⁰ Unrestricted flow via an open DA is essential for those neonates until they can undergo corrective surgery. Histological characteristics of the DA of healthy neonates and those with heart anomalies are identical³ and similarly influenced by PGE.¹¹ This offers a unique opportunity to study DA remodeling using surgical biopsy specimens.

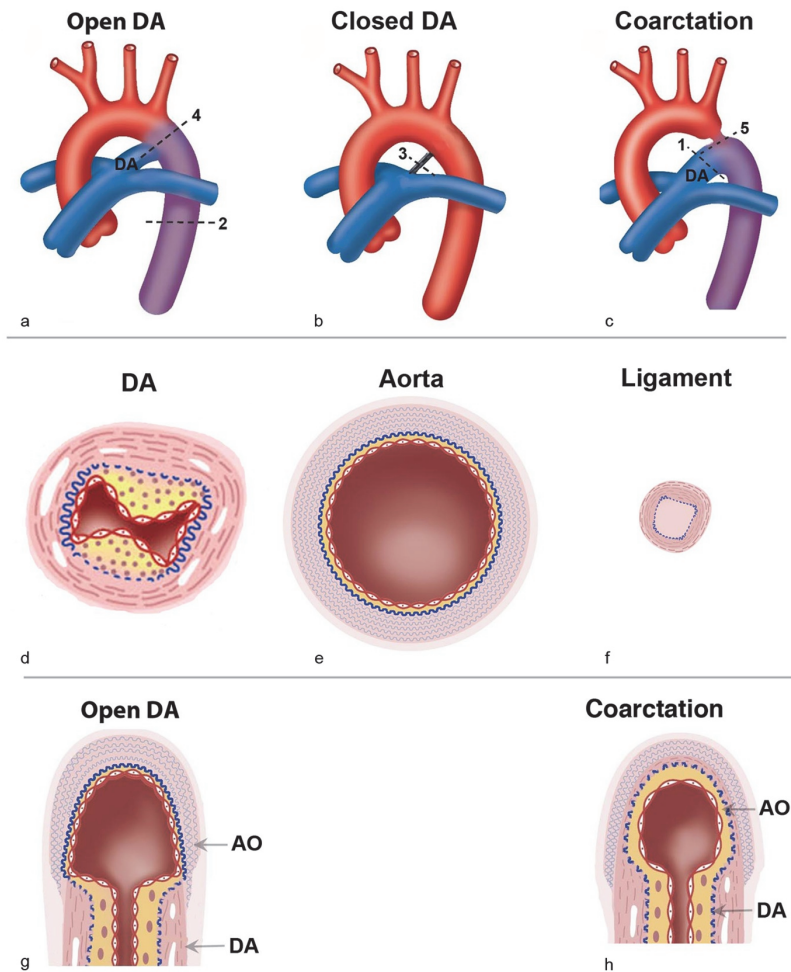


Figure 4.1 Schematic presentations of the great vessels.

Anatomy of DA and the adjacent arteries. (a) A patent DA is found in the fetus before respiration starts. Oxygen-rich blood (in red) from the placenta passes through the aorta. Oxygen-poor blood from the fetus (in blue) passes the DA entering the descending aorta (in purple). (b) After the start of respiration the DA regresses into a ligamentum arteriosum. Aortic (red) and pulmonary (blue) circulations are disconnected. (c) In aortic coarctation, a left-sided heart malformation, the DA is kept open by administration of PGE allowing blood to enter the systemic circulation. Dashed lines indicate the plane of tissue sections shown in d-h.

Transverse sections of the muscular DA (d; 1 in c), the elastic aorta (e; 2 in a), and the ligamentum arteriosum (f; 3 in b). Intimal thickening in the DA is indicated in yellow. Areas with CN are indicated in white and the wavy elastic lamellae with blue. Endothelial cells (red outlines) line the inside of the vessels.

Longitudinal sections through the DA (4 in a, providing Fig. 1g, and 5 in c providing Fig. 1h). (g) DA tissue and aorta tissue are merging and can be separately distinguished by their characteristic histology. (h) In coarctation, inner media and intimal thickening of the DA extend into the aorta sometimes almost encircling the lumen of the aortic arch at that site (as depicted). The aortic tissue will always be present in the roof of the coarctation.

Children with Hutchinson-Gilford Progeria Syndrome (HGPS) develop progressive arterial occlusive disease and will eventually die due to severe atherosclerosis, see¹². HGPS is caused by a mutation in *LMNA*, which leads to accumulation of a truncated lamin A protein, progerin, at the nuclear lamina.¹³ Arteries of HGPS patients exhibit intimal thickening with dense fibrosis and thinning of the adjacent media.¹⁴ Relative to lesions in typical atherosclerosis, the atheromatous core tends to be smaller¹⁴ or even absent¹⁵ in HGPS arteries. Reduced numbers of SMCs in the aortic media was a prominent finding in a murine HGPS model.¹² Marked adventitial fibrosis in arteries and veins has been added to the spectrum of vascular lesions in HGPS.¹⁴ The anatomical remodeling process that converts the muscular DA vessel of the fetus and neonate into the fibrous structure of the ligamentum arteriosum is characterized by intimal thickening without an atheromatous core. Furthermore, loss of SMC in the inner media and intima are due to CN.^{4,5,9} Although degeneration during closure of the DA and vascular pathology in HGPS is not identical, the similarities in intimal thickening and loss of VCMCs warrant the hypothesis that progerin may also play a role in the DA closure. Here, we show that progerin is specifically expressed during the closing process of the DA in areas with developing CN, while Lamin A/C is hardly expressed in these regions. The molecular and cellular implications of these novel findings are discussed.

Materials and Methods

Ethics statement

The medical ethics committee of the Leiden University Medical Center exempted this study from review because tissues normally discarded were analyzed anonymously and waived the need for consent due to the fact that linkage of the samples received with individual patients was not possible.

Tissue samples

Postnatal human DA tissues were obtained during surgical correction of cardiac anomalies. The resection of the DA was part of the surgical strategy for aortic arch reconstruction in all cases (see Figure 4.1c,h, and dashed line 5 for site and orientation of the dissected biopsies). Fetal tissues were acquired from post-mortem specimens after legal or spontaneous abortion after PGE induction. All

tissues were collected and stored at the department of Anatomy and Embryology of Leiden University. Tissue type, diagnosis and age were recorded for this study.

Tissue samples were directly fixed in 4% phosphate-buffered formaldehyde or 2% acetic acid in absolute ethanol at room temperature. Fixed samples were embedded in paraffin and sectioned at 5 μm . For RNA extraction or cryostat sections, samples were placed in Tissue-tek, and were directly frozen in liquid nitrogen and stored at minimal -20°C . Fetal material was obtained from 2 cases (14 and 18 weeks gestation) providing aorta and DA wall tissue. Neonatal tissue was studied in 16 DA biopsies and two descending aortas. The neonates (aged 3-21 days, median 10 days) were born after 38 ± 2 weeks of gestation. All of them received a low dose of PGE ($0,0125 \mu\text{g/kg/min}$) because of left-sided cardiac defects with aortic arch anomalies (Figure 4.1c,h, dashed line 5). The biopsy of a PDA came from a 2-years-old child. A part of the descending aorta was collected from a 4-month-old infant during coarctation repair and served as a negative control (not shown).

Immunohistochemistry microscopy and image quantification

For a morphological overview sections were briefly (5 seconds) counterstained with haematoxylin eosin. Antibodies used in this study are: monoclonal lamin A/C (Chemicon International (Temecula, CA, USA); 1:50), a monoclonal anti-lamin A G608 (progerin) antibody, kindly provided by K. Djabali (Dpt. of Dermatology, Columbia University, NY, USA) and mouse monoclonal antibody against smooth muscle alpha-actin 1A4 (1: 3000; DAKO A/S Glostrup, Denmark). Antibody incubation was performed in PBS containing 0.05% Tween 20 (PBS-T) and 1% bovine serum albumin overnight in a humidified chamber with the primary antibody at room temperature. The first antibody was detected with either HRP-conjugated secondary antibody (Vector Laboratories, Inc. Burlingame, CA, USA) or with a fluorescent- conjugated secondary antibody (Goat-anti-mouse/Alex 488, Invitrogen, Corp. Carlsbad, CA, USA, Donkey-anti-mouse/Cy3, Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The fluorescent conjugated secondary were mounted in Citifluor (Agar Scientific) containing 400 $\mu\text{g/ml}$ DAPI (Sigma-Aldrich) and examined with a confocal laser scanning microscopy (Leica SP5) or a Fluorescence microscope (Leica DM RXA), 63X and 100X lens NA 1.4 plan Apo objective. Apoptotic cells were determined using the in situ Cell Death Detection Fluorescein kit from Roche Diagnostics (Almere, Nederland) following the

manufacturer's instructions. Significance of statistical analysis was performed with the unpaired T-test with Welch's correction.

RNA isolation and RT-PCR reaction

Total RNA was extracted from frozen biopsies using the RNA-Bee RNA extraction kit as per the manufacturer (AMS Biotechnology, Abingdon, U.K.). 1 µg of RNA from each sample was submitted to cDNA synthesis using the RevertAid™ First Strand cDNA Synthesis Kit (Fermantas, Ontario, Canada) or with the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). 13.4 ng of cDNA was used for PCR using primers located in exon 9 and 12 of *LMNA* (forward: 5' ACCCCGCTGAGTACAACCT, and reverse: 5' TGCAGTTCTGGGGGCTCT. The primers cover progerin cryptic site at position 2215 in *LMNA* mRNA, and are 2 bp longer than these that were previously described. Our set of primers gives less non-*LMNA* PCR products in DA and aorta as compared those reported in³¹. PCR conditions were 35 cycles, each cycle consisting of 30-sec denaturation at 94°C, 30-sec annealing at 60°C, and 30-sec polymerization at 72°C. 15 µl of the reaction was analyzed on 2% agarose gel and stained with ethidium bromide. PCR products were eluted from gel and were sequenced at www.lgtc.nl. Primers for GUSB amplification are: forward- 5'CTCATTGGAATTTTGCCGATT 3'; reverse- CCGAGTGAAGATCCCCTTTTA.

Results

To determine whether progerin is expressed in neonatal DA, reverse transcriptase (RT) PCR was performed on fresh and frozen biopsies of DA that were collected during corrective cardiac surgery. The expression of progerin mRNA was analyzed in neonatal DA sections using primers that cover the progerin cryptic site.¹⁶ The full length mRNA PCR product is 270 bp (Figure 4.2A). In addition a 185 bp product representing the delta10 isoform was amplified in the DA and aorta tissues. Importantly, in the neonatal DA but not in aorta tissue a 120 bp PCR product was found (Figure 4.2A). These PCR products were evaluated by sequencing analysis which confirmed that the 120 bp fragment corresponds to progerin whereas the 270 bp fragment is of *LMNA* full length mRNA (Figure 4.2B). It is relevant to note that in the analyzed biopsies this primer set led to unspecific PCR products (i.e. a 100 bp fragment; Figure 4.2A). Therefore, it was essential to confirm *LMNA* gene

products by sequencing of the PCR products. The housekeeping beta-glucuronidase (*GUSB*) gene was used as a control¹⁷ and PCR product was found in all tissues. In total, progerin PCR product was found in all 6 DA samples analyzed, but was not detected in the two aorta samples. Aorta and DA are physically connected, while remodeling in neonates is limited to the DA, suggesting an association between progerin and DA remodeling.

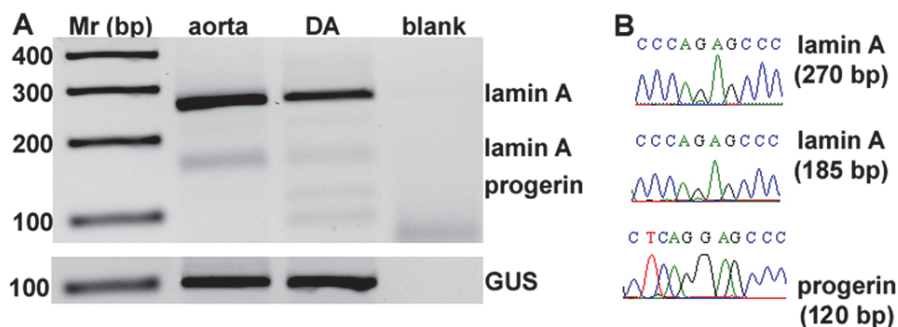


Figure 4.2 RT-PCR reveals expression of progerin mRNA in DA.

A. RT-PCR analysis of LMNA and progerin mRNA in neonatal aorta and DA was performed with primers that amplify 270 and 185 bp of LMNA, and 120 bp of progerin. GUSB PCR product was used as a control. Aorta tissue was obtained from a 4-month patient and DA tissues from 6 neonates 8 days after birth.

B. Direct sequencing of PCR fragments 270, 185 and 120 bp. Shown are the sequence histograms from position 2212 bp.

The low level of progerin mRNA in DA indicates that the protein would be expressed at very low levels or in only part of the cells. Progerin and lamin A/C protein expression was analyzed with immunohistochemistry using antibodies that specifically recognize progerin or lamin A/C.¹⁶ Due to irreversible farnesylation at the C-terminus, progerin accumulates at the nuclear lamina.¹⁸ In contrast, lamin A/C is also found in the nucleoplasm.¹⁸ Indeed, confocal microscopy revealed progerin expression (Figure 4.3A,c), at the nuclear lamina, while lamin A/C exhibited an additional nucleoplasm distribution (Figure 4.3A,b). This confirms that the two antibodies recognize different proteins.

Progerin expression in neonatal DA was found in a large number of cells, while in the aorta its expression was less prominent (Figure 4.3B b,e). We determined that the mean fraction of progerin expressing cells in the DA was $16.2\% \pm 7.2$ while in

the aorta it was only $2.5\% \pm 1.5$ (Figure 4.3C,a) indicative for a tissue-specific expression of progerin. Since the major histological difference between the DA and aorta in neonates is degeneration of the inner media and the intima, we determined progerin expression across the DA layers. A higher fraction of progerin positive cells was found in the intima and inner media ($25\% \pm 15.0$), compared to the outer media ($11\% \pm 11.2$) (Figure 4.3C,b). This difference in the spatial distribution of progerin was statistically significant. TUNEL staining of the neonatal DA, indicative of apoptosis, was predominantly found in the inner media bordering the intima (Figure 4.4a-d). As cytolytic necrosis will initially develop in cells of the intima and inner media, the high expression of progerin in these layers supports a functional relationship with cell death. The spatial overlap in the DA between progerin expression and TUNEL, suggests a causal relation between progerin expression and apoptosis in the development of CN.

Progerin expression was analyzed in 16 neonatal DA biopsies (including 2 with adjacent aortic tissue) with various degrees of CN development. Furthermore, specimens from a 14- and an 18-week-old fetus and a PDA from a 2-year-old child were studied. In the fetal DA CN is not developed, and, as expected, uniform SM-actin staining was found across the DA layers (Figure 4.5A). Regression of the normal neonatal closing DA is characterized by a reduced expression of contractile proteins, such as alpha-SM-actin in the inner media.^{5,9} In the DA from neonate I (Figure 4.5B) SM-actin expression in the inner media was less intense compared to the adjacent wall layers. In the same area from the 10-day-old infant II (Figure 4.5C), however, prominent CN in the inner media was present showing a zone without cells and complete absence of alpha-SM-actin expression (Figure 4.5C). These represent two subsequent stages in the degeneration of the neonatal DA. Spatially unchanged SM-actin staining was exhibited in the PDA (Figure 4.5D) similar to the fetal DA. In both the fetal and PDA without CN, progerin expression was not detected (Figure 4.5A,D). In contrast, in the DA of both neonates progerin expression was found in the intima and inner media but not in the outer media (Figure 4.5B,C), where SMCs remain present throughout ligament formation.

Since progerin is a splicing variant of the lamin A gene, we investigated the expression pattern of lamin A/C in the DA. Lamin A/C expression was found in all nuclei in the fetal DA (Figure 4.5A), but in neonatal DA the expression was markedly less in areas of developing CN (Figure 4.5B,C). Lamin A/C was found in all layers in the PDA (Figure 4.5D) indicating a spatiotemporal association between lamin A/C expression and the development of CN in the normal closing neonatal DA.

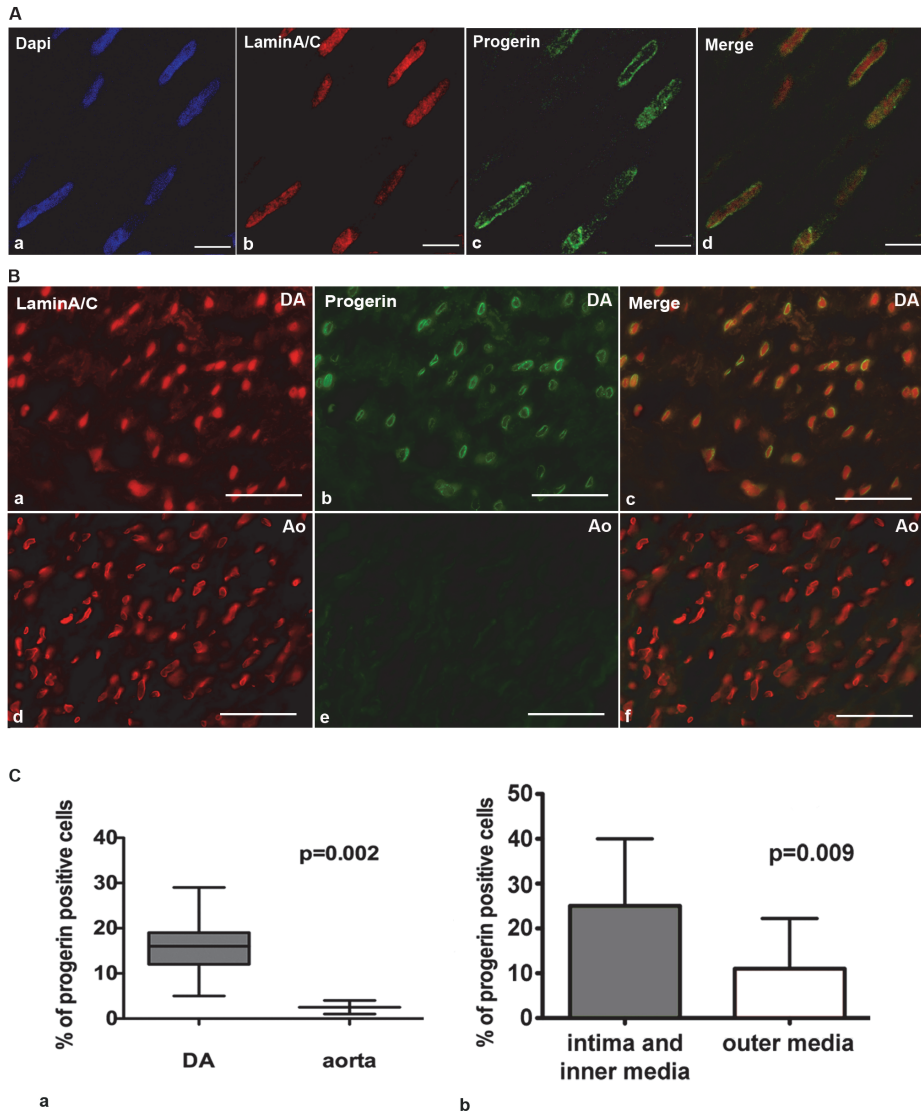


Figure 4.3 Spatial changes of progerin expression in a neonatal DA.

