Part VII

Ductus Arteriosus

Perspective

Susumu Minamisawa

The ductus arteriosus (DA), an important fetal artery that connects the main pulmonary artery and the aortic arch, closes immediately after birth. Therefore, closure of the DA is a symbolic event of the change from fetal to neonatal circulation. When the DA remains open after the first 3 days of life in humans, the condition is called patent DA (PDA), and it usually causes a left-to-right shunt. If the shunt volume significantly increases, neonates may exhibit pulmonary edema and respiratory distress. Especially in preterm infants, PDA could be a serious life-threatening risk. The incidence of PDA unaccompanied by any other cardiovascular abnormality has been estimated to be 0.06 % of term infants, and the incidence sharply increases in premature infants. Symptomatic PDA cases have been found in 28 % of infants with very low birth weight (<1500 g) and 55 % of infants with extremely low birth weight (<1000 g). On the other hand, PDA is lifesaving in some patients with congenital heart diseases such as hypoplastic left heart syndrome and pulmonary atresia. The DA must be open to maintain blood flow in the systemic or pulmonary circulation. Current pharmacological treatment for closing or maintaining PDA is limited to the agents that control the vasodilatory effect of prostaglandin E_2 (PGE_2), such as cyclooxygenase inhibitors or PGE_1 preparations.

DA closure is thought to occur in response to a combination of two different mechanisms. One mechanism is an acute response of smooth muscle constriction within the first several hours of life, known as functional closure of the DA. The other is a relatively chronic response of structural change in the DA during the

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perinatal period, known as anatomical closure or vascular remodeling, such as progression of neointimal thickening and impaired elastic fiber formation. After shutting down blood flow, progressive apoptosis and fibrotic changes occur in the DA, resulting in permanent DA closure and a remnant structure known as the ligamentum arteriosum. In this part, four studies highlight the recent advances in molecular mechanisms underlying DA closure from the perspective of functional and anatomical closure.

The primary driving force behind functional DA closure is an increase in oxygen tension and a decrease in circulating PGE_2. The DA is an oxygen-sensitive vessel. Several potassium channels are known to play an important role in the oxygen-sensing system. Momma et al. show that ATP-sensitive potassium channels (K_{ATP} channels) are another oxygen sensor. They show that sulfonylureas, which are commonly used to treat diabetes, inhibit K_{ATP} channels to constrict the DA, suggesting that sulfonylureas can be used for patients with PDA. The response to oxygen is weaker in premature DA than in mature DA. Hayama et al. investigate the developmental changes in the contractile apparatus and sarcoplasmic reticulum in the DA and its connecting arteries that may contribute to the oxygen-sensitive mechanism.

Anatomical closure of the DA is associated with distinct differentiation of the vessel wall. Intimal thickening is the most prominent phenotypic change, and it involves several processes: (a) an area of subendothelial extracellular matrix deposition, (b) the migration of undifferentiated medial smooth muscle cells (SMCs) into the subendothelial space, and (c) the disassembly of the internal elastic lamina and the loss of elastic fibers in the medial layer. Yokoyama et al. demonstrate that chronic activation of the PGE_2 receptor EP4 plays a pivotal role in neointima formation and impaired elastic fiber formation in the DA. Therefore, PGE_2 signaling has dual roles in functional and anatomical DA closure. DA remodeling is known to resemble the vascular remodeling of aging arteries. Gittenberger-de Groot et al. demonstrate that progerin, an alternative splice variant of lamin A/C, is highly expressed in the closing DA as well as in premature aging of vessels in children with Hutchinson progeria syndrome, suggesting that progerin plays a role in vascular remodeling of the DA.

These studies show that development of a new strategy to treat DA closure or opening first requires understanding of the precise molecular mechanisms underlying both functional and anatomical DA closure.

Progerin Expression during Normal Closure of the Human Ductus Arteriosus: A Case of Premature Ageing?

34

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Abstract

The ductus arteriosus (DA) is a fetal vessel bypassing the still nonfunctional lungs. Closure of the DA at birth is essential for the transition from a fetal to a neonatal circulation. This closing process begins with a physiological contraction followed by definitive anatomical closure. The latter process starts already before birth by development of intimal thickening followed after birth by degeneration of the inner media, including cytolytic necrosis and apoptosis. The DA will remain patent when there is insufficient maturation in prematurely born babies or when there is a structural abnormality as seen in persistent DA (PDA). The histological changes during normal DA closure resemble the features seen in the premature ageing vessels in children with the Hutchinson progeria syndrome. The latter syndrome is caused by a mutation in the lamin A/C gene resulting in accumulation of the progerin splice variant. We studied human DA biopsies from the fetal to the neonatal period to investigate whether lamin A/C and progerin might be involved in the DA closure process. The results

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show an increase in the intima and inner media of progerin in the normal neonatal DA, while expression of lamin A/C is diminished. In the non-closing aorta, the fetal DA and the PDA, no or hardly any progerin expression was found. We postulate that the lamin A/C to progerin balance is important during normal anatomical closure of the DA presenting a unique case of physiological premature vascular ageing.

Keywords
Lamin A/C Progerin • Atherosclerosis • Apoptosis • Vascular biology • Persistent ductus arteriosus

34.1 Introduction

The ductus arteriosus (DA) is a fetal vessel that connects the pulmonary trunk to the distal aortic arch. The patent DA is functional before birth directing oxygen-rich blood from the placenta to the systemic circulation bypassing the still immature lung vascular bed. After birth, the DA contracts, and this physiological closure is followed by definitive anatomical closure. Ultimately, the DA will be remodeled into a fibrous strand [1, 2]. The physiological closure is regulated by many substances in which prostaglandins play an important role [3]. The onset of anatomical closure in the human fetus starts already in the second trimester of gestation with blebbing of the endothelium and formation of intimal thickening [2]. The degenerative changes in the media, in which cytolytic necrosis and apoptosis play an important role [4], start immediately after birth. By then, marked intimal thickening supports the closure of the DA upon contraction. This maturation process of ductal closure (Fig. 34.1) follows a temporo-spatial pattern. Upon premature delivery of the baby, the DA closure process may not be effective already resulting in an immature patent DA. Usually, this resolves spontaneously within a few days to weeks after birth. The process might be enhanced by providing prostaglandin inhibitors like indomethacin [2]. Another reason for non-closure resulting in a persistent DA (PDA) may be structural abnormalities, in most cases based on an elastin deposition problem [3]. The histological features of normal DA closure resemble atherosclerotic changes in the vessel wall albeit without the atheroma component [5]. The mature DA vessel wall structure is highly similar to the premature ageing histopathology seen in vessels of children with the Hutchinson-Gilford syndrome (HGPS) [6]. This syndrome is based on a genetic defect in the lamin A (*LMNA*) gene which leads to pathological accumulation of the splice variant progerin [7, 8]. Increased progerin expression has also been reported during normal vascular ageing [9, 10]. Based on the observed similarities, we investigated the role of an *LMNA*/progerin balance during normal perinatal closure of the DA.