A. (b) Confocal images of immunofluorescence show even nuclear distribution of lamin A/C (red) and (c) nuclear envelope localization of progerin (green) in the media of DA. (a) Nuclei are counterstained with DAPI. (d) Merging of the staining patterns. Scale bars: 5 μ m.

B. Microscopic image of immunofluorescence in DA and aorta (Ao) show (a,d) the tissue specific expression of lamin A/C and (b,e) progerin. (c,f) in the media. Nuclei expressing both proteins as shown in the merged images. Scale bars: 20 μ m.

C. (a) Box-plot shows the percentage of progerin positive cells in DA and aorta. Statistical analysis represents 1302 nuclei in DA and 302 nuclei in aortas from 5 individuals. P-value indicates significant difference in progerin expression between DA and aorta. (b) Histograms show the average ratio of progerin positive cells in intima and inner media *versus* the outer media. Statistical analysis represents 4 samples from each of the 5 individuals. P-value indicates that intima and inner media contain significantly more progerin expressing cells than the outer media.

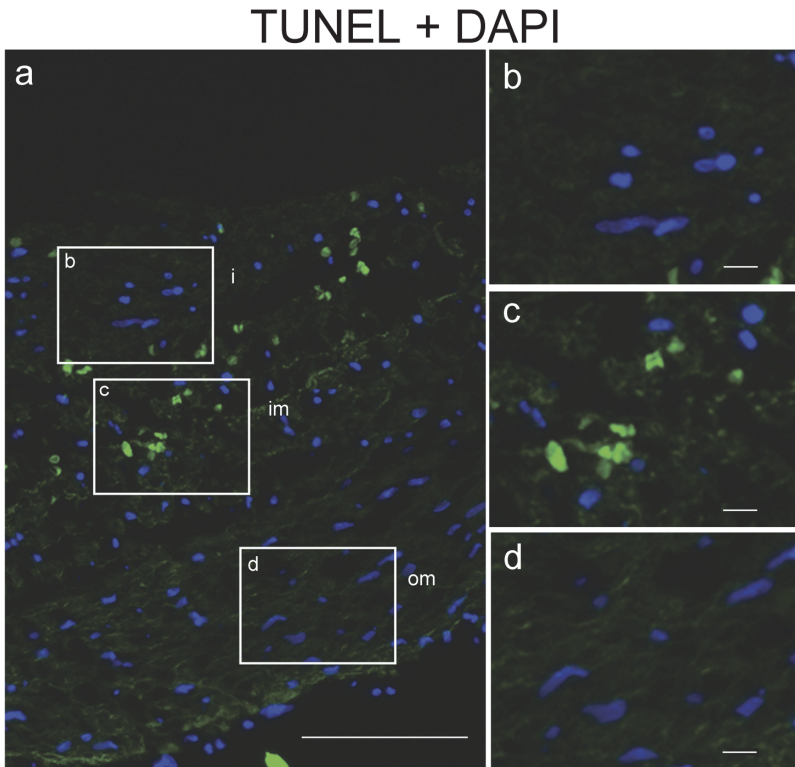


Figure 4.4 Spatial distribution of TUNEL staining in the DA
(a) A sister section of the neonatal DA from figure 3A that was stained with TUNEL (green). Details of the staining are shown in magnifications of the boxed areas b-d. TUNEL staining is predominantly found in the inner media (c) and the bordering intima (b). TUNEL positive nuclei were not found in the outer media (d). Nuclei were counterstained with DAPI. Scale bars are: scale bar 100 μm (a); 10 μm (b,c,d).

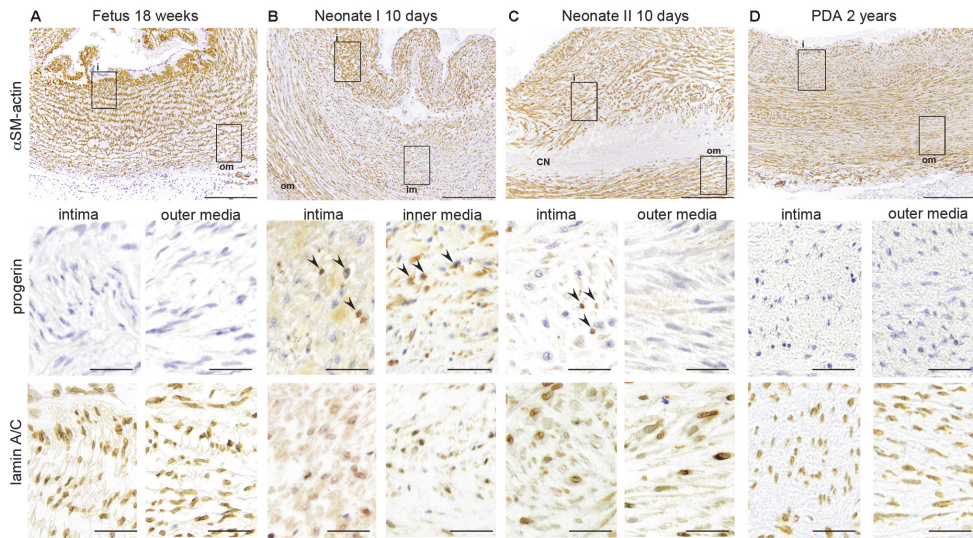


Figure 4.5 Spatial and temporal expression of progerin is associated with development of CN in the DA. A. Overview of the wall of the DA of an 18-week-old fetus depicting α -smooth muscle actin (α SM actin) staining of the vascular smooth muscle cells. Details of intima (i) and outer media (om) show the absence of progerin and the overall expression of lamin A/C in the cells. B. Overview of the DA of a 10-day-old neonate with onset of degeneration of the inner media (im). In this layer there is loss of α -SM actin staining. Details of intima and inner media show progerin expressing nuclei (arrowheads) and reduction of lamin A/C expressing cells compared to the fetal DA in A. C. Overview of the DA of a 10-day-old neonate with marked cytolitic necrosis (CN) in the inner media with loss of cells. Smooth muscle cells are still detectable in the intima and outer media. Detail of the intima shows some progerin expressing nuclei (arrowheads) and absence of these nuclei in the outer media. Lamin A/C expression is found in the inner and outer media. D. Overview of a non-closing persistent DA (PDA) of a 2-year-old infant. In all layers there is abundant α -SM actin expression. Details of the intima and outer media show absence of progerin and presence of lamin A/C expressing cells. Scale bars: overviews A-D 200 μ m, details 50 μ m.

Discussion

The molecular mechanisms that are involved in DA closure are poorly understood. Here we report, for the first time, progerin expression in the neonatal DA. We demonstrated expression of progerin predominantly in the neonatal but not in the fetal DA, and in a higher number of cells in intima and inner media, compared to the outer media and to the aorta. This spatial and temporal progerin expression pattern highly correlates with CN development in the degenerating neonatal DA implying a functional role for progerin in CN development. Abnormal DA closure was not reported so far in HGPS patients or in the HGPS mouse models. To

confirm a role for progerin in DA closure detailed studies in neonatal HGPS models and patients are relevant.

Cell death in the DA is accompanied by activation of apoptosis.^{9,19} We show a spatial correlation between progerin expression and frequency of apoptosis. We postulate that progerin plays a role in cell death of smooth muscle cells in the DA. Reduction of alpha-SM-actin was found in arteries of HGPS patients in cells expressing progerin¹³ indicating that spatial and temporal expression of progerin correlates with development of apoptosis. Apoptosis frequency is increased in fibroblasts from HGPS²⁰ and elevated apoptosis in the mouse model for progeria is reported.¹² The role of progerin in initiating apoptosis of cells in the inner media and intima of the DA should be further investigated.

Elevated progerin expression was spatially associated with reduced lamin A/C expression in the DA. These spatial changes between lamin A/C and progerin expression in the inner media and intima suggest an association with alternative splicing of *LMNA* gene products during DA remodeling. Activation of *LMNA* alternative splicing was found during cell differentiation and cell cycle progression.²¹ During postnatal cardiac development splicing variants of *ASF/SF2* and *SERCA2* are activated^{22,23} to change cellular function. Lamin A/C was recently shown to play a role in cardiac disease. In heterozygous *LMNA*+/- knockout mice, a 50% reduction in lamin A/C protein levels cause myocardial apoptosis and cardiac abnormalities.²⁴ Deregulation of lamin A/C processing correlates with atherosclerotic lesions from aged individuals. Pre-lamin A accumulates in medial SMC from aged individuals and in atherosclerotic lesions correlating with the down-regulation of lamin A/C processing enzymes.²⁵ Based on these studies we hypothesize that changes in lamin A/C expression and activation of splicing variants are an alternative mechanism to regulate cardiovascular development. Alternative splicing is affected by oxidative stress.²⁶ Whether oxygen levels alter *LMNA* splicing during DA closure is yet to be determined. Anatomical definitive sealing of the DA is preceded by contraction of SMCs in which many molecular pathways are involved¹ e.g. activated by reactive oxygen species. Accumulation of reactive oxygen species has been described in both the onset of DA closure²⁷ as well as in vascular ageing.²⁸ Progerin expression in coronary arteries of non-HGPS individuals, which significantly increases throughout life has been associated with vascular ageing.¹⁴ Farnesyltransferase inhibitors (FTIs) can in part correct cellular defects caused by progerin.^{29,30} FTIs reverse the permanent farnesylation of progerin or pre-lamin A and, thus repair structural and functional nuclear defects. Given our finding that progerin is expressed in the neonatal DA, it will be relevant

to study whether FTIs can interfere with local vascular remodeling and prevent anatomical DA closure.

Our study indicates a novel role for progerin in a programmed cellular process although a relation between DA closure and the process of ageing needs further clarification.

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Chapter 5

Laser-capture microdissection and comparative microarray expression analysis identified *Rgs5* and *Dlx1* as vessel-specific molecular markers of the ductus arteriosus in fetal rats

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Abstract

Rationale: Closure of the ductus arteriosus (DA) is a crucial step in the transition from the fetal to the postnatal circulation. Patent DA is one of the most common cardiovascular anomalies in children causing morbidity especially in premature infants. Little is known about the molecular mechanisms regulating DA maturation and closure.

Objective: We aimed to identify genes that specify the DA in the fetus and differentiate it from the non-closing aorta.

Methods and results: Comparative microarray analysis of laser-captured microdissected endothelial (ECs) and vascular smooth muscle cells (SMCs) from the DA and aorta of fetal rats identified vessel-specific transcriptional profiles. The linear model of microarray analysis (LIMMA) revealed a strong age-dependency of gene expression in the samples from embryonic day 18 and 21. Among the DA-dominant genes the regulator of the G-protein coupled receptor 5 (*Rgs5*) and the homeobox transcription factor *Dlx1* exhibited the highest and most significant level of differential expression. The aorta showed a significant preferential expression of the Purkinje cell protein 4 (*Pcp4*) gene. The results of the microarray analysis were validated by real-time quantitative PCR. Finally, immunohistochemistry at day 21 documented differential expression of the proteins encoded by the three newly identified genes in DA and aorta.

Conclusion: Our study confirms a DA and aorta-specific transcriptional profile in ECs and SMCs. For the first time we recognized the preferential expression of *Rgs5* and *Dlx1* in the fetal DA. These genes represent novel molecular targets for the regulation of fetal DA maturation and postnatal DA closure.

Introduction

The ductus arteriosus (DA) is a highly specialized fetal blood vessel that connects the pulmonary trunk with the descending aorta. In the fetal circulation the DA bypasses the fluid-filled lungs by shunting blood from the pulmonary trunk into the systemic circulation. At birth, with the transition from placental oxygen supply to air breathing, this connection becomes unnecessary and closes. DA closure is a crucial step in the transitional circulation as it ensures the efficient delivery of oxygenated blood from the lungs to all organs.

Although sharing the same embryologic origin and environment the DA shows a deviant development and differentiation compared with other pharyngeal arch artery derivatives.^{1,2} Early in fetal development the DA acquires its typical muscular morphology while the adjoining aorta differentiates into an elastic vessel by elastin deposition and concomitant loss of actin expression in the media.² Already at this early stage the DA media is composed of highly differentiated vascular smooth muscle cells (SMCs)³ compared to the aorta. From the second trimester of pregnancy onwards endothelial cells (ECs) and SMCs in the inner part of the DA undergo a ductus-specific remodeling process.⁴ As a result of this process the mature DA exhibits intimal thickening, which facilitates sealing of the constricting vessel immediately after birth. Early (functional) closure of the DA is triggered by the postnatal increase of oxygen partial pressure and prostaglandin withdrawal.⁵ The contraction in response to oxygen is intrinsic to the SMCs of the DA and highly conserved among different species. Oxygen-induced inhibition of Kv channels, activation of L-type voltage-gated Ca-channels, increased endothelin synthesis and redox-dependent activation of ROCK⁶ and other calcium sensitization pathways⁷ have been documented in the DA of humans and rabbits. After the functional closure the definitive anatomical closure of the DA is initiated. In the subsequent postnatal period the DA degenerates due to cytolytic necrosis and apoptosis from the muscular artery into a fibrous remnant,⁸ while the adjoining elastic vessels remain open.

Although various anatomical, physiological and biochemical aspects of DA closure have been studied during the last decennia reviewed in Bökenkamp et al.⁵ little is known about the molecular mechanisms controlling the unique remodeling process of the DA. A recent study documented the expression of the truncated lamin A protein “progerin”, the underlying cause of Hutchinson-Gilford Progeria syndrome, related to the cytolytic necrosis process in the DA. This finding suggests

involvement of alternative splicing of lamin A (*LMNA*) in the vascular remodeling of the normal neonatal DA.⁹

Human disease states offer insights into the transcriptional regulation of certain physiological processes. Regarding DA closure Char syndrome and patent DA in preterm infants are instructive. The patent DA and mild facial and hand anomalies in Char syndrome are caused by mutations in the neural crest-related transcription factor AP2B gene (*TFAP2B*).¹⁰ Sequence polymorphisms in the same gene are associated with isolated non-syndromic patent DA in preterm infants¹¹⁻¹³ and consequently suggest a role for *TFAP2B* in the transcriptional control of normal DA closure. Targeted deletions of *Tfap2B* in mice confirm the critical role of *TFAP2B* in DA development and limb patterning.^{14,15}

Two previous studies on transcriptional profiles of the DA and aorta in fetal rats revealed characteristic differences between both vessels^{16,17} although these studies are hardly comparable in the analysis and finality because of the differences in perspective and reference.¹⁸ Upregulation of sarcomeric genes, characteristic for the fetal DA in the study of Costa et al.¹⁶ was found in the aorta by Jin et al.¹⁷ Growth hormone receptor (*Ghr*) exhibited the most significant upregulation in the DA among all DA-specific genes in the study of Jin et al.¹⁷ but was not differentially expressed in the dataset of Costa et al.¹⁶ Both studies used pooled whole vessel preparations of DA and aorta for the microarray analysis.

In microarray analyses of whole vessel extracts the specific role of different cell populations, i.e. ECs and SMCs of the DA and descending aorta, cannot be determined. We have chosen for a selective technique. Gene expression profiling of laser-captured microdissected cells offers the opportunity to study gene expression profiles in cells of different embryonic origin, with different reactions on the same environmental conditions and different functions during development. By analyzing the profiles from late fetal (day 18) and near term (day 21) rats we were able to study the change in ECs and SMCs during the DA remodeling process at late gestation. We hypothesized that both cell-types of the DA and aorta bear a distinct transcriptional profile characterizing the artery that will close after birth and the neighboring vessel that will remain open for the duration of life. We postulate that transcriptional profiles in ECs and SMCs from DA and aorta reflect either a different origin or function of these cells, which might be detected in the perinatal period accompanying the radical changes of environment and phenotype.

Failure of DA closure leads to one of the most common cardiovascular anomalies in children causing morbidity and mortality especially in premature infants.^{19,20} Inhibition of DA closure on the other hand is critical for the immediate postnatal

survival of infants with various types of severe structural heart disease.²¹ The present study aims to increase the knowledge of the molecular mechanisms governing physiological DA closure and to improve the available pharmacological strategies to treat both groups of patients.

Animals and Methods

Animals

Pups from 6 timed-pregnant Wistar rats were delivered after respectively 18 and 21 days of gestation (spontaneous birth occurs at 22 days of gestation) by hysterotomy through a median abdominal incision. For the time of delivery the dams were anesthetized with sevoflurane 2.5% room air subsequently the dams were euthanized. Care was taken to keep the time between the delivery of the first and the last pup within 5 minutes. The litter size was approximately 12 pups per dam. Out of these 12 the first 3 pups were used for the microarray experiment. Isolation of the fetal thorax was carefully performed in RNase free conditions, frozen in tissue tek (Sakura Finetek USA, Torrance, CA, USA), and stored at -80 C. One half of the remaining pups were processed in the same way in order to provide spare material for additional microarray experiments. The other half of the pups were fixed for 24 hours in 4% buffered paraformaldehyde and embedded in paraffin for conventional and immunohistochemical evaluation. The feasibility of the experiments was tested in a separate pilot experiment using 4 Wistar rat embryos after 21 days gestation. Data from the pilot experiment are presented in the supplemental figures 3 and 5.

Both experiments were approved by an independent Institutional Animal Care and Use committee at the LUMC in accordance with the Helsinki convention for the use and care of laboratory animals.

Laser-capture microdissection

Cryostat sections (8 µm) were attached to RNase free (Superfrost™) microscopic slides and immediately placed on dry ice. The slides were preserved in -80°C conditions. Processing of separate batches of tissue sections of a specified embryo was performed at random. Three slides of each embryo were immunohistochemically stained in one batch. RNase free PBS was used for the appropriate dilution of antibodies and washing steps. Furthermore, Suprase (AM2696,

Ambion, Austin, TX, USA) was added to the diluted antibody solution (1U/ μ l). After fixation in cold acetone (4°C) during 2 minutes, cold (4°C) conditions were maintained during further immunohistochemical staining. The three slides could easily be air-dried after acetone fixation. 30 μ l of cold PBS was applied to each tissue section and drained off. In subsequent order, 30 μ l of Biotin-conjugated mouse anti-rat CD31 (BD 555026) (5 ug/100ul), Cy3 Streptavidin (1:100) and alpha smooth muscle Actin antibody (1A4) FITC (Abcam ab8211) (1 μ g/30 μ l) were applied. In order to further limit RNase activity, each antibody solution was only applied for 2 minutes. Subsequently, the antibody was gently drained off with 200 μ l of cold PBS. The slides were dehydrated at room temperature in 75% EtOH (30 sec), 95% EtOH (30 sec), 100% EtOH (30 sec), 100% EtOH (120 sec), xylene (180 sec). Dehydration was immediately followed by laser capture microdissection (LCM) (Veritas Microdissection Instrument. Arcturus Bioscience Inc., Mountain View, CA, USA). 100 to 200 cells from three tissue sections were dissected from the DA and descending aorta of each embryo. ECs were defined as cells lining the lumen and staining positive for anti-rat CD31. Alpha-smooth muscle actin positive cells were identified as SMCs. The microdissected cells were transferred to a Gene Amp tube (Applied Biosystems, Foster City CA, USA) containing 75 μ l of RNeasy lysis buffer (RLT; Qiagen, Hilden, Germany) containing 0.14 M beta-mercaptoethanol and 200 ng Polyinosinic Acid (Sigma Aldrich, USA). The samples were incubated at 42°C during 20 minutes and stored at -80°C.

RNA isolation, amplification, labeling, and microarray hybridization

The samples were processed further in one batch after thawing. RNA was isolated, cleaned up and eluted in 14 μ l of RNase-free water according to the RNeasy Minelute protocol (Qiagen, Hilden, Germany). Two rounds of linear mRNA amplification were performed (modified Eberwine protocol): two-cycle cDNA synthesis and synthesis of biotin-labeled aRNA was performed according to the GeneChip Eukaryotic Sample and Array Processing Manual (Affymetrix, Santa Clara, CA, USA). MEGAscript T7 kit (Ambion, Austin, TX, USA) was used for *in vitro* transcription of the second cDNA strand in the first round of amplification. The second round of amplification, starting from 100 ng of first round aRNA, was finalised during the GeneChip *in vitro* transcription (IVT) labelling. Despite immunohistochemic double-staining and laser-capture dissection sufficient amounts of RNA could be isolated from these cells and amplified for the microarray

experiment. (see supplement (Table S5.1, S5.2) for cRNA yields). Labeled RNA was hybridized to GeneChip Rat Genome 230 2.0 Array. Hybridization was performed using 12.5 µg of biotin-labeled RNA at 45°C for 16 hours under continuous rotation. Arrays were stained in Affymetrix Fluidics stations using streptavidin-phycoerythrin (SAPE), followed by staining with anti-streptavidin antibody and a second SAPE staining. Subsequently, arrays were scanned with an Affymetrix Gene Chip 3000 Laserscanner (Affymetrix, Santa Clara, CA, USA).

Real time quantitative PCR analysis

First-strand cDNA synthesis was performed on the cRNA from the microdissected cells using random hexamer primers and Superscript III RT (Invitrogen, Grand Island, NY, USA). 50 ng of first-strand cDNA was used for subsequent reactions. Quantitative PCR (qPCR) was performed on an ABI Prism 7900 cycler (Applied Biosystems, Foster City, CA, USA) using a Taqman PCR kit (Applied Biosystems, Foster City, CA, USA). Serial dilutions of cDNA were used to generate standard curves of threshold cycles versus the logarithms of concentration for *PGK1/PP1B* and the genes of interest. See supplemental Table S5.3 for sequences and TaqMan® gene expression assays.

Statistical analysis

The Affymetrix probe level data were summarized using FARMS (Factor Analysis for Robust Microarray Summarization). Raw intensities were $\log_2$ transformed to get data normally distributed. First, an unsupervised multivariate projection method, Spectral Map Analysis, was applied to reduce the complexity of highly dimensional data (n genes versus p samples). Spectral Map Analysis provides an unbiased identification of the predominant clusters of genes and subjects that are present in the data set. Second, we modeled the expression data in LIMMA (Linear Models for Microarray Data)^{21,22}, a method that is designed to get stable analyses even for experiments with small number of arrays. In LIMMA, we used a model that includes the three dichotomous factors of cellular origin (anti-CD31cells versus anti-alpha smooth muscle actin cells), tissue origin (aorta versus DA), and time (day 18 versus day 21), as well as all two-way interactions between these factors. In this model, we performed tests for differential gene expression between the two cellular origins. Third, differences between DA and aorta in expression profiles over embryonic age were investigated by testing for the presence of a two-way interaction of tissue origin and time, using the same model in LIMMA. Models like

LIMMA assume that all the samples have been randomly and independently collected. This was the case in our separate experiments. In all our LIMMA analyses, we tested for the differences corresponding to each factor both globally over all samples to obtain increased power, but also separately for subsets of the samples corresponding to specific values of the other factors, to be able to detect whether effects are consistent for different values of the other factors. We corrected each of the analyses separately for multiple testing, using the false discovery rate (FDR) and corrected all p-values for multiplicity. Multiplicity-adjusted p-values below 0.05 were considered significant. The complete set of data is available in the GEO database.

Immunohistochemistry

Immunohistochemistry was used as a qualitative technique to confirm protein expression in tissue sections. By rigorously standardizing the methodology (ensemble staining of sections using the same aliquots, timing, temperature we were able to validate differences in staining intensity between sections, albeit not the degree of difference.

The experiments were performed on sections from 4% paraformaldehyde fixed tissue routinely processed for paraffin immunohistochemical investigation. Primary Antibodies against RGS5 (Santa Cruz Biotechnology, CA, USA, SC-28492) 1:200-1:400, PCP4 (Santa Cruz Biotechnology, CA, USA, SC—98549) 1:200-1:400, DLX-1 (Aviva Systems Biology, San Diego, CA, ARP32866) 1:1000, TCFAP2beta (Abcam, Cambridge, MA, USA ab181113) 1:200 were used. The primary antibodies were dissolved in phosphate buffered saline (PBS)-Tween-20 with 1% Bovine Serum Albumin (BSA, Sigma Aldrich, USA). Slides were incubated overnight. Between subsequent incubation steps all slides were rinsed in PBS 2x and PBS Tween-20 1x. The slides were incubated with secondary antibodies for 45 min: for RGS 5 with Biotinylated Horse anti Goat (H+L) (Vector laboratories, USA BA-9500), 1:200 and 1:66 normal horse serum (Brunschwig Chemie, Switzerland, S-2000), for PCP4 and DLX1 with Biotinylated Goat anti Rabbit (H+L) (Vector laboratories, USA, BA-1000), 1:200, for TCFAP2B with Biotinylated Horse anti Mouse (H+L) (Vector laboratories, USA, BA-2000), 1:200 and 1:66 normal horse serum (Brunschwig Chemie, Switzerland, S-2000). The slides were incubated with ABC-reagent (Vector laboratories, USA, PK-6100) for 45 min. For visualization the slides were incubated with 400µg/ml 3-3'di-aminobenzidin tetrahydrochloride (DAB, Sigma-Aldrich Chemie, USA, D5637) dissolved in Tris-maleate buffer pH 7.6 to

which 20 µl H₂O₂ was added for 8 minutes. Counterstaining was performed with 0,1% haematoxylin (Merck, Darmstadt, Germany) for 5 sec., followed by rinsing with tap water for 10 min. All slides were dehydrated and mounted with Entellan (Merck, Darmstadt, Germany)

Results

First we explored the microarray data of all 48 samples by the unsupervised spectral map analysis (SPM). This analysis revealed evident differences between the 24 samples from day 18 and the 24 samples from day 21 (Figure 5.1A). The SPM biplot illustrates the results of this unsupervised analysis. The first component of the principal component analysis explained 29% of the total variance in the dataset of the 11484 genes that were reliably detected, and separated the samples from day 18 from those from day 21. The second component explained 8% of the total variance and separated ECs from SMCs. Next we analyzed the dataset with a supervised test. With the linear model for microarray data analysis (LIMMA) (*P*-values <0,05 adjusted for multiple testing using the False Discovery Rate (FDR)) we compared all samples by gestational age (ie. day 18 versus day 21), by cell-type (i.e. ECs versus SMCs) and by vascular origin (i.e. DA versus aorta). LIMMA confirmed the strong age-dependency of the gene expression detected by the unsupervised test. In the gene-by-gene analysis 3239 genes were differentially expressed between the 24 samples from day 18 and the 24 samples from day 21. Compared to the other factors as cell-type and vascular origin gestational age had the largest effect on the distribution of the data.

Comparing ECs and SMCs we found differential expression of 858 genes in the gene-by-gene analysis. Examples of these differences were found among well-known endothelial-specific genes such as tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (*Tie1*), endothelial tyrosine kinase, (*Tek*), platelet/endothelial cell adhesion molecule 1 (*Pecam1*) and von Willebrand factor (*vWf*). The latter was found to be the highest upregulated gene (5.1 logfold) in ECs (adjusted *P*-value 1.8e-06) in the LIMMA analysis. The upregulation of *vWf* was also validated by rtqPCR confirming that the LCM procedure was successful for the enrichment of EC.

The comparison between the DA and the aorta revealed 16 genes that were differentially expressed between both vessels (Figure 5.1B). Five out of these 16 genes were significantly upregulated in the aorta. Among these genes *Pcp4*

showed the highest (3.2 log-fold, adj. $P < 0.001$) and the most significant upregulation. Out of the nine ductus-dominant genes *Rgs5* (3.1 log-fold, adj. $p = 0.045$) had the highest expression level. Table 5.1 lists the 16 genes together with their functional categories. Apart from the two genes involved in intracellular signaling *Pcp4* and *Rgs5* none of the other genes had earlier been detected in connection with DA remodeling.

Table 5.1 The 16 most significant differentially expressed genes between DA and aorta. Functional categories, probe set identification numbers (ID Affymetrix) of the GeneChip Rat Genome 230 2.0. Array, gene name according to UniGene, gene symbols, logfold changes (LogFC) between DA en aorta and the adjusted p-value are shown.

Probe set ID Affymetrix	Gene Name (UniGene)	Symbol	LogFC DA:Ao	P-value
<u>Structural System</u>				
<i>Membrane protein</i>				
	Lipidphosphate phosphatase-related protein type 4 (LPPR4)	<i>Prg1</i>	-0.753	<0.050
<i>DNA, RNA protein processing</i>				
294864_at	Chaperonin containing TCP1	<i>Cct5</i>	0.509	<0.045
295284_at	RNA binding motif protein 8A	<i>Rbm8_predicted</i>	0.388	<0.050
<i>Cytoskeleton and matrix components</i>				
316758_at	Laminin alpha 1	<i>Lama1</i>	1.42	<0.050
304021_at	Collagen type 8 alpha	<i>Col8a1_predicted</i>	-2.081	<0.001
1383708_at	Integrin, beta-like protein 1 (Itgbl1)	<i>LOC498564</i>	-2.216	<0.050
<i>Intracellular Signaling</i>				
<i>Transcription factors</i>				
296500_at	Distal-less homeobox1	<i>Dlx1</i>	1.741	<0.050
<i>Intracellular signaling</i>				
25510_at	Purkinje cell protein 4 (also known as neuron specific protein PEP-19)	<i>Pcp4</i>	-3.185	<0.001
54294_at	Regulator of G-protein signaling 5	<i>Rgs5</i>	3.075	<0.050
1378988_at	mab-21-like 2	<i>LOC680102</i>	0.947	<0.050
<u>Tissue Modeling</u>				
<i>Regulators of cell cycle and apoptosis</i>				
25203_at	Cyclin B1	<i>Ccnb1</i>	0.587	<0.050
296883_at	Replication protein A3	<i>Rpa3_predicted</i>	0.642	<0.045
85246_at	Growth arrest specific protein 7	<i>Gas7</i>	-0.676	<0.021
<u>Metabolic system</u>				
293652_at	NADH dehydrogenase (ubiquinone) Fe-S protein 8	<i>Ndufs8_predicted</i>	0.573	<0.045
<u>Immune response</u>				
29199_at	Hemochromatosis gene	<i>Hfe</i>	0.426	<0.050
<u>Others</u>				
1372066_at	Family with sequence similarity 103, member A1	<i>RGD1310022</i>	0.709	<0.045

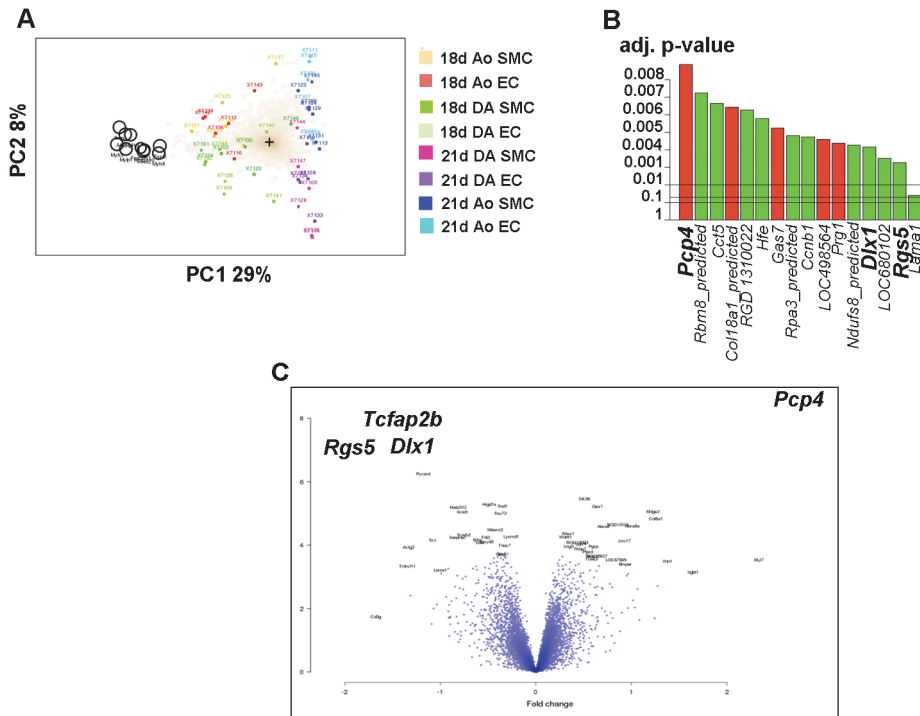


Figure 5.1 **A:** Spectral map biplot. The first principal components (PC) of the weighted spectral map analysis (SPM) of normalized microarray data are plotted. Colored squares with numbers depict different samples, while circles depict genes. Distances between the squares are a measure for similarity between samples. Genes that do not contribute to the differences between the samples are indicated as dots in the cloud around the centroid (represented by the cross). The ten most significantly contributing genes to the differences between samples are positioned in the largest distance from the centroid and annotated by their gene symbol. The first PC represented on the x-axis explains 29% of the variance in the dataset and discriminates samples from day 18 ($n = 24$) from those of day 21 ($n=24$). The second PC represented on the Y-axis explains 8% of the variance and discriminates between ECs and SMCs. Among the genes that contribute to the differences are the muscle proteins myosin heavy chain 3 (*Myh3*), myosin light chain, phosphorylatable (*Mylpf*), myosin heavy chain 8 (*Myh8*) and tenascin N (*Tnn2*). **B:** Histogram showing the 16 most significant differentially expressed genes between DA and aorta. The annotation of the genes is on the x-axis. The adjusted P-values are shown on the y-axis. Red bars represent genes that are upregulated in the aorta. Green bars represent genes that are upregulated in the DA. **C:** Volcano plot. The volcano plot constructed with LIMMA analysis summarizes the fold changes between the two types of samples (i.e. DA versus aorta) and the log₁₀ transformed p-values. The negative log₁₀ transformed p-values (y-axis) are plotted against the log ratios between the samples (log₂ fold change). For our study we selected 4 genes. The position of these genes in the upper left (*Rgs5*, *Tcfap2b*, *Dlx1*) or upper right (*Pcp4*) is the result of a high ratio of differential expression in combination with a high significance level. This suggests biological relevance of these genes.

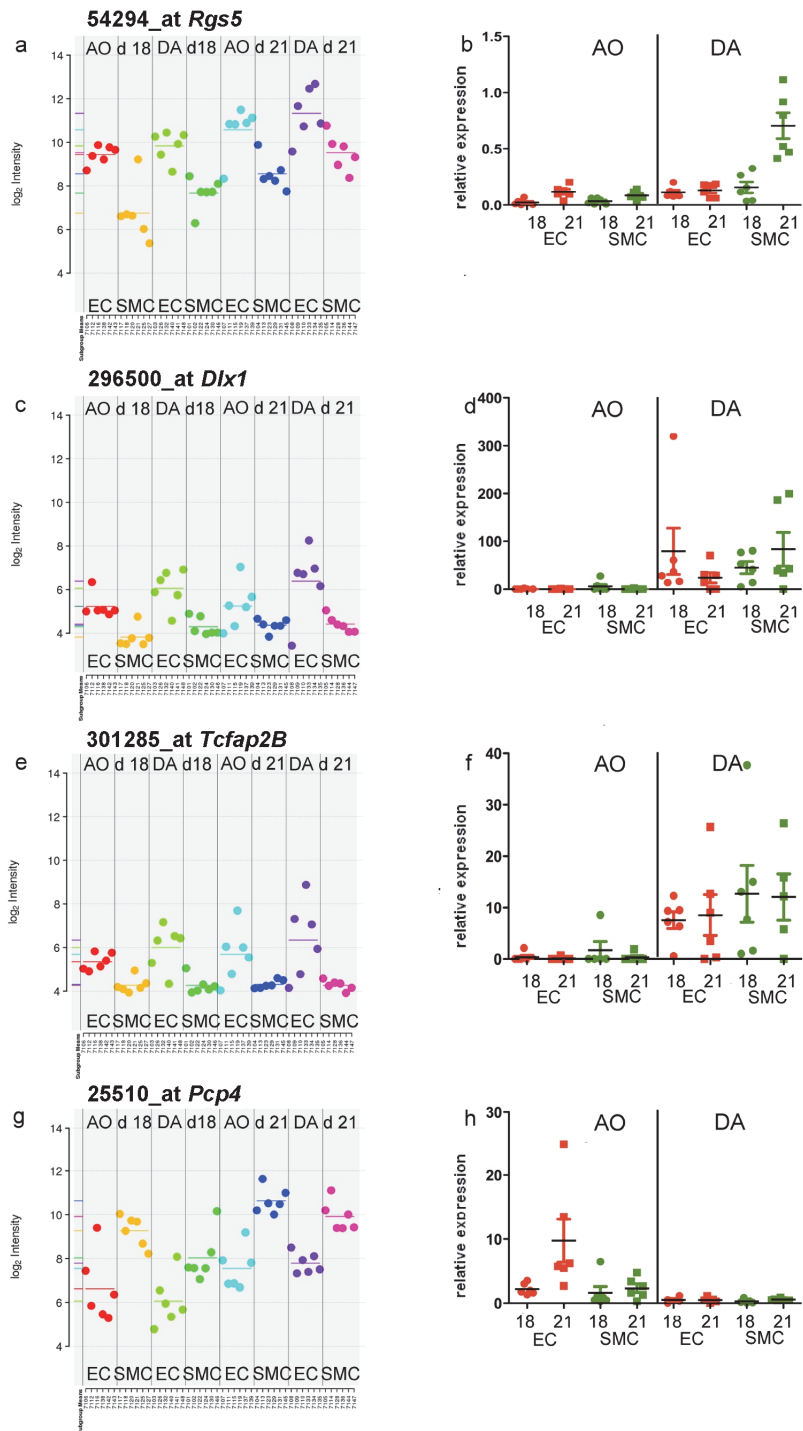


Figure 5.2 Gene expression of *Rgs5*, *Dlx1*, *Tfap2B*, and *Pcp4* by microarray and quantitative RT-PCR. Expression levels are expressed as fluorescent signal intensity measured on the array after normalization (a,c,e,g). Expression levels are shown for individual samples. The colors correspond to the colors used in Figure 5.1. The colored horizontal lines represent the means. The graphs on the right side show the relative quantification of mRNA of (b) *Rgs5*, (d) *Dlx1*, (f) *Tfap2B*, (h) *Pcp4* by quantitative RT-PCR (qRT-PCR) normalized against *Ppib*. The red symbols represent ECs, the green SMCs. All individual samples are presented. The horizontal lines indicate the means. The same mRNA preparations were used for microarray and the qRT-PCR. (a,b) *Rgs5* was detected by microarray and qRT-PCR as a DA-dominant gene. The microarray revealed the highest *Rgs5* expression in the ECs of the DA at 21 days. (b) qRT-PCR detected the largest relative expression in the SMCs of the DA at 21 days. (c) *Dlx1* was identified as DA-dominant gene with high levels of expression in EC of the DA at day 18 and day 21 by the microarray. (d) Quantitative RT-PCR confirmed the dominance in all cell-types of the DA. In the aorta *Dlx1* was virtually absent. (e) *Tfap2B* expression levels by microarray were higher in EC than in SMC with the highest levels in the DA at day 18 and 21. (f) qRT-PCR demonstrated *Tfap2B* expression almost exclusively in the DA. There was no significant difference between the two time-points (18 versus 21 days) and cell-types (ECs versus SMCs) of the DA. (g,h) *Pcp4* was identified as aorta- dominant with both techniques. (g) By microarray higher expression levels of *Pcp4* were found in SMCs than in ECs. (h) By qRT-PCR the highest relative *Pcp4* expression was detected in the ECs at day 21. At day 18 the expression levels were comparable in ECs and SMCs of the aorta.