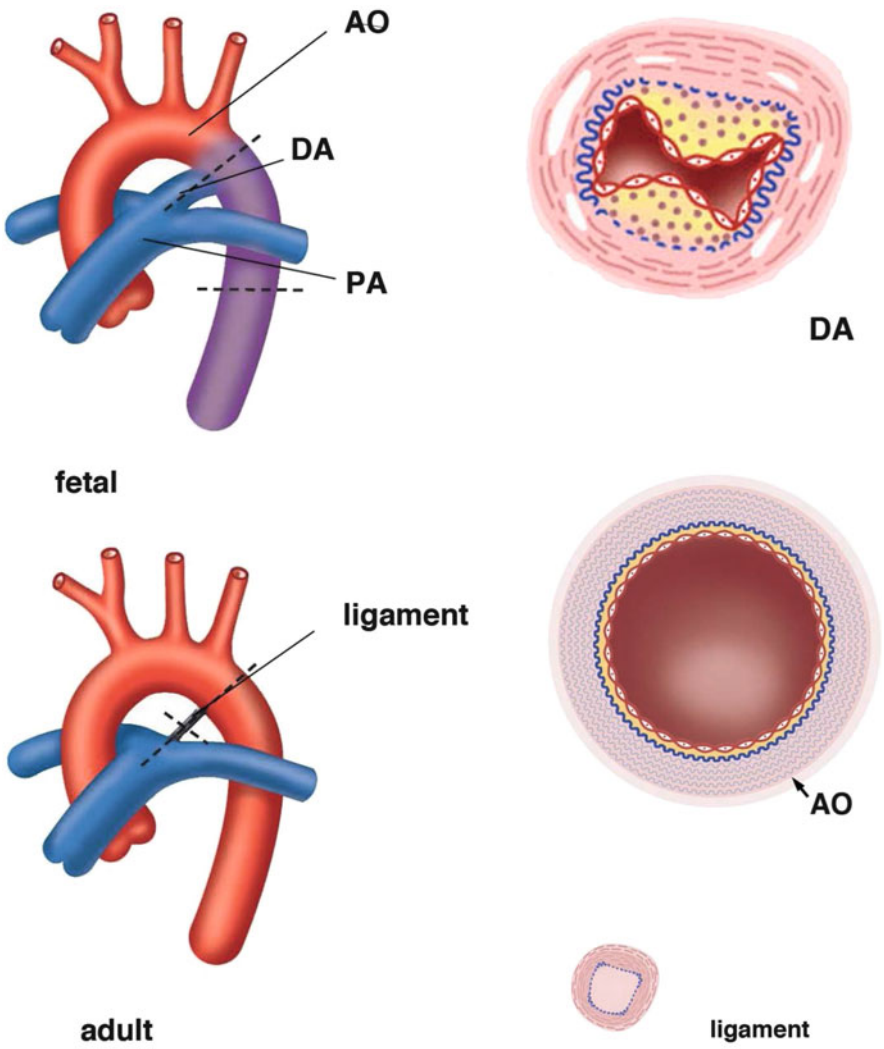

Fig. 34.1 Schematic representation of the aortic arch and the cross sections of the muscular ductus arteriosus with intimal thickening (DA) and the elastic aorta (AO) and pulmonary artery (PA), during the fetal, neonatal, and adult stage of DA, in which the latter is remodeled into a ligament

34.2 Material and Methods

Following aortic arch reconstruction and surgical closure of the DA, a biopsy specimen of human neonatal DA ($n = 16$) and adjoining descending aorta ($n = 2$) was obtained. The fetal DA (one of 14 weeks' and one of 18 weeks' gestation) was

acquired from postmortem fetal specimen after legal or spontaneous abortion. One biopsy specimen was obtained from a 2-year-old child with PDA. Details on sectioning, fixation, and immunohistochemistry have been described as well as the technique for RNA isolation and RT-PCR reactions [11].

34.3 Results

The RT-PCR studies of the aorta and neonatal DA revealed that next to lamin A also progerin was detected. Lamin A was seen both in the aorta and neonatal DA, while low levels of progerin were only seen in the neonatal DA [11]. The elastin expression showed clearly that the DA is a muscular artery, while the aorta is an elastic artery (Fig. 34.2a, d, e, j). Immunohistochemistry of the tissue sections