Volcano plots, displaying the magnitude of the gene expression ratio on the x-axis and the significance of the difference on the y-axis are provided (Figure 5.1C and supplemental Figure S5.2). The volcano plot (Figure 5.1C) shows the comparison between DA and aorta in all 48 samples. From this plot we selected four genes (*Rgs5*, *Dlx1* and *Tfap2B* in the DA and *Pcp4* in the aorta) for further study. These four genes were found in the left (*Pcp4*) or right (*Rgs5*, *Dlx1*, *Tfap2B*) upper part of the plot. The high ratio of differential expression combined with a high significance level is responsible for the position of the genes in the plot and suggests biological relevance of these genes. In volcano-plots showing the comparison between DA and aorta separately for each cell-type and gestational age (supplemental Figure S5.2) the four selected genes were found among the highest and most significantly differentially expressed genes.

Rgs5

Rgs5 was identified as a DA-dominant gene by the microarray. LIMMA revealed a significant up-regulation of *Rgs5* in all four cell-types of the DA at both time-points (Figure 5.2a) showing higher expression levels at day 21 as compared to day 18. In order to confirm the findings we performed rtqPCR analyses (Figure 5.2b). Using validated fluorogenic TaqMan® gene expression array normalized against the *Ppib* we confirmed the upregulation of *Rgs5* in the DA in comparison to the aorta. The highest relative expression of *Rgs5* was found in the SMCs of the DA at day 21. At day 18 *RGS5* was significantly lower expressed in the SMC of the DA confirming

also the strong age-dependency of this gene in the DA. ECs of the DA expressed comparably low levels of *Rgs5* at day 18 and 21. In the aorta *Rgs5* was below the detection level in both cell-types at day 18 and remained at a very low level at day 21.

Dlx1

Dlx1 was detected as a DA-dominant gene in the microarray. Globally *Dlx1* exhibited a 1.75 log-fold upregulation in the cells of the DA when compared to the cells from the aorta (Figure 5.1b). Expression levels of DLX1 were higher in all samples of ECs than in those of SMCs (Figure 5.2c). The DA-dominance was significant in ECs and SMCs at day 18 and in SMC at day 21. In ECs at day 21 the differential expression of *Dlx1* did not reach statistical significance. RtpPCR analysis normalized against *Ppib* confirmed the DA-dominant *Dlx1* expression (Figure 5.2d). The clear expression difference was present in all cell-types. *Dlx1* was virtually absent in aortic ECs of both gestational ages and in aortic SMCs of day 21 (0.3-0.4 fold-difference). In the DA at day 18 and day 21 both ECs and SMCs had a high magnitude of *Dlx1* expression (ranging between 24 and 84 fold-difference). An increase of the expression levels with gestational age was present in the SMCs but not detected in ECs.

Tfap2B

Tfap2B was selected because of its differential expression between DA and aorta shown in the volcano-plot (Figure 5.1C). An upregulation of *Tfap2B* was documented in both cell-types of the DA at both time-points (Figure 5.2e) showing higher expression levels at day 21 as compared to day 18. Using validated fluorogenic TaqMan® gene expression array normalized against the *Ppib* we confirmed the ductus-specific expression in ECs en SMCs at day 18 and day 21 (Figure 5.2f). Differences between the expression levels in the SMC and the EC of the DA were not significant. In cells from the aorta *Tfap2B* expression was below or just slightly above detection level.

Pcp4

Pcp4 was recognized as an aorta-dominant gene with the highest and most significant level of differential expression between aorta and DA by LIMMA in ECs and SMCs at both time-points (Figure 5.2g). Furthermore, the expression level of

Pcp4 was higher in all samples from day 21 as compared to day 18. LIMMA analysis also revealed the significant up-regulation in the aorta (log-fold 3.2, adj. $P < 0.001$) globally and also separately for gestational age and cell-type. Because of its high and significant up-regulation in the aorta, we selected *Pcp4* for confirmatory rtqPCR. The validated fluorogenic TaqMan® gene expression array normalized against the *Ppib* gene also showed an up-regulation of this gene in the aorta compared to the DA (Figure 5.2h). The highest and most significant up-regulation (10 fold-difference) was found in the ECs of the aorta at day 21 while the magnitude of expression was only 2 fold increased in aortic ECs at day 18. SMCs of the aorta did not show an increase of *Pcp4* expression with gestational age. In all samples of DA cells relative *Pcp4* expression was detected at very low levels (0.3-0.7 fold difference) confirming the microarray data.

Immunohistochemistry

The expression of RGS5, PCP4, and DLX1 protein was visualized by immunohistochemistry in tissue samples of the DA and aorta (Figure 5.3). As the highest levels of mRNA expression of these genes were observed at day 21 we used this stage for the analysis of protein expression. Immunohistochemical staining revealed expression of RGS5, PCP4, and DLX1 in all samples. Although immunohistochemistry is an essentially qualitative technique, we could confirm quantitative differences. The differential protein expression in DA and aorta confirmed the results of the microarray and rtqPCR. RGS5 was expressed in the cytoplasm of EC and SMC. The highest intensity of staining was detected in SMC of the DA localized in the subendothelial part of the intima (Figure 5.3a). ECs and SMCs in other parts of the DA wall were also more intensely stained than the aortic ECs and SMCs in the descending aorta (Figure 5.3b). DLX1 staining resulted in cytoplasmatic positivity in most of DA and aorta (Figure 5.3c, d). The highest intensity of DLX1 staining was found in the EC of the DA and as a whole the DA (Figure 5.3c) was more intensely stained than the descending aorta (Figure 5.3d). The expression of TFAP2B protein was not identified by immunohistochemistry in the DA or aorta by absence of available selective antibodies. Sections stained against PCP4 exhibited a strong nuclear positivity and a weaker cytoplasmatic staining (Figure 5.3e, f). The strong nuclear staining was most prominent in the EC of the aorta (Figure 5.3f).

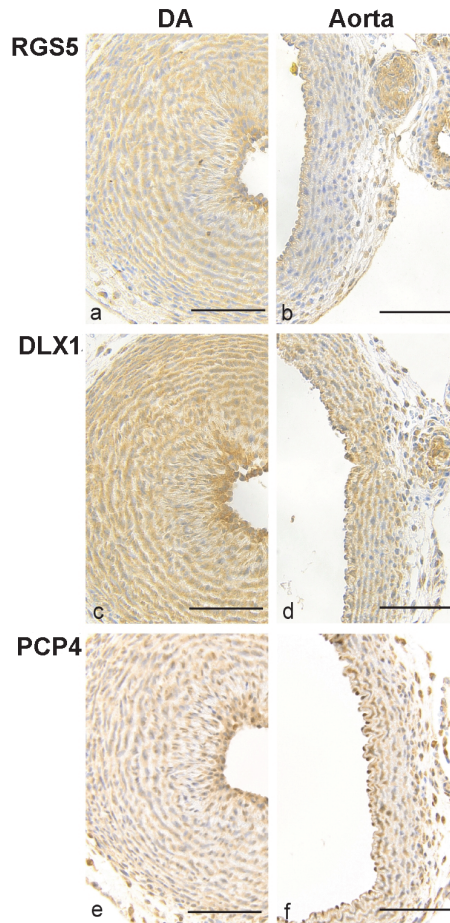


Figure 5.3 Protein expression of RGS5, DLX1 and PCP4 by immunohistochemistry. Photomicrographs of representative transverse sections show (a,b) RGS5 (c,d) DLX1 and (e,f) PCP4 expression in DA and aorta. The DA (a) shows a more intense cytoplasmatic staining against RGS5 in ECs and SMCs than the aorta (b). This is most clearly seen in the EC and the subendothelial layer of SMCs which were also studied in the microarray experiment. Dlx1 is predominantly expressed in the DA (c) where EC and the innermost layers of SMC show the most intense staining. The EC in the aorta (d) are almost negative for DLX1. PCP4 is predominantly expressed in the aorta (f). The staining is most intense in the nuclei of the EC of the aorta. In comparison with the aorta (f) the DA (e) shows less stained nuclei and cytoplasm of ECs and SMCs. Scale bars: 100 μ m.

Discussion

DA maturation and closure is regulated by the interaction of many genes in different cell-types. We performed a genome-wide microarray analysis searching

for genes that are differentially expressed between DA and aorta of fetal rats at day 18 and day 21. Laser-capture microdissection was used to isolate ECs and SMCs as an efficient and precise method for the sampling of single cells or subgroups of cells in heterogeneous tissues.²³ This selective approach minimizes the noise of the microarray introduced by the variation in tissue composition.²⁴ We selectively amplified mRNA from ECs and SMCs from DA and aorta and intentionally did not pool the various samples. Analysis of the microarray data revealed for the most part DA- and aorta- dominant genes that were not detected by former studies^{16,17} in which microarrays of the complete wall of the aorta were performed. Among 16 differentially expressed genes between DA and aorta detected by LIMMA, this study for the first time identified *Rgs5* and *Dlx1* as DA-dominant molecular markers in the fetus. Together with *Pcp4*, the most significant aorta-dominant gene in our analysis, and *Tfap2B* these genes were studied more extensively with other techniques. In all analyses a significant time-dependency for *Rgs5*, *Dlx1* and *Pcp4* was observed. This strengthens the initial observation that gestational age had the largest influence on the differential gene expression between all samples.

The small overlap in detected genes between the current study and former studies is not surprising as differences are unavoidable when comparing transcriptional profiles by the microarray technique as Coceani et al.¹⁸ emphasized commenting on the large variance between two former studies.^{16,17} Therefore it was suggested that any such comparison should consider cohorts of genes for distinct functions rather than single genes.¹⁸ Cell populations were rigorously separated in the current study enabling us to discuss single genes that were most significantly differentially expressed between ECs and SMCs in DA and aorta in a selective microarray study.

Rgs5 is a DA-dominant gene in ECs and SMCs at the end of gestation. Furthermore we document the maturation-state dependent upregulation of the *Rgs5* expression from day 18 to day 21 in the DA. *Rgs5* is coding for a protein regulating the function of vasoactive G-protein coupled receptors (GPCRs).²⁵ Many GPCRs are active during fetal maturation and postnatal closure of the DA such as EP2, 3, 4²⁶, angiotensin¹⁶ and ET-1 receptors.²⁷ *Rgs5* is the most highly and differently expressed Rgs-R4 subfamily member in arterial smooth muscle, suggesting that *Rgs5* is a candidate for regulating arterial contractility,²⁸ probably under control of platelet-derived growth factor (PDGF) that represses *RGS5*.²⁵ *Rgs5* is downregulated in pericytes and vascular smooth muscle cells of *Pdgfr* null mice.²⁹ The maturation-dependent upregulation of this DA-dominant-gene in the newborn was not categorized in the fetal cohort.¹⁶ Recently it was shown, that the

homogeneous *RGS5* expression in the aorta of neonatal mice changed into a mosaic pattern in adult animals.³⁰ The lowest expression levels were found in the ascending aorta and carotid arteries. Interestingly a localized high *Rgs5* expression was documented at the site of the former DA insertion³⁰ suggesting that an origin-specific epigenetic program resulting in differential *RGS5* promoter methylation is involved in the regulation of *Rgs5* expression. A likely explanation for differences in *Rgs5* expression levels between DA and descending aorta relates to the origin of the SMCs as we selected cells derived from different embryonic sources. The DA SMCs derive from neural crest cells populating the 6th pharyngeal arch, while the SMCs from the descending aorta are derived from the adjacent somites. We did not study the role of epigenetic factors causing differential *Rgs5* promoter methylation as shown in mice.³⁰

Microarray analysis also revealed *Dlx1* as a DA-dominant gene. It discriminates the DA from the aorta at day 18 and day 21 and is highly enriched in both ECs and SMCs of the DA as documented by quantitative rtPCR. This is a novel finding, Costa et al.¹⁶ and Jin et al.¹⁷ did not identify DA dominance of this gene. *Dlx1* is part of a subfamily of vertebrate homeobox-containing genes that are structurally similar to the *Drosophila* distal-less gene. Six members in this family have been identified in three bigene clusters in mammals (*Dlx1*, *Dlx2*, *Dlx3*, *Dlx5*, *Dlx6*, and *Dlx7*). *Dlx1* plays a role in the development of pharyngeal arch elements including jaws and teeth, and during neurogenesis in the forebrain.³¹ *Dlx* genes have been detected in several cancer cell lines³² where deregulation of *Dlx* genes was related with tumor progression.³² *Dlx1* and 2 are expressed in the embryonic caudal pharyngeal arch complex including the sixth, in which the ductus arteriosus develops.³³ This is also the case for *Msx* and *Prx* genes that interact with *Drx*.³⁴ Interestingly, DA-specific expression of *Prx2* in developing chicken DA contrasts with *Prx1* in the adjoining vessel parts³⁵ whereas the *Prx1/2* double null results in DA malformations.³⁶ The interactions between *Msx*, *Prx2* and *Dlx1* genes in DA development have yet to be elucidated. In addition to its role in embryogenesis *DLX1* has been reported in hematopoietic cells³⁷ as a regulator of multiple signals from TGF-beta superfamily members.³⁸ *DLX2* the counterpart of *DLX1* in the bigene cluster is expressed depending on reactive oxygen species (ROS) in response to glucose deprivation.³⁹ Based on our data and the information from other biological contexts we propose that *Dlx1* is involved in late gestational vascular remodeling of the DA.

The differential expression of *Tfap2B* shown in volcano-plots let us select this gene for further validation by rtqPCR. We confirmed its DA-dominance as we reported

high levels of *Tfap2B* in both cell-types of the DA at day 18 and 21 with a predominance of this gene in the SMC. The AP-2B transcription factor is involved in the regulation of SMC development in the DA in a transcriptional network with ET-1 and HIF2a.¹⁴ *Tfap2B* was shown to be necessary for proper ET-1 signaling in the DA.¹⁴ Therefore it has been suggested that patent DA in *Tfap2B* deleted animals could partly be attributed to the lack of ET-1 signaling. Recently Zhao et al¹⁵ described the phenotype of *Tfap2B* knockout mice and documented *Bmp2* and *Bmp4* as downstream targets of *Tfap2B*. These mice are characterized by a PDA, postaxial accessory digits and enhanced apoptotic cell death of renal epithelial cells.^{15, 40} Interestingly, *DLX1* directs the expression of *BMP4* in various biological contexts.³⁸ The DA-dominant expression of both transcriptionfactor genes *Dlx1* and *Tfap2 B* suggests a vessel-specific regulation of *Bmp4* signaling during fetal DA remodeling.

Pcp4 was identified as the most significant aorta-dominant gene. From day 18 to day 21 a increase in *Pcp4* levels was observed. The late gestational up-regulation of *Pcp4* provides evidence for a biological function of *Pcp4* in the aorta during this period. However, the role of *Pcp4* and its protein has not been characterized sufficiently in the vascular system. *Pcp4* has mainly been studied in neuronal tissues where it regulates calcium/calmodulin interactions⁴¹ and inhibits calcium induced neuronal cell-death by apoptosis.⁴² By analogy to the neuronal tissues we postulate that up-regulation of *Pcp4* in the aorta might protect the aorta around birth against apoptosis. Accordingly, the relatively lower expression of *Pcp4* in the DA might render the EC and SMC in the DA more susceptible for apoptosis, which plays an important role in postnatal DA closure.⁵ Interestingly, a study on the parturition of mice linked another apoptosis driven process – the softening of the cervix at the end of gestation with a relative decline in *Pcp4* expression in cervix tissue.⁴³ This supports a role for *Pcp4* as anti-apoptotic regulator. The results of Jin et al¹⁷ are in concordance with our results and categorized *Pcp4* as aorta-dominant gene in the fetus. Costa et al¹⁶ detected a postnatal up-regulation of the same gene in the DA in comparison to the aorta. Additionally a cluster of other calcium-linked genes was identified in the neonatal DA, while its postnatal up-regulation was related to a modified calcium homeostasis and increased drive of the DA to contraction.¹⁶ Our analysis of mRNA from ECs and SMCs from fetal DA did neither identify *Pcp4* nor one of the other members of the cluster of calcium-linked genes described in the neonatal DA.

In conclusion, our study confirms a DA-specific transcriptional profile in ECs and SMCs and thereby offers a basis to unravel the molecular regulation of fetal DA

maturation. For the first time we recognized *Rgs5* and *Dlx1* as DA-dominant genes. These genes represent novel molecular targets for the regulation of fetal DA maturation and postnatal DA closure.

Non-standard abbreviations and acronyms

ABC-reagent	avidin-biotin complex reagent
anti-rat-CD 31	antibody against CD 31 also known as PECAM-1 (platelet endothelial cell adhesion molecule)
aRNA	antisense RNA), which is mRNA derived
BMP	bone morphogenetic protein
cDNA	complementary DNA synthesized from mRNA
DA	ductus arteriosus
DLX1-7	distal-less homeobox1-7, homeobox transcription factor similar to the drosophila distal-less 1-7
<i>DRX</i>	retinal homeobox gene
ECs	endothelial cells
ET-1	Endothelin-1
FARMS	factor analysis for robust microarray summarization
FDR	false discovery rate
TaqMan	real-time PCR assay using hydrolysis probes relying on the activity of taq polymerase
GHR	growth hormone receptor
GPCRs	G protein-coupled receptors
Kv-channel	voltage-gated potassium channel
LCM	laser-capture microdissection
LIMMA	linear models of microarray
LMNA	lamin A/C
MSX	muscle segment homeobox, homeobox transcription factor similar to the Drosophila muscle segment homeobox
MYH3	myosin heavy chain 3
MYH8	myosin heavy chain 8
MYLPF	myosin light chain, phosphorylatable
PCP4	purkinje cell protein 4
PPIB	peptidylprolyl isomerase B (cyclophilin B)
PDGF	platelet-derived growth factor
PECAM1	platelet endothelial cell adhesion molecule
PRX1/2	paired-related homeobox transcription factor 1/2
qPCR	quantitative polymerase chain reaction
RGS5	regulator of G-protein signaling 5
RGS-R4	regulator of G-protein signaling 5-receptor 4

RLT-buffer	lysis buffer supplied with RNeasy kit®
ROCK	Rho-associated, coiled-coil containing protein kinase 1
ROS	reactive oxygen species
RT	reverse transcriptase
SMCs	(vascular) smooth muscle cells
SPM	spectral map analysis
TEK	tyrosine kinase, endothelial
TFAP2B	transcription factor AP-2 beta (activating enhancer binding protein 2 beta)
TGF-beta	transforming growth factor beta
TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains1
TNN2	tenascin N
VWF	von Willebrand factor

Human: Gene symbols generally are italicised, with all letters in uppercase.