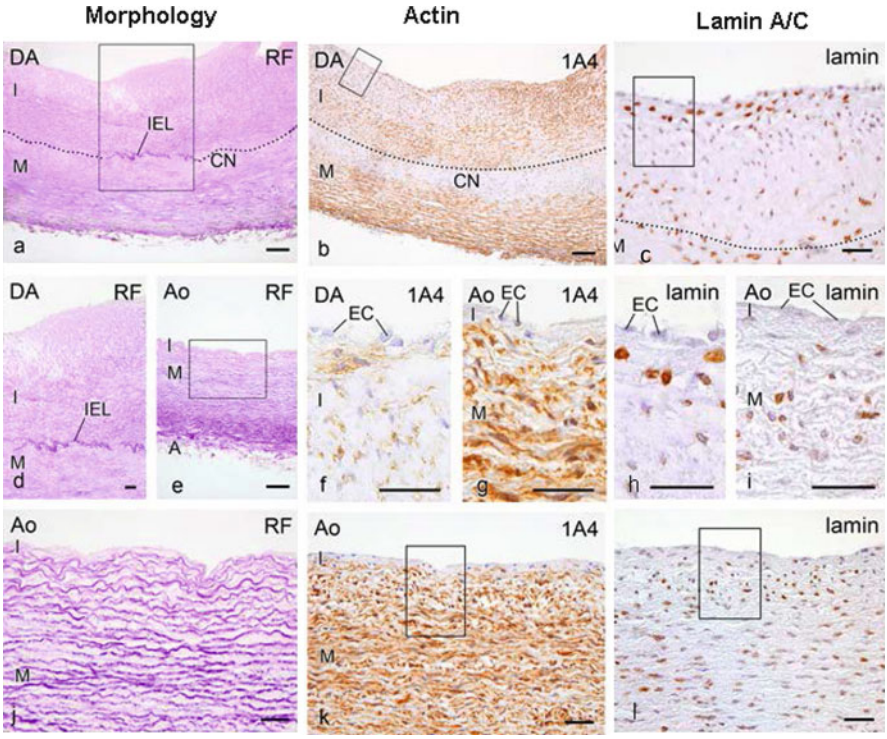

Fig. 34.2 Transverse histological sections of the ductus arteriosus (DA) and the aorta (AO). (**a**) Overview of the DA wall with a resorcin-fuchsin staining (RF) showing the morphology of a muscular artery with an internal elastic membrane (IEL) (**a**, **d**) between the intima (I) and the media (m). In the inner media, there is cytolytic necrosis (CN), which is better appreciated by the alpha smooth muscle actin loss (actin) in the smooth muscle cells (**b**). The endothelial cells (ECs) are negative for actin, also in the AO (**f**, **g**). (**e**) Overview of the elastic wall of the AO with a regular manifestation of elastic lamellae (**j**) and marked actin staining in the smooth muscle cells (**k**). There is no CN in the aorta. (**c**, **h**, **i**, **l**) In both the DA and AO lamin A/C, positive cells are found. ECs are negative for lamin A/C. Adventitia: A

revealed alpha smooth muscle actin to be present in all cases studied both in the intima (when thickened) and in the media. The expression level of alpha smooth muscle actin became less in the cases of a normal closing neonatal DA where inner media degeneration was seen with developing cytolytic necrosis (Fig. 34.2a, b). Furthermore, the expression of lamin A/C was found in all DA and the aorta with exception of the 14-week fetal DA. There was a clear difference with regard to progerin expression (Fig. 34.3). This was only found in the normal closing neonatal DA. If the latter was already fully developed including apoptosis and loss of nuclei, the progerin expression was diminished. This shows that progerin expression precedes apoptosis and degeneration. Progerin was not detected in the fetal 18-week DA, being also absent in the non-closing PDA and aorta.

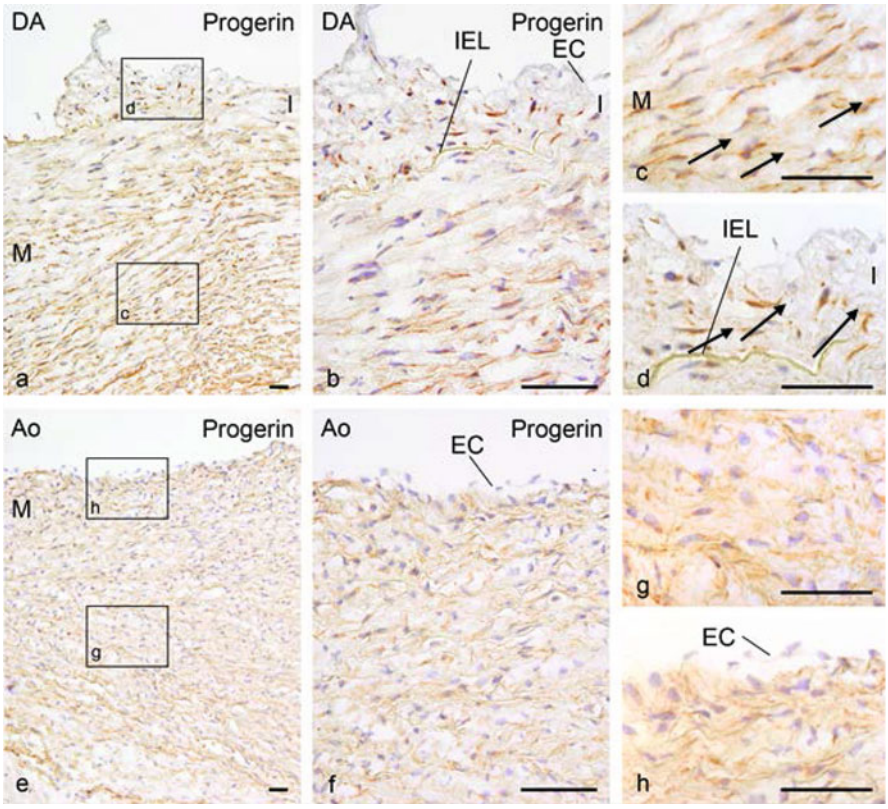

Fig. 34.3 (**a**) Overview of the intima (I) and media (M) of a 10-day-old neonatal ductus arteriosus (DA) with faint cytoplasmic and strong nuclear and perinuclear expression of progerin in the vascular smooth muscle cells of the media (**c**) and intima (**d**). The endothelial cells (ECs) are negative (**b**, **d**). (**e**) In the aortic wall (AO), no regions with strong progerin expression are found (**g**, **h**). ECs are also negative (**f**, **h**). *IEL* internal elastic lamina. *Bars* = 30 μm

34.4 Discussion

Normal ductal closure is regulated by many molecular pathways [3]. In our study, we introduced for the first time a role for lamina A/C and progerin [11]. This observation was triggered by the histopathology found in the vessel wall of young adolescents with the HGPS [8], in which premature atherosclerosis was observed. The physiological development of prenatal intimal thickening and the development of cytolytic necrosis in the DA showing contractions after birth mimic the degenerative changes seen in the HGPS. Alterations in the farnesylation process can be the cause of accumulation of progerin at the nuclear membrane. This process is associated with the initiation of apoptosis in these progerin-expressing cells [12], being characteristic for degeneration in the DA media [13]. The cause for progerin increase in the closing DA needs further investigation, but it is known that alternative splicing is affected by oxidative stress [14] which is activated during normal DA closure [15]. Accumulation of reactive oxygen species has also been observed during vascular ageing [10] in which an increase of progerin has been described in the smooth muscle cells of the coronary vessels of elderly persons as well as a gradual increase of progerin-expressing cells in other vessels with upclimbing age [7, 10]. It is noteworthy that the non-closing PDA, although only one case has been studied, and the aorta did not show progerin increase in the neonatal stage. This aspect needs further study as in families with bicuspid aortic valve (BAV) a correlation with PDA [16] has been reported. Unpublished data from our group show a diminished progerin expression in the dilated ascending aorta in BAV as compared to dilation of the aorta in tricuspid valves. The latter shows an increase in progerin indicative of advanced ageing.

Potentially, ductal closure based on increased progerin expression could lead to fetal demise in HGPS; this has, however, not been reported.

34.5 Future Directions and Clinical Applications

Further research is necessary to elucidate the role of the lamin A/C to progerin balance in vascular pathology, ageing, and the unique observation in selective DA closure at birth.

Clinical applications might be related to the use of farnesyltransferase inhibitors which may prevent onset and progression induced by the accumulation of progerin [17]. It cannot be excluded that inhibition of physiological progerin expression might prevent anatomical closure of the DA, which can be relevant in ductus-dependent congenital heart disease.

References

1. Gittenberger-de Groot AC. Persistent ductus arteriosus: most probably a primary congenital malformation. Br Heart J. 1977;39:610–8.
2. Gittenberger-de Groot AC, van Ertbruggen I, Moulaert AJMG, et al. The ductus arteriosus in the preterm infant: histological and clinical observation. J Pediatr. 1980;96:88–93.
3. Bokenkamp R, DeRuiter MC, van Munsteren C, et al. Insights into the pathogenesis and genetic background of patency of the ductus arteriosus. Neonatology. 2009;98:6–17.
4. Gittenberger-de Groot AC, Strengers JLM. Histopathology of the arterial duct (ductus arteriosus) with and without treatment with prostaglandin E1. Int J Cardiol. 1988;19:153–66.
5. Slomp J, van Munsteren JC, Poelmann RE, et al. Formation of intimal cushions in the ductus arteriosus as a model for vascular intimal thickening. An immunohistochemical study of changes in extracellular matrix components. Atherosclerosis. 1992;93:25–39.
6. Goldman RD, Shumaker DK, Erdos MR, et al. Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome. Proc Natl Acad Sci U S A. 2004;101:8963–8.
7. Olive M, Harten I, Mitchell R, Beers JK, Djabali K, et al. Cardiovascular pathology in Hutchinson-Gilford progeria: correlation with the vascular pathology of aging. Arterioscler Thromb Vasc Biol. 2010;30:2301–9.
8. Stehbens WE, Wakefield SJ, Gilbert-Barness E, et al. Histological and ultrastructural features of atherosclerosis in progeria. Cardiovasc Pathol. 1999;8:29–39.
9. McClintock D, Ratner D, Lokuge M, et al. The mutant form of lamin A that causes Hutchinson-Gilford progeria is a biomarker of cellular aging in human skin. PLoS One. 2007;2:e1269.
10. Ragnauth CD, Warren DT, Liu Y, et al. Prelamin A acts to accelerate smooth muscle cell senescence and is a novel biomarker of human vascular aging. Circulation. 2010;121:2200–10.
11. Bökenkamp R, Raz V, Venema A, et al. Differential temporal and spatial progerin expression during closure of the ductus arteriosus in neonates. PLoS One. 2011;6:e23975.
12. Bridger JM, Kill IR. Aging of Hutchinson-Gilford progeria syndrome fibroblasts is characterised by hyperproliferation and increased apoptosis. Exp Gerontol. 2004;39:717–24.
13. Tananari Y, Maeno Y, Takagishi T, et al. Role of apoptosis in the closure of neonatal ductus arteriosus. Jpn Circ J. 2000;64:684–8.
14. Kajimoto H, Hashimoto K, Bonnet SN, et al. Oxygen activates the Rho/Rho-kinase pathway and induces RhoB and ROCK-1 expression in human and rabbit ductus arteriosus by increasing mitochondria derived reactive oxygen species: a newly recognized mechanism for sustaining ductal constriction. Circulation. 2007;115:1777–88.
15. Slomp J, Gittenberger-de Groot AC, Glukhova MA, et al. Differentiation, dedifferentiation, and apoptosis of smooth muscle cells during the development of the human ductus arteriosus. Arterioscler Thromb Vasc Biol. 1997;17:1003–9.
16. Glancy DL, Wegmann M, Dhurandhar RW. Aortic dissection and patent ductus arteriosus in three generations. Am J Cardiol. 2001;87:813–5.
17. Capell BC, Olive M, Erdos MR, et al. A farnesyltransferase inhibitor prevents both the onset and late progression of cardiovascular disease in a progeria mouse model. Proc Natl Acad Sci U S A. 2008;105:15902–7.

The Multiple Roles of Prostaglandin E_2 in the Regulation of the Ductus Arteriosus

35

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Abstract

The ductus arteriosus (DA) is a shunt vessel between the aorta and the pulmonary artery during the fetal period. It is well recognized that prostaglandin E_2 (PGE_2) dilates the DA through activation of its receptor EP4 and subsequent cyclic AMP (cAMP) production during the fetal period and that oxygen constricts the DA by inhibiting potassium channels immediately after birth. In addition to the regulation of vascular tone, morphological remodeling of the DA throughout the perinatal period, such as prominent intimal thickening and poor elastogenesis, has been demonstrated.

We recently identified the molecular mechanisms of the acquisition of unique morphological remodeling in the DA during development. During the fetal period, PGE_2-EP4 signaling decreases elastic fiber formation through degradation of the cross-linking enzyme lysyl oxidase (LOX) and increases hyaluronan-mediated intimal thickening in the DA. This remodeling is mediated by activation of the EP4 receptor via diverse downstream intracellular signaling pathways. Hyaluronan-mediated intimal thickening was induced by the EP4-Gs protein-cyclic AMP-protein kinase A pathway. The attenuation of elastogenesis is mediated through a non-cyclic AMP signaling pathway, such as c-src-phospholipase C (PLC). These data suggest that placental PGE_2-mediated vascular remodeling via different signaling pathways orchestrates the subsequent luminal DA reorganization, leading to complete obliteration of the DA.