Protein designations are the same as the gene symbol, but are not italicised; all letters are in uppercase. Genomics 2002;79:464-470

Mouse and rat: Gene symbols generally are italicised, with only the first letter in uppercase and the remaining letters in lowercase. Italics are not required on web pages. Protein designations are the same as the gene symbol, but are not italicised and all are upper case. According to the 2011 Guidelines of the International Committee on Standardized Genetic Nomenclature for Mice and Rat Genome and Nomenclature Committee

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Appendix

Supplemental figures and tables

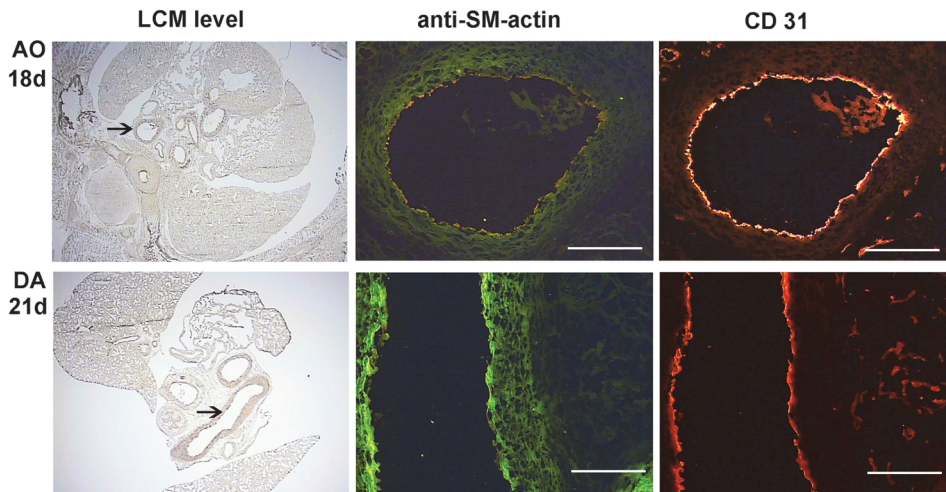


Figure S5.1 Sections used for laser-capture microdissection after immunohistochemical double-staining. The arrows represent the sites of laser dissection in the aorta (AO) of a fetal rat at 18 days (18d) gestation and the ductus (DA) of a fetal rat at 21 days (21d) gestation. Smooth muscle cells are labelled with anti-SM-actin in green (b,e) and endothelial cells are labelled with CD31 in red (c,f). Bars: 100 μ m

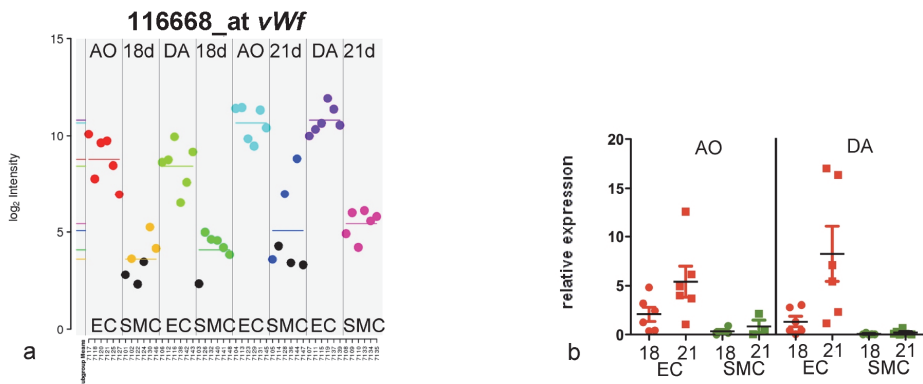


Figure S5.2 Gene expression of von Willebrand Factor (*vWf*) by microarray and RT-PCR. Expression levels are expressed as fluorescent signal intensity measured on the array after normalization (a). Expression levels are shown for individual samples. The colors correspond to the colors used in Figure 5.1. The colored horizontal lines represent the means. Black dots represent samples that are not reliably detected. Note the high expression level of *vWf* in all EC samples. The graph (b) shows the relative quantification of mRNA of *vWf* by quantitative RT-PCR (qRT-PCR) normalized against PGK1. The red symbols represent EC, the green SMC. All individual samples are presented. The horizontal lines indicate the means. The same mRNA preparations were used for microarray and the qRT-PCR. The PCR results confirm the microarray results by showing a high relative expression of *vWf* in EC while its mRNA is just above detection level in SMC of the aorta.

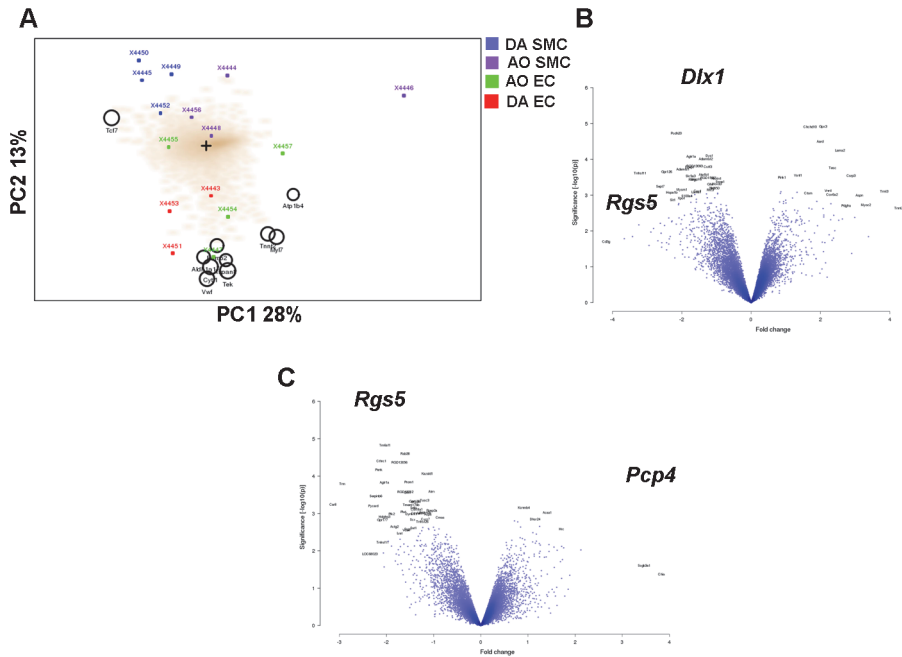


Figure S5.3 A: Spectral map biplot of samples of day 21 in the independent pilot experiment. The first principal components (PC) of the weighted spectral map analysis (SPM) of normalized microarray data are plotted. Colored squares with numbers depict different samples, while circles depict genes. Distances between the squares are a measure for similarity between samples. Genes that do not contribute to the differences between the samples are indicated as dots in the cloud around the centroid (represented by the cross). The ten most significantly contributing genes to the differences between samples are positioned in the largest distance from the centroid and annotated by their gene symbol. The first PC represented on the x-axis explains 28% of the variance of the dataset and discriminates between DA and aorta. The second PC represents 13% of the variance and discriminates between ECs and SMCs. Note *vWf* among the genes that contribute to this difference. **B:** Volcano plot of the pilot experiment. Differential expression between samples of SMC of the DA and the aorta at day 21. The volcano plot constructed with LIMMA analysis summarizes the fold changes between the two types of samples (i.e. DA versus aorta) and the log 10 transformed p-values. The negative log 10 transformed p-values (y-axis) are plotted against the log ratios between the samples ($\log_2$ fold change). From the four selected genes of our study *Dlx1* and *Rgs5* are found in the upper left of the plot. **C:** Volcano plot of the pilot experiment. Differential expression between EC of the DA and aorta. *Dlx1* (upper left) and *Pcp4* (upper right) both show a high ratio of differential expression in combination with a high significance level. This suggests biological relevance of these genes

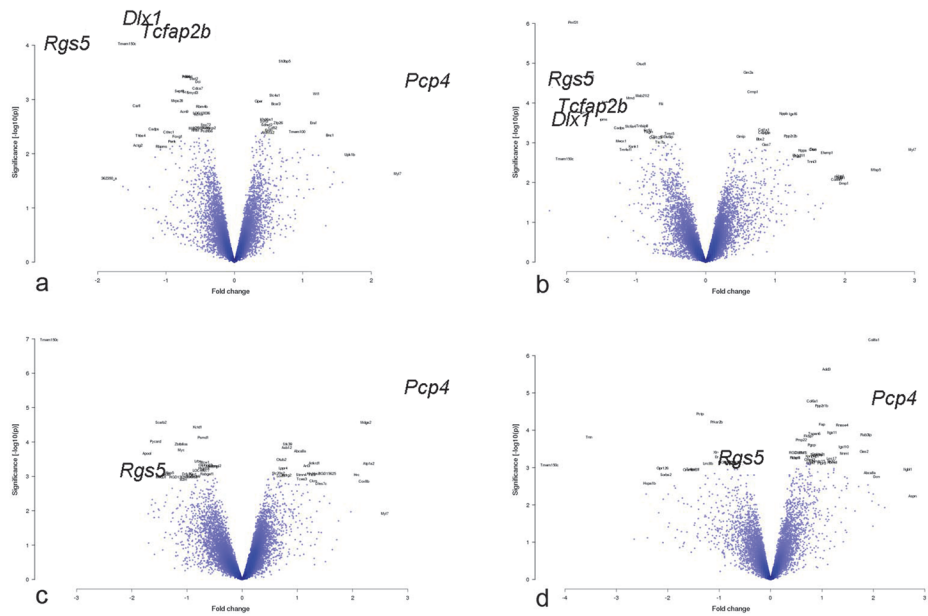


Figure S5.4 Volcano plots constructed with LIMMA analysis plotted separately for (a) EC at day 18, (b) SMC at day 18, (c) EC at day 21 and (d) SMC at day 21. The four selected genes *Rgs5*, *Dlx1*, *Tcfap2B* and *Pcp4* are all identified with high ratios of differential expression in combination with a high significance level in (a) and partly in (b,c,d).

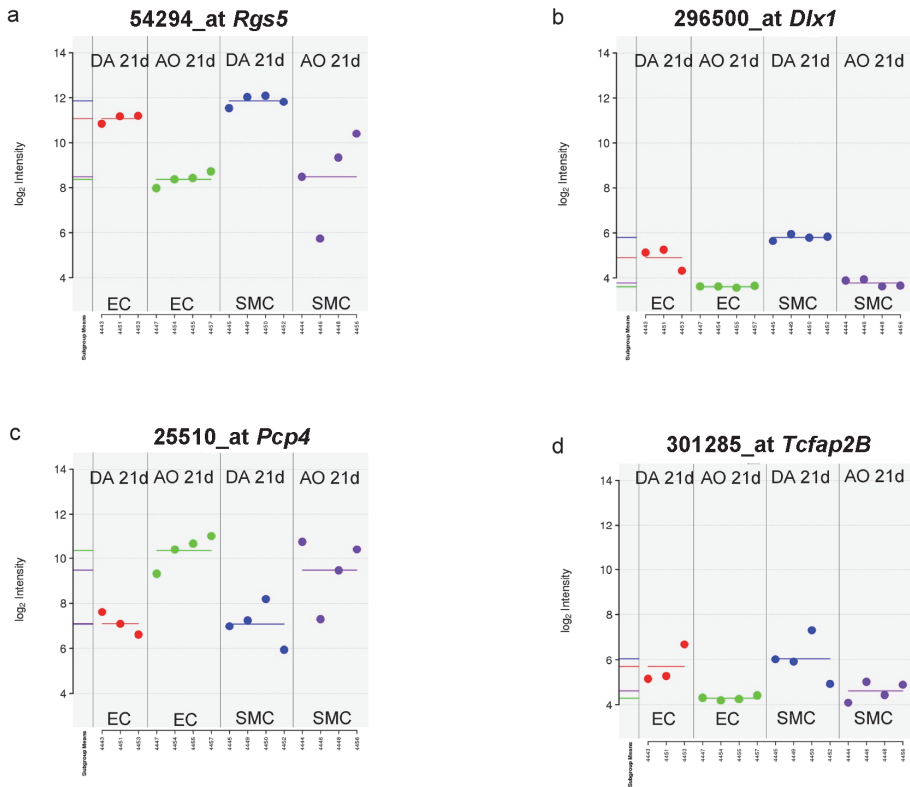


Figure S5.5 Gene expression of *Rgs5* (a), *Dlx1* (b), *Pcp4* (c), and *Tcfap2B* (d) at day 21 by microarray in the independent pilot experiment using 4 DA and 4 aorta samples. One DA sample was excluded because of mRNA degradation. Expression levels are expressed as fluorescent signal intensity measured on the array after normalization. Expression levels are shown for individual samples. The colors correspond to the colors used in Figure 5.3. The colored horizontal lines represent the means. This experiment identifies *Rgs5* as a DA-dominant gene with the highest expression in SMC of the DA. *DLX1*, also DA-dominant, shows the highest expression in the SMC of the DA. *Pcp4* is found to be aorta dominant. The highest expression levels were documented in EC of the aorta. *Tcfap2B* is predominantly found in the DA with a little higher expression level in SMC than in EC. All findings from the pilot experiment concerning predominant expression of *Rgs5*, *Dlx1*, *Pcp4*, and *Tcfap2B* are confirmed in the larger study by microarray and/or quantitative RT PCR.

Chip ID	Random no.	Sample	aRNA concentration ng/ml	aRNA yield ng	cRNA concentration ng/ml	cRNA yield mg	cRNA concentration mg/ml
7101	1	8399 E Aorta SMC 18d	24,69	493,8	2007,89	120,47	2,01
7102	2	8469 B Aorta SMC 18d	11,01	220,2	2347,47	93,90	2,35
7103	3	8469 B DA SMC 18d	13,44	268,8	1950,83	78,03	1,95
7104	4	8462 M Aorta EC 21d	21,74	434,8	1601,12	64,04	1,60
7105	5	8462 M Aorta SMC 21d	23,9	478	2558,39	102,34	2,56
7106	6	8469 F DA EC 18d	19,87	397,4	1989,22	79,57	1,99
7107	7	8412 I DA EC 21d	15,64	312,8	2462,99	98,52	2,46
7108	8	8412 I DA SMC 21d	15,09	301,8	2633,82	105,35	2,63
7109	9	8462 A DA SMC 21d	22,19	443,8	1677,63	67,11	1,68
7110	10	8462 C DA SMC 18d	20,47	409,4	1149,93	46,00	1,15
7111	11	8462 A DA EC 21d	26,47	529,4	1842,4	110,54	1,84
7112	12	8469 D DA EC 18d	18,07	361,4	1480,23	59,21	1,48
7113	13	8412 I Aorta EC 21d	24,9	498	802,44	32,10	0,80
7114	14	8412 I Aorta SMC 21d	39,51	790,2	756,09	30,24	0,76
7115	15	8462 M DA EC 21d	21,72	434,4	851,29	34,05	0,85
7116	16	8469 B DA EC 18d	13,94	278,8	2894,31	115,77	2,89
7117	17	8399 H Aorta EC 18d	25,61	512,2	2060,53	82,42	2,06
7118	18	8399 F Aorta EC 18d	23,97	479,4	2323,01	92,92	2,32
7119	19	8412 D DA EC 21d	21,7	434	1118,98	44,76	1,12
7120	20	8469 B Aorta EC 18d	35,78	715,6	2078,61	83,14	2,08
7121	21	8399 E Aorta EC 18d	15,19	303,8	1422,11	56,88	1,42
7122	22	8399 F Aorta SMC 18d	18,95	379	1376,75	55,07	1,38
7123	23	8462 C Aorta EC 21d	19,41	388,2	2273,48	90,94	2,27
7124	24	8469 D Aorta SMC 18d	9,62	192,4	2184,54	87,38	2,18
7125	25	8469 F Aorta EC 18d	32,07	641,4	1919,89	115,19	1,92
7126	26	8469 F DA SMC 18d	25,8	516	1370,44	54,82	1,37
7127	27	8469 D Aorta EC 18d	22,88	457,6	2054,34	82,17	2,05
7128	28	8412 A Aorta SMC 21d	21,71	434,2	578,04	23,12	0,58
7129	29	8412 A Aorta EC 21d	26,51	530,2	690,37	27,61	0,69
7130	30	8469 F Aorta SMC 18d	17,82	356,4	1998,24	79,93	2,00
7131	31	8412 D Aorta EC 21d	15,42	308,4	694,46	27,78	0,69
7132	32	8399 F DA SMC 18d	27,22	544,4	1912,01	76,48	1,91
7133	33	8412 D DA SMC 21d	19,43	388,6	769,08	30,76	0,77
7134	34	8462 M DA SMC 21d	12,37	247,4	1649,3	65,97	1,65
7135	35	8412 A DA SMC 21d	27,78	555,6	1688,72	67,55	1,69
7136	36	8412 D Aorta SMC 21d	11,91	238,2	733,54	29,34	0,73
7137	37	8412 A DA EC 21d	23,3	466	1181,48	47,26	1,18
7138	38	8399 F DA EC 18d	28,29	565,8	1977,44	79,10	1,98
7139	39	8462 C DA EC 21d	15,28	305,6	1607,9	64,32	1,61
7140	40	8399 H DA SMC 18d	23,61	472,2	1787,44	107,25	1,79
7141	41	8469 B DA SMC 18d	51,29	1025,8	1668,08	66,72	1,67
7142	42	8399 E DA EC 18d	27,55	551	1998,44	119,91	2,00
7143	43	8399 H DA EC 18d	39,05	781	2076,34	124,58	2,08
7144	44	8462 A Aorta SMC 21d	23	460	2112,28	84,49	2,11
7145	45	8462 A Aorta EC 21d	30,43	608,6	1278,07	51,12	1,28
7146	46	8399 H Aorta SMC 18d	20,26	405,2	1528,84	61,15	1,53
7147	47	8462 C Aorta SMC 21d	21,94	438,8	1654,4	66,18	1,65
7148	48	8399 E DA SMC 18d	20,8	416	2694,16	107,77	2,69

Table S5.1 RNA yields of amplified RNA (aRNA) and biotin-labeled antisense mRNA (cRNA). Chip ID = Chip identification number, Individual samples are described by a four-digit number followed by a capital letter, sample site, cell type and gestational age. Aorta = descending aorta, DA= ductus arteriosus, EC = endothelial cells, SMC = smooth muscle cells, 18d = 18 days, 21d = 21d.

Assay ID	Gene Symbol
Rn00564515_m1	Purkinje cell protein 4, Gene rCG52610 Celera Annotation
Rn01443930_m1	Purkinje cell protein 4, Gene rCG52610 Celera Annotation
Rn01511929_m1	transcription factor AP-2 beta, Gene rCG43555 Celera Annotation
Rn01492194_s1	Von Willebrand factor homolog, Gene rCG29743 Celera Annotation

Table S5.2 Showing a list of the pre-designed assays (Applied Biosystems) that were used for quantitative rt-PCR. As the validated Applied Biosystems assays for *Dlx1* will all detect sequences 5' upstream of the 3' sequence detected by the Affymetrix microarray probes. Therefore, primers/ probes designed against this 3' sequence were used. Forward 5'-AGG TCG CTT GAA AGT CAG AC-3'. Reverse 5'-ACA ACG ATT AGG TCC TGC AC-3'

Chapter 6

Aortic disease and recurrence of congenital heart disease in first and second-degree relatives of patients with PDA

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Abstract

Aims: Persistent ductus arteriosus (PDA) and familial thoracic aneurysm and dissection (TAAD) are genetically heterogeneous. Data on the prevalence of aortic disease including TAAD, the familial recurrence of PDA, and other congenital heart disease (CHD) provide essential information for the investigation of genetic factors of these diseases.

Methods and results: Retrospective patient-based study using a questionnaire assessing the prevalence of cardiovascular disease in family members of children with PDA. A thorough family history provided information on three generations. The data were analyzed by a binominal test. The prevalence in the general population was derived from a Dutch population-based study.

Over the last 30 years 320 patients older than 3 months with isolated PDA have been treated in our institution. Data on 175 patients and their 2336 family members were available. The prevalence of aortic disease was higher in the study group compared to the general Dutch population. Aortic disease was reported in $4/590 = 0.7\%$ of first grade relatives and $29/1339 = 2\%$ of second grade relatives. In second grade relatives the prevalence of TAAD was increased to 0.7% ($9/1339$) compared to 0.016% in the general population ($p < 0.001$). The recurrence rate of CHD was higher (2.7%) in first-degree relatives than in second-degree relatives (0.79%).