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Keywords
Ductus arteriosus • Prostaglandin E • Intimal thickening • Smooth muscle • Elastic fiber

35.1 Introduction

The ductus arteriosus (DA) normally closes immediately after birth. Although the DA is a normal and essential fetal structure, it becomes abnormal if it remains patent after birth. DA closure occurs in two phases: functional closure of the lumen in the first hours after birth by smooth muscle constriction and anatomic occlusion of the lumen over the next several days due to extensive neointimal thickening in human DA [1–3]. There are several events that promote DA constriction immediately after birth. Increasing oxygen tension and a dramatic decrease in circulating PGE_2 promote muscular constriction of the DA. In addition, DA remodeling is also necessary for its complete closure. Remodeling is characterized by (a) an area of subendothelial deposition of extracellular matrix [4], (b) the disassembly of the internal elastic lamina and loss of elastic fiber in the medial layer [5], and (c) migration into the subendothelial space of undifferentiated medial smooth muscle cells (SMCs). Some of these changes begin about halfway through gestation, and some occur after functional closure of the DA in the neonate [3, 6]. In addition to the well-known vasodilatory role of PGE_2, our findings revealed the role of PGE_2 in the anatomical closure of the DA.

35.2 The Molecular Mechanisms of Intimal Thickening of the Ductus Arteriosus

35.2.1 Hyaluronan-Mediated Intimal Thickening

PGE_2 plays a primary role in maintaining the patency of the DA via its receptor EP4. However, previous studies have demonstrated that genetic disruption of the PGE receptor EP4 paradoxically results in fatal patent DA in mice [7, 8]. In addition, double mutant mice in which cyclooxygenase (COX)-1 and COX-2 are disrupted also exhibit patent DA [9]. We found that intimal thickening was completely absent in the DA of EP4-disrupted neonatal mice [3]. Moreover, a marked reduction in hyaluronan production was found in EP4-disrupted DA, whereas a thick layer of hyaluronan deposit was present in wild-type DA. PGE_2-EP4-cyclic AMP (cAMP)-protein kinase A (PKA) signaling upregulates hyaluronan synthase type 2 mRNA, which increases hyaluronan production in the DA. Accumulation of hyaluronan then promotes SMC migration into the subendothelial layer to form intimal thickening [3].

EP4 is a Gs protein-coupled receptor that increases intracellular cAMP by adenylyl cyclases (ACs) consisting of nine different isoforms of membrane-bound forms of ACs (AC1 through AC9). We found that AC2 and AC6 are more

highly expressed in rat DA than in the aorta during the perinatal period [10]. Our data using AC subtype-targeted siRNAs and AC6-deficient mice suggest that AC6 is responsible for hyaluronan-mediated intimal thickening of the DA, whereas AC2 inhibits AC6-induced hyaluronan production. The activation of both AC2 and AC6 induces vasodilation.

35.2.2 Epac-Mediated SMC Migration

In addition to PKA, a new target of cAMP that is an exchange protein activated by cAMP has recently been discovered; it is called Epac [11]. Epac is a guanine nucleotide exchange protein that regulates the activity of small G proteins and has been known to exhibit a distinct cAMP signaling pathway that is independent of PKA [12]. There are two variants: Epac1 is expressed in most tissues, including the heart and blood vessels, whereas Epac2 is expressed in the adrenal gland and the brain. Although both Epac1 and Epac2 are upregulated during the perinatal period, Epac1, but not Epac2, acutely promotes SMC migration and thus intimal thickening in the DA [13]. Since Epac stimulation does not increase hyaluronan production, the effect of Epac1 on SMC migration is independent of that of hyaluronan accumulation, which operates through a mechanism different from that underlying PKA stimulation.

35.2.3 Regulation of Elastogenesis

Elastic fiber formation begins in mid-gestation and increases dramatically during the last trimester in the great arteries. However, the DA exhibits lower levels of elastic fiber formation [5], which may contribute to vascular collapse and subsequent closure of the DA after birth. We found that EP4 significantly inhibited elastogenesis and decreased lysyl oxidase (LOX) protein, which catalyzes elastin cross-links in DA SMCs but not in aortic SMCs. In EP4-knockout mice, electron microscopic examination showed that the DA acquired an elastic phenotype that was similar to the neighboring aorta. More importantly, human DA and aorta tissues from seven patients showed a negative correlation between elastic fiber formation and EP4 expression, as well as between EP4 and LOX expression. Together with in vitro experiments, these data suggest that PGE_2-EP4 signaling inhibits elastogenesis in the DA by degrading LOX protein. The EP4-cSrc-PLC-γ-signaling pathway, a signaling pathway that has not previously been recognized, most likely promoted the lysosomal degradation of LOX [14, 15].

35.3 Future Direction and Clinical Implications

The persistently patent DA after birth is a major cause of morbidity and mortality, especially in premature infants, that can lead to severe complications, including pulmonary hypertension, right ventricular dysfunction, postnatal infections, and

respiratory failure [16]. The incidence of DA patency has been estimated to be 1 in 500 in term newborns [17]. In preterm babies with birth weights <1,500 g, the incidence of patent DA exceeds 30 % [18]. Therefore, it is important to improve current pharmacological therapy through understanding the precise mechanisms of the regulation of the DA. Since both vascular contraction and remodeling are required for complete DA closure, pharmacological therapies that promote vasoconstriction and remodeling would be ideal for premature infants with persistently patent DAs. On the other hand, vasodilation and inhibition of intimal thickening are required for DA-dependent congenital heart diseases.

Our data suggest that PGE_2-EP4-cAMP signaling promotes hyaluronan and Epac-mediated intimal thickening and that the EP4-PLC pathway attenuates elastogenesis in the DA. These cascades of events via different signaling pathways are thought to orchestrate the subsequent luminal DA reorganization (Fig. 35.1),

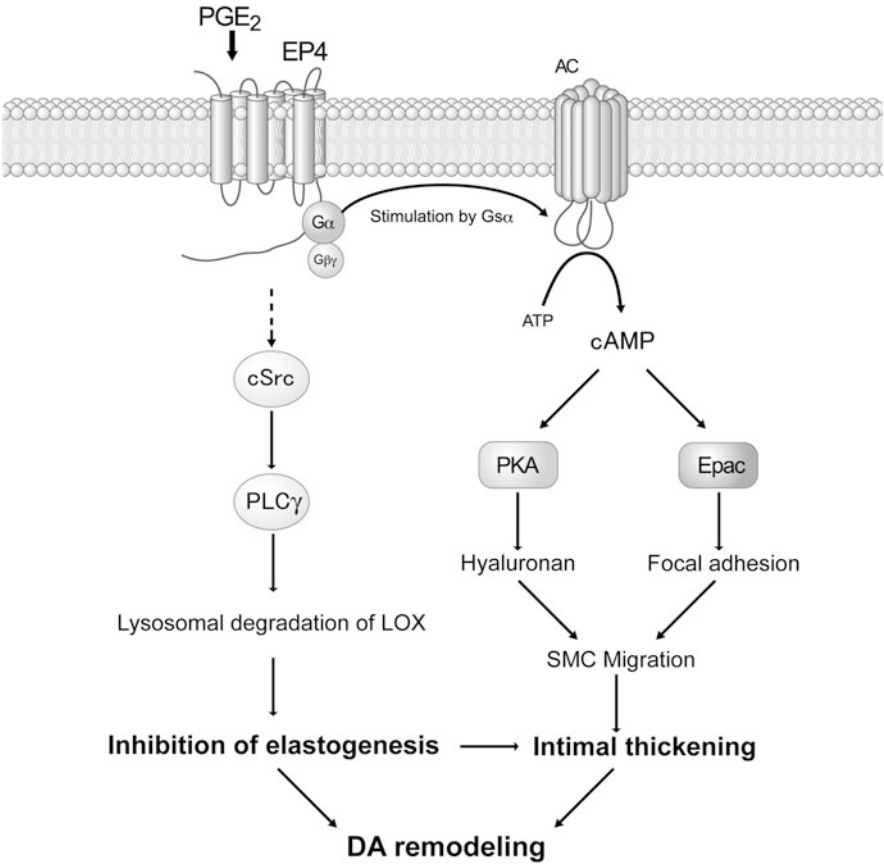

Fig. 35.1 The diverse EP4 signaling pathways. Both PKA and Epac synergistically promoted intimal cushion formation in the DA, but they work in two distinct ways. The cSrc-PLC pathway inhibited elastogenesis via degrading LOX proteins

leading to complete obliteration of the DA. In addition to its role in controlling vascular tone in the functional closure of the DA, the vascular remodeling of the DA is now attracting considerable attention as a target for novel therapeutic strategies for patients with persistently patent DA and DA-dependent cardiac anomalies.

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References

1. Smith GC. The pharmacology of the ductus arteriosus. Pharmacol Rev. 1998;50:35–58.
2. Clyman RI. Mechanisms regulating the ductus arteriosus. Biol Neonate. 2006;89:330–5.
3. Yokoyama U, Minamisawa S, Quan H, Ghatak S, Akaike T, Segi-Nishida E, Iwasaki S, Iwamoto M, Misra S, Tamura K, Hori H, Yokota S, Toole BP, Sugimoto Y, Ishikawa Y. Chronic activation of the prostaglandin receptor EP4 promotes hyaluronan-mediated neointimal formation in the ductus arteriosus. J Clin Invest. 2006;116:3026–34.
4. Gittenberger-de Groot AC, Strengers JL, Mentink M, Poelmann RE, Patterson DF. Histologic studies on normal and persistent ductus arteriosus in the dog. J Am Coll Cardiol. 1985;6:394–404.
5. De Reeder EG, Girard N, Poelmann RE, Van Munsteren JC, Patterson DF, Gittenberger-De Groot AC. Hyaluronic acid accumulation and endothelial cell detachment in intimal thickening of the vessel wall. The normal and genetically defective ductus arteriosus. Am J Pathol. 1988;132:574–85.
6. Slomp J, Gittenberger-de Groot AC, Glukhova MA, Conny van Munsteren J, Kockx MM, Schwartz SM, Koteliansky VE. Differentiation, dedifferentiation, and apoptosis of smooth muscle cells during the development of the human ductus arteriosus. Arterioscler Thromb Vasc Biol. 1997;17:1003–9.
7. Segi E, Sugimoto Y, Yamasaki A, Aze Y, Oida H, Nishimura T, Murata T, Matsuoka T, Ushikubi F, Hirose M, Tanaka T, Yoshida N, Narumiya S, Ichikawa A. Patent ductus arteriosus and neonatal death in prostaglandin receptor EP4-deficient mice. Biochem Biophys Res Commun. 1998;246:7–12.