Conclusion: These data provide evidence for a genetic association of PDA and aortic disease. The prevalence of PDA and other CHD is increased in first grade relatives of the index patient, suggestive of common genetic factors in the determination of CHD and aortic disease.

Introduction

Isolated persistent ductus arteriosus (PDA) is a common minor congenital cardiac anomaly. The prevalence varies between about 0.8 and 10.2 per 1000 live births.^{1,2} Symptoms and therapeutic consequences of the persisting fetal connection between aorta and pulmonary artery vary with patient's age and the size of the PDA. The large variability of available data is explained by the difficulty to differentiate clinically between the delayed closing ductus arteriosus (DA) and the structurally abnormal PDA in young infants, mild or absent symptoms in patients with small PDA, and the increasing survival of premature babies. The present study focuses on the families of patients treated for an isolated PDA above the age of 3 months, in whom we considered PDA as a primary congenital malformation. Nowadays, it is possible to treat most of the affected patients by catheter techniques.³ Surgery, considered the golden standard for PDA treatment in the past, is restricted to patients who are not suitable for catheter interventional therapy. Clinical problems, caused by the left-to-right shunt between aorta and the pulmonary system, disappear after closure. Therefore, patients with isolated PDA are in general not followed after successful treatment has been performed and documented.⁴

Isolated PDA beyond the neonatal age is considered a sporadic and multifactorial disease. Examples for environmental factors include maternal exposure to the rubella virus⁵, and several drugs that increase the incidence of PDA.⁶⁻⁸ Bokenkamp et al.⁹ reviewed a correlation of PDA with some monogenic defects, such as Char syndrome and specific genetic defects including trisomy 21, 18 and CHARGE syndrome. In addition, populations with increased parental consanguinity have been reported with autosomal recessive inheritance with variable penetrance.¹⁰ Although familial cases of PDA are rare the recurrence rate of 5% in sibs¹¹ suggests a genetic component also in less consanguineous populations.

The association of thoracic aorta aneurysm and dissection (TAAD) with PDA has been documented in two families carrying mutations in genes encoding smooth muscle cell (SMC)- specific beta-myosin (*MYH11*)¹² or aortic smooth muscle alpha-actin (*ACTA2*)¹³. This suggests that unimpaired SMC contractility is a prerequisite for proper DA remodeling in addition to its critical role in maintaining the structural integrity of the aorta.¹⁴ Recently it has been documented that *ACTA2* mutations predispose patients to a variety of diffuse and diverse vascular diseases, including TAAD, premature coronary artery disease, ischemic strokes, and Moyamoya disease.¹⁵ Whether other cases of familial TAAD and PDA,¹⁶ can be attributed to

anomalies in contractile proteins is unclear. Considering the embryonic processes responsible for the formation of vessel walls in general and the DA in particular, all mutations impairing endothelial (EC) and SMC function as well as elastogenesis might be responsible for PDA and TAAD. DA closure and normal aortic distensibility both depend on proper interaction between EC and SMC and involve turnover and repair of extra cellular matrix substances.¹⁷

Different from earlier studies on the familial segregation of vascular diseases in families with TAAD mutations^{12,13} we investigated whether aortic pathology, specifically TAAD, occurs more frequently in families of children with isolated PDA of unknown origin. In addition to the determination of the prevalence of aortic disease in these families we aimed to study the familial recurrence of PDA and other types of CHD in first and second-degree relatives of patients with PDA.

Methods

Study population

For this study 322 patients treated for PDA after 3 months of age have been identified from the patient databases of the Centre of Anomalies of the Heart Amsterdam/Leiden (CAHAL) covering a time-span of 30 years.

The 322 patients, or in case of patients younger than 16 years, their parents or guardians, were sent a questionnaire appropriate to the patient's age and familial situation. (Supplement questionnaire) 175 questionnaires were returned resulting in a response rate 54%. These index patients provided information spanning 2336 family members with a mean of 14.3 family members per patient. Data from 634 first-degree relatives (parent, sibling or offspring) and 1390 second degree relatives (grandparent, uncle, aunt, nephew, niece, half-sibling) were included in our study.

Recorded data included age, sex, cardiovascular morbidity and mortality and general mortality in the families. All cardiovascular interventions and a positive family history for connective tissue disorders were noted. Aortic disease was concluded from answers that were suggestive for any type of aortic disease including aortic aneurysms, acute or chronic dissections, aortic root or valve disease. A more detailed description of the aortic disease including "ruptured aorta" or "dilated aorta" was necessary to conclude that a relative had TAAD. The recurrence rate of PDA and CHD in general was analyzed in the cohort of first-

degree relatives. The recurrence rate was defined as percentage of patients affected related to the number of index cases. The family history was part of a more extended study on the elastic properties of the aorta in PDA patients and had been approved by the medical ethical board of the LUMC.

Statistics

SPSS 19.0 was used for analysis of the family history data. The prevalence of TAA was based on data describing a prevalence of 0.0016 in the Dutch population.^{18,19} A Wald test was used to compare the proportions of patients and family members with aortic disease to the prevalence in the Dutch population. To take into account a possible dependence within families, robust standard errors were computed by using the generalized estimating equations module in SPSS and assuming an independence working correlation. A p-value <0.05 was considered as statistically significant. Cardiovascular morbidity and mortality data were compared to data published for the Dutch population in the same study period.²⁰

Results

Baseline characteristics of the patients are summarized in (Table 6.1). Our study cohort consisting of 175 patients who responded to the questionnaire showed a male preponderance (male to female ratio of 1.76). The majority of the patients (142, 81.1%) were treated by catheter techniques. Treatment for PDA took place at a mean age of 2.8 (0.3-46.5) years.

Table 6.1 Baseline characteristic of the study population.

Characteristics	Total population	PDA patients
Number of patients	2511	175
Male, n (%)	1020 (40.6)	111 (63.1)
Female, n (%)	1052 (41.9)	63 (35.8)
Unknown, n (%)	439 (17.5)	1 (1.1)
mean age at inclusion (in years)	42.8 (0-110)	12.0 (1-50)
mean age at PDA treatment (in years)	n.a.	2.8 (0.3-46.5)
PDA surgery, n (%)	n.a.	29 (16.6)
PDA catheter treatment, n (%)	n.a.	142 (81.1)
PDA treatment unknown, n (%)	n.a.	4 (2.3)

n.a. = not applicable

The distribution of aortic disease among the PDA patients and relatives is depicted in Table 6.2A and 6.2B. Thirty-six relatives of PDA patients fulfilled our definition of

aortic disease (Table 6.2A). In 11 out of these 36 relatives we concluded that the aortic disease was TAAD. According to the binominal test for proportions TAAD was significantly more frequent in second- degree relatives of PDA patients than in the Dutch general population. Results are provided in Table 6.2B.

Table 6.2 Distribution of aortic disease in the PDA patient group and their relatives.

A					
Degree of relationship	Aortic disease	n	Observed Prop.	Test Prop.	p
PDA patients	no	113	0.974	0.998	0.002
	yes	3	0.026		
	total	116	1.0		
First-degree relatives	no	586	0.993	0.998	0.045
	yes	4	0.007		
	total	590	1.0		
Second-degree relatives	no	1309	0.978	0.998	<0.001
	yes	30	0.002		
	total	1339	1.0		
B					
Degree of relationship	TAAD	n	Observed Prop.	Test Prop.	p
PDA patients	no	116	1.0	0.998	0.793
	yes	0	0		
	total	116	1.0		
First-degree relatives	no	588	0.997	0.998	0.454
	yes	2	0.003		
	total	590	1.0		
Second-degree relatives	no	1330	0.993	0.998	<0.001
	yes	9	0.007		
	total	1339	1.0		

Observed Prop. = observed proportion of absence (no) or presence (yes) of aortic pathology or of TAAD in the study group, Test Prop. = test proportion absence of aortic pathology or absence of TAAD in the Dutch population, p = p-value of the binominal test

We observed an increased prevalence of CHD in first and second-degree relatives of patients with PDA (2.73% resp. 0.79%) as compared to the Dutch population exhibiting 0.43% CHD (confidence interval 0.35 and 0.54). The frequency and the type of congenital heart disease (CHD) among relatives of PDA patients are summarized (Table 6.3). CHD was present in 17 (2.73%) first-degree relatives, including isolated PDA in 9 and the combination of PDA with atrial septal defect (ASD), ventricular septal defect (VSD) or arrhythmia in 5. In addition, 3 other first-degree relatives had non-specified valve anomalies (2) and non-specified septal defect (1). Eleven (0.79%) second-degree relatives were diagnosed with CHD, among those were VSD in 2, valve anomalies in 7 and transposition of the great arteries in 2 cases.

Table 6.3 Frequency and the type of congenital heart disease (CHD) among relatives of PDA patients.

Degree of relationship	Diagnosis	n	Percentage (%)
First-degree relatives	PDA, isolated	9	1.4
	PDA + ASD	2	0.3
	PDA + VSD	2	0.3
	ASD of VSD	1	0.2
	PDA + arrhythmia	1	0.2
	valve anomaly	2	0.3
	PDA total	14	2.2
Second-degree relatives	all anomalies	17	2.7
	VSD	1	0.1
	TGA	2	0.1
	valve anomaly	7	0.5
	all anomalies	10	0.7

ASD = atrial septal defect, VSD = ventricular septal defect, TGA = transposition of the great arteries

The familial recurrence of CHD was further analyzed in the cohort of first-degree relatives. The recurrence rate of CHD in general was 9.7%. Isolated PDA recurred in 9/17 first-degree relatives conform to a recurrence rate of 5%. As all 14 cases with PDA, regardless whether the PDA was isolated or associated with other lesions, the recurrence rate of PDA was 8%. Compared to the general population exhibiting a frequency of PDA of 0.05%.²¹ PDA was more frequent in first degree-relatives of PDA patients (1.4% isolated PDA and 2.2% all types of PDA) as extrapolated from our data.

Even higher than the direct concordance of 52% (9/17) (i.e. another family member with an isolated PDA) is the partial concordance for CHD (i.e. another family member with a CHD) of 82% (14/17) in first degree-relatives. In the group of second-degree relatives no PDA was reported. 2/10 individuals with various CHD were born with a transposition of the great arteries, which is more than the expected birth prevalence of 0.2/1000.²² One index patient was exposed to maternal rubella infection. Apart from one case of trisomy 21 no other genetic syndromes were diagnosed in index patients. Out of the 18 deaths in the group of 626 first-degree relatives 2 were reported as a consequence of CHD in infancy. Among the 283 deaths of second-degree relatives just one was related to CHD in an infant.

We analyzed the data on the frequency of and mortality due to CVDs to get insight in the total prevalence of cardiovascular diseases (CVDs) according to the definition of the WHO. This includes coronary, cerebrovascular, peripheral arterial,

rheumatic, congenital disease and deep vein thrombosis and pulmonary embolism in families of PDA patients. Among the first degree-relatives 42/553 (6.6%) had CVDs. The mortality due to CVDs was 0.2%. As expected among second-degree relatives the prevalence 172/1174 (12.4%) and the mortality due to CVDs (2%) was higher, most probably due to the increased age of these individuals at inclusion. With respect to coronary disease, we did not find a higher prevalence in relatives of PDA patients than in the general Dutch population. Table 6.4 provides an overview of the distribution of coronary disease in comparison to the prevalence in the population as provided by the Netherlands Heart Foundation.²⁰

Table 6.4 Overview over the frequency of coronary disease in the study population compared to the prevalence in the Dutch population.

Relation to the indexpt.	Mean age (y)	Coronary disease (n)	Coronary disease (%)	Prevalence
Maternal grandmother	67.0	10	5.7	86.97/1000
Maternal grandfather	66.4	24	13.7	192.06/1000
Paternal grandmother	69.9	4	2.3	126.06/1000
Paternal grandfather	69.4	16	9.1	192.6/1000
Mother	41.5	0	0	4.45/1000
Father	44.9	2	1.1	27.29/1000
Sister	16.0	0	0	0/1000
Brother	13.7	0	0	0.3/1000
Maternal aunt	39.9	0	0	4.54/1000
Maternal uncle	37.9	2	1.2	3.97/1000
Paternal aunt	40.7	0	0	4.54/1000
Paternal uncle	42.8	0	0	8.1/1000
Child	5.3	0	0	0/1000

Indexpt. = indexpatient, y=years, prevalence = prevalence in the Dutch population after.²⁰

Discussion

The most important result of our study is that aortic disease and especially TAAD occurs more frequently in relatives of patients with PDA. As expected from data on familial patterns of TAAD²³ significantly more cases than in the general population were found in the cohort of second-degree relatives, which included the generation of the grandparents whose mean age ranged between 66.4 and 69.9 years. The association of PDA and TAAD indicates a common molecular mechanism that might be responsible for both diseases. This might put PDA patients, usually treated in childhood, and their families at a higher risk to develop aortic disease later in life.

As DA closure and normal aortic distensibility both depend on proper interaction between EC and SMC involving turnover and repair of extra cellular matrix

substances¹⁷, all mutations impairing EC and SMC function and elastogenesis are candidate culprits. Only a small number of mutations affecting contractile proteins have been documented in studies on the familial segregation of vascular diseases in families with TAAD mutations.^{13,24} To our knowledge studies taking the PDA-patient as starting-point to study the incidence of aortic disease and the underlying genetic background have not been published yet.

Various mutations under which *MYH 11* and *ACTA 2* mutations might link PDA with TAAD or aortic disease in our study. Defects in the transcription factor myocardin diminish the contractile function of vascular SMC and associate PDA with aortic aneurysms in animal models.^{25,26} Mutations in *TFAP2B* impair the differentiation of SMC during fetal development²⁷ and are responsible for PDA in Char syndrome,²⁸ although aortic aneurysms have not been described. The lack of aortic disease in Char syndrome could be explained by a specific spatial overexpression of *Tfap2B* in the DA as documented in rats (data submitted).

Aortic aneurysms and dissections are the leading clinical problem in genetic syndromes impairing elastogenesis such as Marfan and Loeys-Dietz syndrome (LDS). As the loss of intimate spatial association between elastin deposits and vascular smooth muscle cells is the histological hallmark of LDS it is plausible that patients with LDS show an increased incidence of PDA.²⁹ In the Brown-Norway rat small PDA and aortic fragility including aneurysms is related to an abnormal distribution of elastic fibers and diminished elastin content.³⁰⁻³² In our study population only one family member, but no index patient, was diagnosed with Marfan syndrome and Char syndrome has not been reported. In view of the increased incidence of aortic disease and TAAD in families with PDA we advocate a thorough family history in all PDA patients. In selected cases genetic testing will be needed to define the underlying genetic abnormality in a family.

The high prevalence of CHD (2.7% among first-degree relatives of patients with PDA) corroborates larger population-based studies³³⁻³⁵ and is comparable to the recurrence rate of other CHD in first degree-relatives detected by detailed fetal echocardiography.³⁶ In 5% of our index patients a first-degree relative was diagnosed with an isolated PDA. This recurrence rate is in agreement with other studies^{11,37} and supports the idea of a genetic component in the pathogenesis of isolated non-syndromic PDA.¹⁰ Our study documents a relatively high complete (52%) and partial (82%) concordance for recurrent PDA in first-degree relatives. The degree of concordance for various types of CHD varies between the different studies. So is the concordance for PDA higher than for other CHD based on fetal echoscreening³⁸ or at the same level as described by others.^{36,39} Despite the

relatively high concordance for recurrent PDA in our study, a familial association of PDA with more complex CHD was also present. In this point our data support the international guidelines that recommend a fetal echocardiographic study in all first degree-relatives of patients with PDA. When counseling relatives about prenatal diagnostic tests it must be realized that the DA is a normal fetal structure. An isolated PDA cannot be detected on a fetal echocardiogram. The recurrence rate of 5% and the relatively high complete and partial concordance compared to other lesions justifies a postnatal clinical and echocardiographic investigation in addition to the fetal examination. In second-degree relatives we found an increased frequency of valve anomalies. This observation was made in the older cohort of second –degree relatives and has two potentially interconnected reasons. In the first place the higher frequency might reflect the usually late clinical manifestation and diagnosis of minor structural aortic valve anomalies such as bicuspid aortic valves. Secondly, the valve anomaly might be related to the dilatation of the ascending aorta in patients with aortic disease, which is also more frequent at an older age. We are aware of the limitations inherent to survey research i.e. low response rate and the lack of details in the diagnosis data.⁴⁰ The response rate in our study was 54%, which is more than expected from a mailed questionnaire.⁴⁰ According to the answers we categorized the diseases into CHD and other CVDs. While aortic disease, PDA and CHD were overrepresented in our sample, coronary disease was not more frequent and did not show an earlier onset in our study population as compared to the general population. As assessed by the frequency of coronary disease the study population was a representative sample of the general Dutch population. The premature onset of coronary disease, which might occur with *ACTA2* mutations,¹⁵ was not observed.

In conclusion our data provide evidence for an association of isolated PDA and aortic disease in members of the same family. This links a minor cardiac anomaly usually treated in childhood to serious aortic disease that will manifest later in life. In addition we showed that the prevalence of PDA and other more complex CHD is increased in first-grade relatives of the index patient. This suggests common genetic factors in the determination of CHD and aortic disease in these families. Moreover, searching specifically for mutations in these families could help to identify new genes that may contribute to PDA, other CHD and cause aortic disease.

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Appendix

Supplements

Supplement 1

Questionnaire for patients of 16 years and older:

1. Are your grandparents alive?
2. What is the age of your grandparents? / At what age have your grandparents died?
3. Has or had one or more of your grandparents a heart or vessel disease?
4. If yes, who has/had what kind of heart or vessel disease?
5. Has or had one or more of your parents been operated on his/her heart or vessels?
6. If yes, who had which kind of operation?
7. Has or had one or more of your grandparents a disease of the aorta?
8. Is your father alive?
9. How old is your father or how old was your father when he died?
10. Has or had your father heart or vessel disease?
11. If yes, which kind of heart or vessel disease?
12. Has your father been operated on his heart or vessels?
13. Has or had your father a disease of the aorta?
14. Is your mother alive?
15. How old is your mother or how old was your mother when she died?
16. Has or had your mother heart or vessel disease?
17. If yes, which kind of heart or vessel disease?
18. Has your mother been operated on her heart or vessels?
19. Has or had your mother a disease of the aorta?
20. Do you have brothers or sisters?
21. How many brothers and sisters?
22. How old are your brothers and sisters?
23. Are all your brothers and sisters alive?
24. What was the cause of death?
25. At what age have they died?
26. Has or had one of your brothers or sisters a heart or vessel disease?
27. What kind of heart or vessel disease?
28. Has one of your brothers or sisters been operated on his/her heart or vessels?
29. Has one of your brothers or sisters a disease of the aorta?
30. Do you have children?
31. If yes, how many children do you have? (How many boys how many girls?)
32. How old are your children?
33. Are all of them alive?
34. If not, what was the cause of death?
35. At what age have they died?
36. Has or had one of your children a heart or vessel disease?
37. If yes, which kind of heart and vessel disease?
38. Has or had one of your children a disease of the aorta?
39. Has or had one of your family members a connective tissue disease such as Marfan, Ehlers Danlos syndrome or does one have the syndrome of Char?
40. Does one of your family members sleepwalk?

Supplement 2

1. Questionnaire for mother/father of an index-patient:
2. Are your parents alive?
3. What is the age of your parents? / At what age have your parents died?
4. Has of had one of your parents a heart or vessel disease?
5. If yes, what kind of heart or vessel disease?
6. Has one of your parents been operated on his/her heart or vessels?
7. Has one of your parents a disease of the aorta?
8. Do you have brothers or sisters?
9. How many brothers and sisters?
10. How old are your brothers and sisters?
11. Are all your brothers and sisters alive?
12. What was the cause of death?
13. At what age have they died?
14. Has of had one of your brothers or sisters a heart or vessel disease?
15. What kind of heart or vessel disease?
16. Has one of your brothers or sisters been operated on his/her heart or vessels?
17. Has one of your brothers or sisters a disease of the aorta?
18. Do you have a heart or vessel disease?
19. What kind of heart or vessel disease?
20. How old are you?
21. Have you been operated on your heart or vessels?
22. If yes, what kind of operation?
23. Do you have a disease of the aorta?
24. How many children do you have? How many boys? How many girls?
25. What is the age of your children?
26. Are all your children alive?
27. If not, what was the cause of death?
28. How old was the child that has died?
29. Has one or have more of your children a heart of vessel disease?
30. If yes, what kind of heart or vessel disease?
31. Has one or have more of your children a disease of the aorta?
32. Has or had one of your family members a connective tissue disease such as Marfan, Ehlers Danlos syndrome or does one have the syndrome of Char?
33. Does one of your family members sleepwalk?

Chapter 7

Concluding considerations

Introduction

Why do we need a thesis on the ductus arteriosus (DA) after more than eleven thousand papers containing the term “ductus arteriosus”, which are accessible via the service PubMed in the U.S. National Library of Medicine's® (NLM) bibliographic database “Medline” and other NLM® databases? The amount of 323 papers dealing with the DA in 2011 proves that the topic is still of interest for the medical and biomedical scientific community. Most of these papers (n=273) cover the “patent” or “persistent” DA, a term that is not defined unanimously in literature. The combination of “patent ductus arteriosus treatment” accounts for more than half of these papers (n=145). A small selection of titles of the studies sheds light on the ongoing problems with the definition “How do we define congenital heart defects for scientific studies?”¹, the indication for treatment “Patent ductus arteriosus: to treat or not to treat”² and search for genetic background in animal models “A heart-hand syndrome gene: TFAP2B plays a critical role in the development and remodeling of mouse ductus arteriosus and limb patterning”.³ Mainly in clinical papers the expressions “patent” and “persistent” DA, both abbreviated as “PDA”, are used synonymously. As described in the introduction of this thesis we use “patent DA” as an umbrella term for all situations in which the DA is open either physiologically or pathologically. We define a patent DA beyond the age of three months after full gestation as a persistent DA. This type of DA is in general characterized by a structural anomaly of the vessel wall.⁴

Careful study of the literature revealed many examples of vascular-bed specific differences in the expression of genes and proteins in smooth muscle cells (SMC) during development.⁵ The characterization of the differences among mural cells may lead to understanding tissue- and vascular-bed specific responses to pathophysiological stressors.⁶ The intriguing fact, that the DA reacts completely different to the postnatal changes of the environmental conditions than the adjacent vessels triggered us to search for vessel-specific differences between DA and adjacent aorta at transcriptional and protein level. Our studies are based on the morphological features that differentiate the normal as well as the abnormal DA from the aorta. Studying two successive fetal stages we documented how vessel-specific gene expression patterns develop during normal fetal development and maturation. In the normal closing DA of human neonates we investigated a molecular mechanism underlying the DA-specific cellular process of cytolytic necrosis. The morphological features of the persistent DA and the aorta in the animal model of the BN-rat suggested a link between a structurally abnormal

persistent DA. This observation was the basis for our study on the association between persistent DA, aortic disease and other congenital cardiovascular defects among families. In our experimental work on human and rat tissue samples we used multiple techniques from conventional histology and immunohistochemistry to PCR and DNA sequencing, microarray and quantitative rt-PCR. To study the differential gene expression in the DA and aorta of fetal rats we applied a new technique. We isolated mRNA selectively from the two most important cell-types from the DA and aorta (i.e. ECs and SMCs), which we harvested by laser-capture microdissection after immunofluorescence labeling. In the last part of the thesis we used a survey to investigate recurrent persistent DA and other congenital cardiac and vascular anomalies in first and second-degree relatives of the patients treated for isolated persistent DA.

From the process of normal ductal closure to treatment options

Therapeutic interventions on the neonatal ductus arteriosus became feasible after some of the physiological and anatomical peculiarities of the fetal muscular artery were elucidated. In that regard the constrictive effect of oxygen on the DA of the neonate,⁷ the relaxing effect of prostaglandin (PG) E₂^{8,9} and the acknowledgement of the pronounced intima thickening in human DA at term¹⁰ were hallmarks.

Ductal closure procedures in neonates

The PG-pathway is the basis for the current pharmacotherapy to promote closure of patent DA in the premature infant as well as for the term neonate with a DA-dependent cardiovascular anomaly. In prematures who clinically need DA closure the unselective cyclooxygenase (COX) inhibitors indomethacin and ibuprofen are equally efficacious.¹¹

Ibuprofen has less renal side effects probably due to its lower affinity to the (renal) COX1 and is therefore increasingly used.^{12,13} Most side effects of both drugs are a direct consequence of the reduced level of endogenous prostaglandins. This leads to decreased cerebral, gastrointestinal, and renal blood flow with the most common clinical problems of gastrointestinal perforation, hemorrhage and temporary renal failure. In addition COX-inhibition alters the platelet function, which further increases the risk of bleeding.¹² Based on the risk of potential serious side effects

pharmacologic DA closure is contraindicated in infants with pre-existent renal insufficiency, bleeding, thrombocytopenia and gastrointestinal complications. Symptomatic premature infants in whom COX inhibitors are contraindicated are offered surgical DA closure. Although surgical DA closure is not considered a high-risk surgical procedure it carries the risk of transient hypotension, bleeding and pneumothorax and musculoskeletal deformities as a possible deleterious long-term effect of the thoracotomy^{14,15} As a whole the surgical procedure poses a risk for further compromise of cerebral oxygenation in the sick preterm infants,¹⁶ that is associated with adverse neurodevelopmental outcome after surgical DA closure as documented in long-term studies. Despite the large amount of data published in studies on the indications and the best way of treatment of the patent DA in the premature infant, there is still space for refinement of the current practice. The most recent studies advocate a less aggressive approach that takes the variation of the physiological process of DA closure into account.¹⁷⁻¹⁹ Furthermore, the use of paracetamol or acetaminophen seems a promising option for medical DA closure in premature infants in whom the standard COX inhibitors are contraindicated or have failed.²⁰ This is an example how a slight modification of the approach to the pharmacologic target (e.g. prostaglandinsynthase) can broaden the therapeutic options and potentially decrease side effects. Concurrently it shows that comprehensive knowledge of the physiological process of DA closure is necessary for a rational new drug design and use.

Prevention of ductal closure in neonates

Concerning the efforts to reopen or keep the DA patent in infants with duct-dependent congenital heart disease the physiological process of DA closure has other challenges. In general, the DA in the term neonate with congenital heart disease (CHD) will close functionally within days after birth²¹ and subsequently remodel exhibiting cytolytic necrosis and apoptosis.²² Since it has been established that the application of systemic PGs can counteract the functional part of the closure process the drug has been life-saving for many critical-ill neonates. However, the application of systemic PG has diverse risks and drawbacks that need to be acknowledged. The most relevant risks when initiating the therapy are apnea and hypotension. About 10% of the patients will experience apnea.²³ Systemic vasodilation is inherent to PG treatment and will cause some extent of arterial hypotension. After initiating therapy the children need to be closely monitored. There must be immediate access to ventilation facilities, fluid and

inotropes should be accessible to treat hypotension. If transfer to a specialized cardiac unit is necessary it is in general safer to intubate the child prior to transport. At least when it is decided after 15-30 min of observation after the therapy has started not to intubate the child one should be prepared to do so, en route. Few studies focused on the dosage of PG during transport.^{24,25} Theoretically the optimal PG dose that is needed varies with gestational and postnatal age, maturation of the DA, type, number, and sensitivity of PG receptors. Data on these topics are sparse in humans.^{26,27} In pigs it has been described that newborns have less PGE2 receptors than fetuses.²⁸ In rabbits a maturational difference in the number and distribution of PGE2 receptor subtypes has been shown.²⁹ From morphologic studies in premature infants it is known that maturation does not strictly correlate with gestational age,³⁰ in animal studies PG receptors diminish or become less reactive. In addition, disease specific (DA perfused with blood with high or low oxygen content) and patient specific characteristics (*TFAP2B* polymorphisms in prematures) might play a role. These theoretical considerations lead to the following practical approach to PG therapy in the newborn with duct-dependent heart disease: neonates immediately after birth who were diagnosed prenatally or those who are proven to have an open DA by echocardiography should receive a low dose of 10 nanograms/kg/min with increments to 50 nanograms/kg/min if needed. Higher start doses (100 nanograms/ kg/min) are indicated in children in whom a closed DA needs to be reopened. The initial dose should be lowered to a maintenance dose (5-10 nanograms/kg/min) as soon as successful reopening of the DA has been established in order to avoid dose-dependent side effects. This will lower the risk of major and minor side effects (fever, flushing, jitteriness, hyponatremia, gastric outlet obstruction, convulsions) that might be associated with systemic PG application.³¹ It has not been reported that a DA that does not respond to the high dose of 100 - 400 nanogram/kg/min will reopen with a further increase of the dosage.³² In addition to the systemic side effects of PG there are also vascular side effects. Lacerations of the vessel wall with intramural bleeding³³ indicate PG-related vascular fragility in the DA that also might occur in other arteries. After long-term PG therapy reversible cortical hyperostosis can occur³⁴ due to PG induced osteoblast proliferation and mineralization. However, there are clinical situations that may justify long-term use of PG as part of a hybrid treatment strategy for PA/IVS or HLHS.^{35,36} Long-term oral PG therapy has been described in the 1980ies but is no longer used in clinical practice. It had been shown to be less effective than the intravenous (iv) application.³⁷ In addition the pharmacokinetic properties of the drug necessitate hourly doses, which make its use inconvenient

and impractical. Thus, approaches including sustained PG application are hampered by the need for intravenous application of the drug and the wide range of serious side effects associated with the systemic use of synthetic PG. Furthermore, ongoing intima thickening in the DA is a concern of long-term PG-treatment. The development of a circular obstruction at the aortic end of the DA has been reported after 33-58 days of low-dose PG application.³⁸ The potential obstruction of the DA endangers the life of patients with a DA-dependent circulation and is therefore the major drawback of prolonged PG therapy. Searching for an alternative, DA-stenting has been introduced as a new clinical tool for manipulation of the DA in the 1990ties. It is used as a substitute for a modified Blalock-Taussig-shunt in patients with restricted or fully DA-dependent pulmonary flow^{39,40} and is part of the hybrid strategy for the treatment of HLHS where it guarantees systemic perfusion.⁴⁰ In pulmonary atresia the tortuous course of the DA makes percutaneous stenting technically challenging but could be overcome in most cases with the use of low-profile and easy deliverable coronary stents.⁴¹

Ductal closure procedures in older children and adults

In older children treatment options for the DA include transcatheter closure with various devices and surgical procedures. As PDAs vary in size and morphology⁴² a uniform approach is not possible. Transcatheter implantation of a closure device either from the venous or from the arterial side is the treatment of choice for most PDAs in children and adults.⁴³ The variety of devices on the market allows proper device selection according to the specific morphology in almost all children >6 months. Nowadays, surgery is reserved for very large and short PDAs that might even need patch closure with the use of cardiopulmonary bypass instead of simple ligation. A clear indication for DA closure in children and adults is symptomatic left-to-right-shunt. Left sided cardiac enlargement is considered a prophylactic indication for DA closure in asymptomatic patients, as it prevents future complications.⁴³ The indication for closure of the tiny or “silent” PDA in asymptomatic children and adults remains a matter of debate. Supporters of PDA closure use the risk of clinical endarteritis as an argument to advocate transcatheter closure^{43,44} while the opponents see a greater risk for complications in the procedure itself. In patients with increased pulmonary resistance due to a large PDA meticulous haemodynamic assessment is necessary to determine if the patient will profit from closure of the PDA. Problematic are adult patients with Eisenmenger’s syndrome caused by a large PDA having a pulmonary resistance

above 8 Wood units. Despite the procedural risks and the uncertainty to predict long-term reversibility of the pulmonary vascular disease, some authors advocate a lung biopsy to aid the decision for or against DA closure.⁴⁵

The “ideal” drug for manipulation of the DA

As described manipulation of the DA with drugs and /or devices is used on a routine basis in three different groups of patients: i.e. premature babies, neonates with DA-dependent cardiac defects, and children and adults with PDA. Each group has its special needs in accordance with the underlying pathology.

Ductal closure in neonates

Current therapeutic modalities are associated with many adverse effects as discussed. Premature babies exhibit a DA with less intima cushion formation³⁰ and reduced sensitivity for oxygen but increased sensitivity to PGE₂, nitric oxide (NO), and perhaps endothelin-1.⁴⁶ As functional and anatomical DA closure both depend on gestational maturity the “ideal” drug for DA closure would be a drug that matures all closing mechanisms of the DA without systemic side effects, safe and effectively. The application should be feasible in small infants with gastrointestinal and venous access problems. There is evidence that administration of cortisol to fetal lambs improves the response of the fetal DA to oxygen and PG-inhibition.⁴⁷ The decreased rate of patent DA in premature babies born after antenatal corticosteroid treatment, (current guidelines advocate antenatal corticosteroids to all women at high risk to preterm delivery between 24 and 34 weeks of gestation)⁴⁸ suggests that this might also be the case in humans. Maternal and (long-term) offspring side effects of this approach are still under investigation. In chapter 5 we demonstrated the increased expression of *Rgs5* in the fetal DA compared to the aorta. RGS5 is a negative regulator of G-protein–coupled signaling in the heart and vessels.⁴⁹ As PGE₂ acts through G-protein–coupled receptors (GPCRs) to relax the vascular smooth muscle layers.⁵⁰ RGS5 is a candidate target for the development of alternative drugs to promote DA constriction without the adverse effects of unselective COX inhibition. However, before this concept can be used all regulatory pathways in which RGS5 is involved need to be elucidated.

Prevention of ductal closure in neonates

In neonates with DA-dependent cardiac defects reliable patency of the DA is desired until a shunt or another surgical procedure has been performed. Systemic PG therapy and, if long-term patency is necessary, stents are the therapeutic options for these patients. The broad range of local and systemic side effects of PGs and the potential complications associated with the implantation of stents^{50,51} motivate the search for an alternative concept. Some years ago a prenatal gene-transfer experiment showed that it is possible to engineer a patent DA in lambs by transferring PGE synthase.⁵² The variety of unsolved issues of gene therapy such as the short-lived nature of the therapy, immunogenicity and problems with viral vectors led to strict governmental regulations and prevented further development of this concept for human use. Avoiding the physiological remodeling process completely instead of solely inhibiting postnatal DA contraction would be advantageous and possibly the most efficient way to guarantee prolonged patency of the DA. However, a new strategy targeting on remodeling needs thorough understanding of the underlying process. In chapter 5 we studied the postnatal anatomical remodeling of the DA and detected selective progerin expression in association with remodeling of the DA. Two different classes of drugs are shown to work against the toxic cellular effects of progerin. Farnesyl-transferase inhibitors (FTIs) belong to the first class of drugs that were discovered. FTIs inhibit the farnesylation of progerin and thereby avoiding the toxic effects of progerin on the nucleus.^{53,54} Recent publications show that rapamycin abolishes the characteristic nuclear effects, prolongs the life span, and enhances the turnover of the cultured fibroblasts of HGPS patients.⁵⁵ This is a promising finding not only for HGPS patients as we showed in chapter 4 that DA remodeling is associated with progerin expression and suggested that activation of Lamin A *LMNA* alternative splicing is involved in neonatal DA closure. In addition to a clinical trial with rapamycin or its analogues, which is under consideration for HGPS patients, this family of drugs seems worthwhile to be evaluated for its potential to inhibit DA remodeling. Based on our data both drugs could potentially be useful in the inhibition of anatomical DA remodeling. It is obvious that potential adverse effects of these drugs need to be carefully studied and weighed against existing therapies before its use in neonates can be advocated. With the increasing accessibility and accuracy of fetal echocardiography for the diagnosis of cardiac defects a prenatal approach to prevent the formation of intimal cushions in the DA might also be useful. Although not proven in humans, the chronic activation of the EP4 receptor that is needed to

induce hyaluronic acid mediated cushion formation in rodent fetus⁵⁶ is interesting. Hyaluronic acid synthase (HAS) could be a new target for DA manipulation that might even be accessible in the fetus. Data on the increased incidence of patent DA in infants exposed to antenatal indomethacin⁵⁷ suggest, that chronic activation of the EP4 receptor mediated HAS induction is also needed for proper intima cushion formation in humans.

Future developments in DA stenting

In-stent stenosis due to neointima formation and stenoses caused by DA-tissue that has not been covered by the stent are the main reasons for complications after DA stenting. In-stent stenosis is inherent to vessel stenting and resembles the normal remodeling process of the DA.⁵⁸ In order to attenuate neointima formation rapamycin-impregnated stents have been evaluated, as this drug induces cell-cycle arrest, ultimately resulting in inhibition of SMC proliferation.⁵⁹ In a pig model these stents were efficiently counteracting the initial contraction and the secondary intimal thickening of the DA over a period of 6 weeks. These data were, however, not sufficient to introduce rapamycin-impregnated stents for clinical use in neonates.⁶⁰

There is no stent specifically designed for the use in the neonatal DA on the market. Stents that are used are developed for coronary lesions, peripheral arterial lesions and the use in bile ducts of adults. A ductal stent should be adapted for the tortuous course and length of the DA, easy to manipulate in a vessel with a much weaker and looser wall structure than arteriosclerotic coronary or systemic arteries. One should bear in mind that the prior use of PG will weaken the wall even more.³³ Furthermore the delivery system either a balloon catheter or a catheter carrying a self-expanding stent should be as small and as flexible as possible to avoid additional vascular damage when delivering the stent via the transvenous or transarterial route in a neonate. A stent in a reopened previously stenotic DA needs a higher radial strength than a stent in a widely patent DA. Various stent materials are used in the DA such as cobalt chromium, stainless steel or nickel titanium (nitinol). Stent designs include closed or open cells, wire based or laser cut devices. If the patency of the adjacent potentially stenotic aortic isthmus is a concern, as in hybrid Norwood procedures,⁶¹ an open cell stent is preferable. A stenosis covered by an open cell stent can be reopened by a stent that is implanted retrogradely in the aorta.⁶² Although being potentially superior to bare metal stents

in the DA, local and systemic drug effects need to be further evaluated. Finally, biodegradable stents providing scaffolding for a short period of about 6 months are an interesting option. The ideal characteristics of a biodegradable stent have been defined as (1) sufficient radial strength to prevent vascular recoil, (2) minimal thrombotic and inflammatory response, (3) avoidance of intimal proliferation, (4) reabsorption of stent components within months, (5) no release of toxic products or embolic material during breakdown and (6) easy processing and sterilization. Based on these considerations, two different concepts have evolved: absorbable polymer stents and absorbable metal stents including magnesium and iron stents.⁶³ Besides incidental use of magnesium stents in infants³⁹ none of these stents has been used systematically in children.