8. Nguyen M, Camenisch T, Snouwaert JN, Hicks E, Coffman TM, Anderson PA, Malouf NN, Koller BH. The prostaglandin receptor EP4 triggers remodelling of the cardiovascular system at birth. Nature. 1997;390:78–81.
9. Loftin CD, Trivedi DB, Tiano HF, Clark JA, Lee CA, Epstein JA, Morham SG, Breyer MD, Nguyen M, Hawkins BM, Goulet JL, Smithies O, Koller BH, Langenbach R. Failure of ductus arteriosus closure and remodeling in neonatal mice deficient in cyclooxygenase-1 and cyclooxygenase-2. Proc Natl Acad Sci U S A. 2001;98:1059–64.
10. Yokoyama U, Minamisawa S, Katayama A, Tang T, Suzuki S, Iwatsubo K, Iwasaki S, Kurotani R, Okumura S, Sato M, Yokota S, Hammond HK, Ishikawa Y. Differential regulation of vascular tone and remodeling via stimulation of type 2 and type 6 adenylyl cyclases in the ductus arteriosus. Circ Res. 2010;106:1882–92.
11. de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature. 1998;396:474–7.
12. Bos JL. Epac: a new cAMP target and new avenues in cAMP research. Nat Rev Mol Cell Biol. 2003;4:733–8.
13. Yokoyama U, Minamisawa S, Quan H, Akaike T, Suzuki S, Jin M, Jiao Q, Watanabe M, Otsu K, Iwasaki S, Nishimaki S, Sato M, Ishikawa Y. Prostaglandin E2-activated Epac promotes neointimal formation of the rat ductus arteriosus by a process distinct from that of cAMP-dependent protein kinase A. J Biol Chem. 2008;283:28702–9.
14. Yokoyama U, Minamisawa S, Shioda A, Ishiwata R, Jin MH, Masuda M, Asou T, Sugimoto Y, Aoki H, Nakamura T, Ishikawa Y. Prostaglandin E2 inhibits elastogenesis in the ductus arteriosus via EP4 signaling. Circulation. 2014;129:487–96.
15. Yokoyama U, Iwatsubo K, Umemura M, Fujita T, Ishikawa Y. The prostanoid EP4 receptor and its signaling pathway. Pharmacol Rev. 2013;65:1010–52.
16. Hermes-DeSantis ER, Clyman RI. Patent ductus arteriosus: pathophysiology and management. J Perinatol. 2006;26 Suppl 1:S14–8; discussion S22-13.
17. Mitchell SC, Korones SB, Berendes HW. Congenital heart disease in 56,109 births. Incidence and natural history. Circulation. 1971;43:323–32.
18. Van Overmeire B, Allegaert K, Casaer A, Debauche C, Decaluwe W, Jespers A, Weyler J, Harrewijn I, Langhendries JP. Prophylactic ibuprofen in premature infants: a multicentre, randomised, double-blind, placebo-controlled trial. Lancet. 2004;364:1945–9.

Developmental Differences in the Maturation of Sarcoplasmic Reticulum and Contractile Proteins in Large Blood Vessels Influence Their Contractility

36

Emiko Hayama and Toshio Nakanishi

Keywords
Ductus arteriosus • mRNA expression • Contractile system • Development

Developmental changes in the contractile system of blood vessels such as the ductus arteriosus (DA), pulmonary artery (PA), and aorta (Ao) have not been investigated extensively. We assessed the developmental changes in the expression of genes that regulate vasoconstriction of fetal blood vessels.

DA, PA, and Ao were taken from rabbit fetuses at 21, 27, and 30 days of gestation (full term = 31 days) as well as 2-day-old rabbits. Total DA, PA, and Ao RNA were isolated from pooled segments. Expression of target mRNAs was quantified using absolute quantitative real-time PCR:

1. *Contractile proteins* Contractile activity in smooth muscles is determined primarily by the phosphorylation state of myosin regulatory light chain (MRLC). During muscle contraction, intracellular Ca^{2+} levels increase substantially, and binding of Ca^{2+} to calmodulin activates MLC kinase, which then phosphorylates MRLC. Expression of calmodulin and MLC kinase was not significantly different among the vessels. Expression of tropomyosin 2 was higher in DA compared to PA.
2. *Sarcoplasmic reticulum (SR)* Cytosolic Ca^{2+} levels are increased through Ca^{2+} release from SR and Ca^{2+} entry from the extracellular space via Ca^{2+} channels. Expression of SR cardiac-type ryanodine receptor (RYR) increased throughout fetal maturation and was much higher than skeletal-type RYR. Expression of SR calcium storage protein calsequestrin-2 increased with development in Ao but

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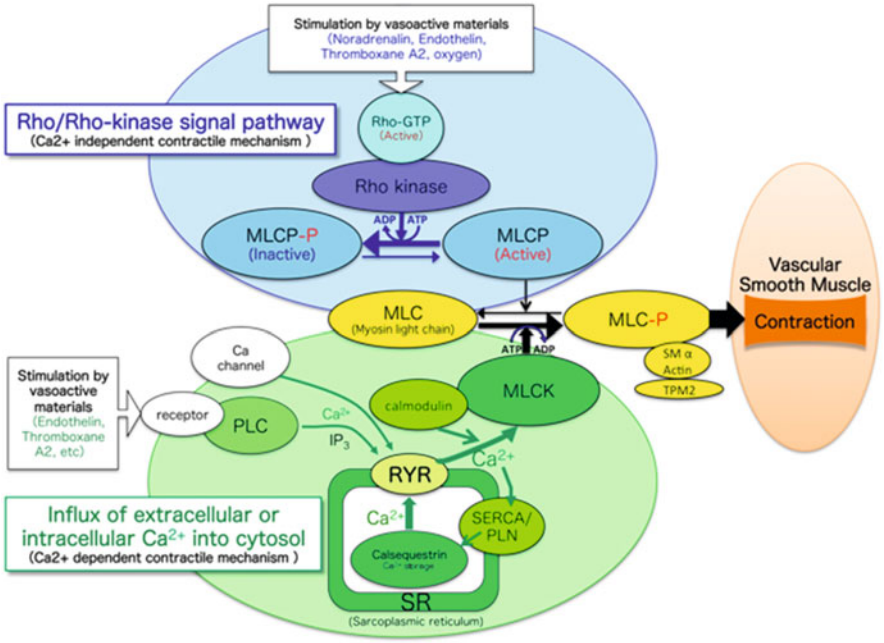

Fig. 36.1 Vascular smooth muscle contraction. Contractile activity in smooth muscle (SM) is determined primarily by the phosphorylation state of myosin regulatory light chain (MRLC). Increase in intracellular Ca^{2+} levels leads to Ca^{2+}-calmodulin binding, which activates MLC kinase (MLCK) to phosphorylate MRLC. Cytosolic Ca^{2+} is increased through Ca^{2+} release from the sarcoplasmic reticulum (SR) and Ca^{2+} entry from extracellular space via Ca^{2+} channels. The Ca^{2+}-independent Rho/Rho kinase pathway inhibits MLC phosphatase (MLCP) activity and promotes phosphorylation of MLC. *RYR* ryanodine receptor, *SERCA* sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, *PLN* phospholamban, *TPM2* tropomyosin 2

not in DA and PA, with expression levels remaining very low in the latter two. Expression of SR Ca^{2+} pump regulator phospholamban increased with development in PA and Ao but remained very low in DA. The expression of SR genes differs significantly at development stages and is vessel dependent, indicating differential maturity of SR in fetal vessels.