A link between PDA and aortic disease

Brown Norway rats differ from other rat strains in vascular elastin content and are prone to develop ruptures in the elastin lamellae of the thoracic and abdominal aorta.^{64,65} We detected that a high proportion (10/12) of adolescent BN rats also showed an inherited PDA.⁶⁶ The small PDA in these rats had an abnormal distribution of elastin lamellae. Therefore we proposed that impaired elastin metabolism is related to the persistence of the DA. In addition this model implicates a genetically determined factor linking PDA with aortic fragility. A study on the genetic basis of the specific arterial phenotype of the BN rat revealed significant quantitative trait loci (QTLs) for PDA on chromosomes 8 and 9 and for aortic fragility a region on chromosome 5 and a locus on chromosome 10, which is syntenic to one linked to a syndrome of thoracic aortic aneurysms (TAAD) with PDA in humans.⁶⁷ Among genes present in these QTLs were large numbers of myosin genes, which is of interest in view of the recently reported involvement of *MYH11* in the human syndrome with aortic aneurysm and PDA.⁶⁸ Mutations in another component of the contractile apparatus, the aortic alpha-smooth muscle actin (*ACTA 2*) gene, are responsible for 14% of familial TAAD.⁶⁹ Interestingly associated findings in families with *ACTA 2* mutations may include livedo reticularis, a bicuspid aortic valve, iris flocculi and PDA.⁶⁹ These associated findings let us set out a study on the familial association of PDA and aortic disease, especially TAAD, presented in chapter 6. The key finding of our survey among patients treated for PDA was a significantly increased prevalence of aortic disease and TAAD in first and second-degree relatives as compared to the general

population. These data provide evidence for a genetic association of PDA and aortic disease and give essential information for the investigation of the genetic background of both lesions.

Three different types of PDA

The first differentiation between types of PDA dates back in the 1980ies.⁴ By the means of histology it was identified, that the PDA in children >3 months differed in structure from the delayed closing DA in the premature infant, in the clinical practice also called a PDA. An aberrant distribution of elastin with a subendothelial elastic lamina was characteristic for the primary congenital malformation of the DA wall in older children with PDA.⁴ Children with isolated PDA and in those with associated CHD showed the same histology. In chapter 4 we documented the lack of progerin expression in the wall of the PDA. Recent genetic data showed that mutations in the neural-crest related transcription factor gene *TFAP2B* are the underlying cause of PDA in Char syndrome⁷⁰ and explain some cases of isolated PDA.^{71,72} Histological data on the PDA in humans harbouring mutations in *TFAP2B* are lacking. However, data from a knock-out mouse model for this disease show an abnormal distribution of elastin lamellae in the DA.⁷³ The increased rate of delayed closing DA in prematures with a specific polymorphism of *TFAP2B* underscores its relevance also for normal DA closure.^{74,75} In chapter 5 we documented the DA-specific expression of the analogue of the *TFAP2B* gene in fetal rats.

In view of the current data presented in this thesis we are inclined to distinguish a third type of PDA from the former two. This third type is a PDA as part of a general vasculopathy. The vasculopathy might be genetically determined and linked to aortic disease. This is the case in families with mutations impairing contractile proteins, or elastogenesis discussed in chapters 2 and 6. PDA and pulmonary artery branch stenoses in cases of congenital rubella represent a virus-induced vasculopathy, characterized by differential retardation of tissue growth in the DA and pulmonary arteries.^{76,77}

As a conclusion of the current thesis we propose to differentiate between three types of PDA, i.e. (1) the immature, (2) the primary congenital malformation and the (3) general vasculopathic type. Each type of PDA needs a different approach to diagnostics, treatment and follow-up depending on gestational age and associated pathology. A vasculopathic type of PDA should be considered in premature babies whose PDA's are refractory to drug treatment, any patient with

PDA and additional vascular anomalies or a positive family history for aortic disease. In these patients a thorough family history, detailed imaging of all cardiovascular structures and in selected cases genetic diagnostic might help to define an underlying general vascular anomaly that might be present in the family and affect adult individuals later in life.

The data presented in this thesis show that the DA is coming of age in several aspects. Starting from the description of the fetal vessel and the understanding of its function in the circulation, basic research has developed its extensions into the fields of molecular and cell biology and genetics. Among others, the use of high-throughput molecular profiling techniques offers the opportunity to study the molecular basis for the DA-specific morphology and function. Progerin, responsible for premature ageing in Hutchinson-Gilford Progeria syndrome and related to vascular ageing in the elderly, has also been identified in the neonatal DA and linked to the maturation and regression of this vessel. The DA is not only of interest in the fetal and neonatal period as in some patients with PDA medical attention should be extended into adult life and to their family members.

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Summary

Summary

The aim of the research described in this thesis was to investigate the genetic background and molecular mechanisms underlying the specific remodeling processes leading to postnatal closure of the ductus arteriosus (DA). To achieve this goal both the normal closing and the persistent ductus arteriosus (PDA) in rats and humans were studied.

In **chapter 2** the literature on the pathogenesis and genetic background of patency of the ductus arteriosus is studied. By describing the animal models used in DA research an overview is given on the regulatory pathways that are involved in physiological DA closure. Studying human genetic syndromes associated with PDA it becomes evident that in virtually all of these anomalies genes are affected, which interfere with the remodeling of the smooth muscle cells (SMC). Therefore the pivotal regulatory role of SMCs in ductal closure is emphasized.

Chapter 3 presents a new animal model of a PDA. The inbred strain of Brown-Norway (BN) rats is characterized by abnormally distributed elastin lamellae in the PDA. The elastin lamellae in the aorta were deficient. Anatomical and histological features of the PDA in the BN rat are documented and compared to the Wistar rat with a normal closing DA. The discussion focuses on the role of elastin in ductal closure and the possible relationship between PDA and aortic aneurysm formation.

In **chapter 4** we investigated the expression of lamin A/C and the defective truncated lamin A form, progerin, in the human neonatal DA. Abnormally expressed progerin destabilize the nuclear membrane and causes the abnormalities that are responsible for the premature ageing in Hutchinson Gilford progeria syndrome. Recently progerin has also been discovered in the skin and vessels of normal ageing individuals. By documenting progerin expression predominantly in the areas of the DA where anatomical remodeling commences we relate progerin with this physiological process in the neonate. As a consequence we propose alternative splicing of the pre-mRNA of the lamin A/C as a molecular mechanism of anatomical remodeling of the DA.

Chapter 5 focuses on genes that are differentially expressed between fetal DA and aorta. By microarray analysis of laser-captured ECs and SMCs we identified for the first time the regulator of the G-protein coupled receptor RGS5 and the homeobox

gene transcription factor *Dlx1* as DA-specific genes in rats at the end of gestation. In the aorta of the same animals the gene encoding Purkinje-cell protein 4 *PCP4* was preferentially expressed. Expression patterns of these genes also validated with quantitative RT-PCR confirmed that DA and aorta-specific transcriptional profiles are present in ECs and SMCs. *RGS5* and *DLX1* represent new molecular targets for the regulation of fetal DA maturation and postnatal closure.

In **chapter 6** we assessed in a patient-based study the prevalence of PDA, aortic disease and other cardiovascular anomalies in first- and second-degree family members of patients treated for PDA. Data of 177 families with 2511 members were available. In second-degree relatives of PDA patients the prevalence of aortic disease was significantly higher than in the general Dutch population. The recurrence rate of CHD was higher (2.7%) in first-degree relatives than in second-degree relatives (0.7%). Our data provide evidence for a genetic association of PDA and aortic disease. In addition the increased prevalence of PDA and other CHD in the studied families is suggestive of common genetic factors determining PDA, CHD and aortic disease.

In the concluding considerations presented in **chapter 7** the topics covered by the chapters 2 to 6 from a clinical perspective are reviewed. The current therapeutic modalities to manipulate the DA and problems associated with medical and catheter-based therapy are discussed. All patient groups including premature infants with delayed closing of the DA, neonates with DA-dependent congenital heart disease, older children or adults with PDA and their families might profit from a better understanding of the mechanisms underlying DA remodeling. The findings presented in this thesis could be seen as a starting-point for the development of new diagnostic and therapeutic strategies for the DA in these patients.

Samenvatting

Samenvatting

Het doel van het onderzoek, dat tot dit proefschrift leidde, was het bestuderen van de genetische basis en de moleculaire mechanismen, die het vaatspecifieke sluitingsproces van de ductus arteriosus (DA) na de geboorte sturen. Om dit doel te bereiken werden zowel de normaal sluitende als de persisterende DA (PDA) bij de rat en bij de mens bestudeerd.

In **hoofdstuk 2** wordt de literatuur over de pathogenese en de genetische achtergrond van het openblijven van de DA na de geboorte onderzocht. Aan de hand van de beschrijving van de diersmodellen, die tot nu toe in het onderzoek naar de ductussluiting gebruikt werden, wordt een overzicht van de regulatiemechanismen gegeven, die betrokken zijn bij de fysiologische ductussluiting. De analyse van de genetische syndromen, die bij de mens met een PDA geassocieerd zijn, maakt duidelijk dat nagenoeg bij al deze syndromen afwijkingen in de genen voorkomen, die de “remodeling” van gladde spiercellen beïnvloeden. Daarom wordt die cruciale rol van gladde spiercellen in de regulatie van de ductussluiting in het bijzonder benadrukt.

In **hoofdstuk 3** wordt een nieuw diersmodel voor een PDA gepresenteerd. De inteelt-stam van de Brown-Norway (BN) rat wordt gekenmerkt door abnormaal verdeelde elastinelamellen in de PDA die deficiënt zijn en een vermindering van het aantal elastinelamellen in de aortawand. De anatomische en histologische bijzonderheden van de PDA bij de BN rat worden beschreven en met de bevindingen bij de Wistar-rat vergeleken, bij welke de DA normaal sluit. De discussie concentreert zich op de rol van elastine bij de ductussluiting en de mogelijke relatie tussen een PDA en de ontwikkeling van aneurysmata van de aorta.

In **hoofdstuk 4** onderzochten wij de expressie van lamin A/C en de verkorte en afwijkende vorm ervan, progerin, in de DA van pasgeboren kinderen. Het afwijkende eiwit progerin destabiliseert de kernmembraan en is daardoor verantwoordelijk voor de vervroegde veroudering bij het Hutchinson-Gilford Progeria syndroom. Kortgeleden werd progerin ook in de huid en de vaten van genetisch normale oudere personen aangetoond. Wij documenteerden progerin in de DA van neonaten vooral in die gebieden waar het anatomische remodelleringsproces van het bloedvat begint. Het specifieke expressiepatroon van

progerin duidt onzes inziens op een verband tussen de fysiologische ductussluiting en progerinexpressie. Daarom menen wij te kunnen concluderen, dat de alternatieve splitsing van het pre-mRNA van het lamin A/C één van de moleculaire mechanismen is, die tot de anatomische “remodeling” van de DA leiden.

Hoofdstuk 5 richt zich op genen, die gedifferentieerd in werking komen in de foetale DA en de aorta. Met behulp van een microarray analyse van door “laser-capture” geïsoleerde endotheel- en gladde spiercellen van de DA konden wij voor het eerst de regulerende werking van de G-protein coupled receptor RGS5 en van de homeobox transcriptiefactor DLX1 als DA-specifieke genen bij de rat aan het einde van de zwangerschap identificeren. In de aorta van dezelfde dieren zagen wij de preferentiële expressie van het gen dat voor het Purkinje-cell protein 4 (PCP4) codeert. Expressiepatronen van deze genen werden ook met een kwantitatieve rt-PCR gevalideerd. Met PCR werd bevestigd, dat er zowel DA als aorta- specifieke genexpressiepatronen in de endotheel- en gladde spiercellen bestaan. Voor de DA zijn RGS5 en DLX1 als nieuwe moleculaire targets te beschouwen, die een rol spelen bij de transcriptionele regulatie van de DA.

In **hoofdstuk 6** analyseerden wij in een patiëntgericht onderzoek de prevalentie van PDA, aortazikten en andere cardiovasculaire anomalieën bij eerstegraads en tweedegraads familieleden van patiënten, die voor een PDA behandeld werden. Er konden gegevens van 177 families met 2511 leden verzameld worden. Bij tweedegraads familieleden van PDA patiënten was de prevalentie van aortaziktes significant hoger dan bij de Nederlandse bevolking in het algemeen. De herhalingskans voor aangeboren hartafwijkingen was met 2,7% hoger bij eerstegraads familieleden dan bij tweedegraads familieleden (0,7%). Onze data leveren enig bewijs voor een genetische associatie van een PDA met aortaziktes. Bovendien duidt de verhoogde prevalentie van PDA en andere aangeboren hartafwijkingen in de onderzochte families op een gemeenschappelijke genetische factor, die PDA, aangeboren hartafwijkingen en aortaziktes bepaalt.

In de concluderende beschouwing, die in **hoofdstuk 7** gepresenteerd wordt, worden de onderwerpen vanuit een klinisch perspectief bekeken. De huidige therapeutische mogelijkheden om de DA open te houden of te sluiten worden besproken. Mogelijke problemen die bij de medicamenteuze en katheter-interventionele behandelingen kunnen optreden worden bediscussieerd. Alle patiënten, van de prematuur geboren met een onrijpe vertraagd sluitende DA,

en de neonaten met ductusafhankelijke aangeboren hartafwijkingen tot de oudere kinderen en volwassenen met een PDA en hun families zouden van een grotere kennis van de basale mechanismen van de fysiologische ductussluiting kunnen profiteren. In deze zin zijn de in dit proefschrift gepresenteerde bevindingen te zien als een uitgangspunt voor de ontwikkeling van nieuwe diagnostische en therapeutische strategieën ten aanzien van de DA bij al deze patiënten.

Zusammenfassung

Zusammenfassung

Das Ziel der hier vorgelegten Arbeit war es die den genetischen Hintergrund und die molekulären Mechanismen zu untersuchen, die dem einzigartigen physiologischen Prozess zugrunde liegen, der zum postnatalen Duktusverschluß mit anschließender anatomischer Remodellierung führt. Um dieses Ziel zu erreichen haben wir sowohl den normal schließenden Duktus als auch den persistierenden Duktus bei der Ratte und beim Menschen untersucht.

In **Kapitel 2** geben wir eine Übersicht über die Literatur, die sich mit der Pathogenese und dem genetischen Hintergrund des Offenbleibens des Duktus befasst. Durch die Beschreibung der für die Erforschung des Duktus verfügbaren Tiermodelle geben wir eine Zusammenfassung der Regulationsprozesse, die beim physiologischen Duktusverschluß bekannt sind. Die Analyse der humanen genetischen Syndrome, die mit persistierendem Duktus (PDA) assoziiert sind, liefert weitere Anhaltspunkte für Gene, die eine Rolle beim Duktusverschluß spielen können. Durch die Syndrome richtet sich unser Augenmerk vor allem auf Gene mit einer Funktion bei der Remodellierung von glatten Gefäßmuskelzellen.

In **Kapitel 3** präsentieren wir ein neues Tiermodell für einen kleinen PDA. Der beschriebene Inzuchtstamm der Brown-Norway-Ratte wird charakterisiert durch die abnormale Verteilung von Elastin im Duktus und ein Defizit an Elastinlamellen in der Aorta. Die anatomischen und histologischen Besonderheiten des PDA der Brown-Norway-Ratte werden mit den Befunden bei der Wistar-Ratte verglichen. In der Diskussion dieses Kapitels wird vor allem auf die Rolle von Elastin bei der duktusspezifischen Remodellierung der glatten Gefäßmuskelzellen (SMCs) eingegangen.

Für **Kapitel 4** untersuchten wir die Expression von Lamin A/C und seiner Spleißvariante Progerin im humanen neonatalen Duktus. Progerin verursacht die Anomalien der Kernmembran, die verantwortlich sind für den frühzeitigen Alterungsprozeß der zum klinischen Bild des Hutchinson-Gilford-Progeriesyndrom führt. Kürzlich wurde Progerin auch in der Haut und den Blutgefäßen genetisch normaler älterer Individuen entdeckt. Unsere Untersuchungen zeigen Progerin erstmals im Duktus des Neugeborenen. Durch die besondere Verteilung des Progerinproteins, das vor allem in den Arealen des Duktus angetroffen wurde, in denen die anatomische Remodellierung des Duktus beginnt, ließen uns auf eine

Bedeutung diese Proteins für den physiologischen Duktusverschluß schließen. Schlussfolgernd gehen wir davon aus, dass alternative Spleißung einer der molekulären Mechanismen ist, der zur anatomischen Remodellierung des Duktus beitragen.

In **Kapitel 5** konzentrieren wir uns auf Gene, deren Expression sich zwischen fötalem Duktus und fötaler Aorta unterscheidet. Mit Hilfe von DNA-Microarrays von durch Laser Capture Mikrodisektion gewonnenen Endothelzellen (ECs) und SMCs identifizierten wir erstmals den Regulator des G-Protein gekoppelten Rezeptor *Rgs5* und den Homeoboxtranskriptionsfaktor *Dlx1* als duktusspezifische Gene bei der Ratte am Ende der Gestationsperiode. In der Aorta derselben Tiere fanden wir die bevorzugte Expression des Genes für das Purkinje-Zellen Protein 4 (*Pcp4*). Die Expressionsmuster der differentiell exprimierten Gene, die auch mit quantitativer RT-PCR Technik verifiziert wurden, bestätigen das Vorliegen von spezifischen Transkriptionsprofilen in ECs und SMCs von sowohl Duktus als auch Aorta. RGS5 and DLX1 stellen Ansatzpunkte für eine neue gezielte Behandlung dar, die in die fötale Duktusreifung und den postnatalen Duktusverschluß eingreift.

In **Kapitel 6** untersuchten wir in einer patientengebundenen klinischen Studie die Prävalenz von PDA, Aortenerkrankungen und anderen kardiovaskulären Anomalien bei Familienmitgliedern ersten- und zweiten Grades von Patienten, die wegen eines isolierten PDA behandelt wurden. Daten von 177 Familien mit insgesamt 2511 Mitgliedern standen uns zur Verfügung. Bei den Verwandten zweiten Grades der PDA-Patienten bestand eine signifikant erhöhte Prävalenz von Aortenerkrankungen im Vergleich zur niederländischen Allgemeinbevölkerung. Die Wiederholungsrate angeborener Herz- und Gefäßfehler war deutlich höher bei Familienmitgliedern ersten Grades (2.7%) als bei denen zweiten Grades (0.7%). Unsere Daten weisen auf eine genetische Assoziation zwischen PDA und Aortenerkrankungen. Die ebenfalls erhöhte Prävalenz von anderen angeborenen Herz- und Gefäßfehlern in den untersuchten Familien deutet außerdem auf einen gemeinsamen genetischen Faktor für angeborene Herz- und Gefäßfehler und Aortenerkrankungen.

In der schlussfolgernden Betrachtung in **Kapitel 7** beurteilen wir die in den Kapiteln 2 bis 4 beschriebenen Untersuchungen aus einer klinischen Perspektive. Die aktuellen Behandlungsmöglichkeiten zur Manipulation des Duktus und deren Probleme werden dargestellt. Alle Zielgruppen der Duktusbehandlung:

Frühgeborene mit verzögert schließendem Duktus, Neugeborene mit duktusabhängigen Herzfehlern, ältere Kinder und Erwachsene mit PDA und deren Familien könnten von einem besseren Verständnis der Mechanismen, die der Remodellierung des Duktus zugrunde liegen profitieren. Die Befunde, die in der vorliegenden Arbeit präsentiert werden, sind als Ausgangspunkt für die Entwicklung neuer diagnostischer und therapeutischer Möglichkeiten für die genannten Patientengruppen zu sehen.

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Curriculum vitae

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Dankwoord

Dankwoord

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J.W. von Goethe

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