3. *Rho/Rho-kinase* The Ca^{2+}-independent Rho/Rho kinase pathway inhibits MLC phosphatase activity and promotes phosphorylation of MLC. Expression of small GTPase RhoB and Rho kinase-1 was higher than that of RhoA and Rho kinase-2. The expression levels of these Rho/Rho kinase pathway genes were similar in fetal and newborn vessels.

In conclusion, contraction of the premature DA, PA, and Ao may be regulated predominantly by the Rho/Rho kinase pathway, owing to the poor expression of the component protein genes in the immature SR. DA contractile systems may be well developed compared with those of the surrounding PA and Ao (Fig. 36.1).

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Fetal and Neonatal Ductus Arteriosus Is Regulated with ATP-Sensitive Potassium Channel

37

Kazuo Momma, Mika Monma, Katsuaki Toyoshima, Emiko Hayama, and Toshio Nakanishi

Keywords

Ductus arteriosus • Fetus • ATP-sensitive potassium channel • Sulfonylurea • Potassium channel opener

The fetal patency and neonatal closure of the ductus arteriosus (DA) are regulated with oxygen and prostaglandins. The proposed oxygen sensors of fetal and neonatal DA include P450-endothelin and the Kv channel [1]. We hypothesized that the ATP-sensitive potassium channel (K_{ATP} channel) is another oxygen sensor [2].

Fetal and neonatal DA was studied with Wistar rats; sulfonylurea drugs including tolbutamide, chlorpropamide, gliclazide, glimepiride, and glibenclamide (K_{ATP} channel inhibitors); diazoxide and pinacidil (K_{ATP} channel openers, KCOs); and rapid whole-body freezing (Fig. 37.1).

Tolbutamide, chlorpropamide, and gliclazide easily passed across the placenta and constricted fetal DA dose-dependently following orogastric administration to near-term pregnant rats. The fetal DA constricted 30 % with clinical doses of sulfonylurea drugs and closed completely with larger doses.

Glimepiride and glibenclamide passed across the placenta minimally and only mildly constricted the fetal DA after maternal administration, but constricted and

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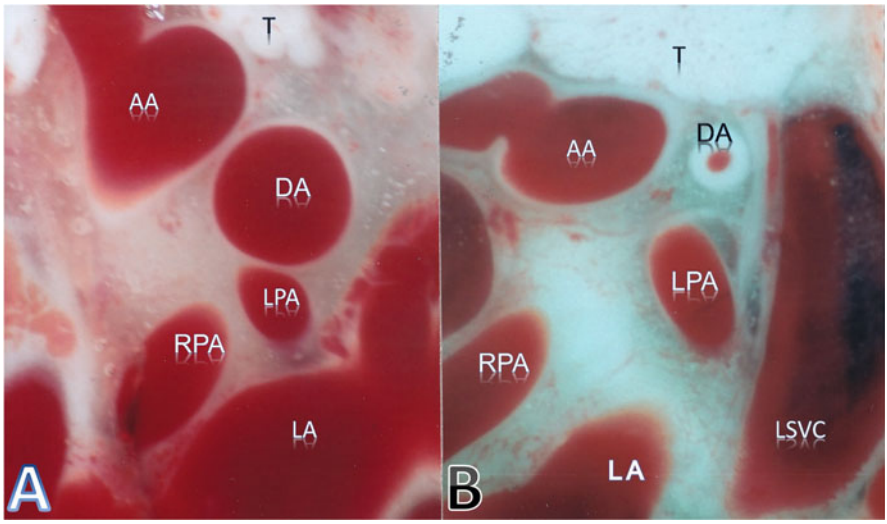

Fig. 37.1 The fetal ductus arteriosus was studied in the near-term fetus or newborn rat following rapid whole-body freezing, cutting on the freezing microtome, with a microscope and a micrometer. The control fetus shows a widely open ductus (**a**), and the fetus with glibenclamide (10 mg/kg; 10–100 times clinical dose injected at 1 h before) shows severely constricted ductus (**b**). *AA* aortic arch, *DA* ductus arteriosus, *LA* left atrium, *LPA* left pulmonary artery, *LSVC* left superior vena cava, *RPA* right pulmonary artery, *T* thymus

closed the fetal DA dose-dependently with direct fetal injection. Fetal DA closure was associated with hydrops and fetal death.

Diazoxide and pinacidil delayed DA closure following neonatal injection immediately postnatally and dilated the closing DA with injection at 60 min postnatally.

All tested sulfonylurea drugs constricted fetal DA dose-dependently and with complete closure at large doses. KCOs dilated the neonatal DA. These results indicate physiological regulation of fetal and neonatal DA with K_{ATP} channels.

This study has several clinical implications. Sulfonylurea-associated fetal death was first reported 50 years ago. The mechanism of death remained unclear prior to this study. Sulfonylureas may be useful for closing patent DA in premature neonates.

Recently reported neonatal DA reopening associated with the use of diazoxide for hyperinsulinemic hypoglycemia has been proved experimentally. DA-dilating effect of KCO drugs may be useful as a bridge to surgery in neonatal DA-dependent congenital heart diseases.

References

1. Coceani F, Baragatti B. Mechanisms for ductus arteriosus closure. Semin Perinatol. 2012;36:92–7.
2. Nakanishi T, Gu H, Momma K, et al. Mechanisms of oxygen-induced contraction of ductus arteriosus isolated from the fetal rabbit. Circ Res. 1993;72:1218–23.

Part VIII

Conduction System and Arrhythmia

Perspective

Michael Artman

One of the fascinating and essential characteristics of the cardiovascular system is that it functions automatically without requiring conscious input. In other words, the normal heart beats regularly and spontaneously regardless of whether or not the individual is thinking about it. Electrical impulses arise in the sinoatrial node, pass through the atria, pause at the atrioventricular node, and then are distributed in a tightly orchestrated spatial and temporal manner to the ventricles for coordinated and effective pumping activity. This elegantly synchronized process repeats itself over and over approximately three billion times during the average human life. Even minor disruptions in this normal process can have devastating consequences. Considerable morbidity and mortality result from abnormalities in intrinsic cardiac pacemaker activity and conduction of impulses. Congenital and acquired arrhythmias remain a significant health problem. Consequently, a clear understanding of the molecular and cellular processes involved in the formation and maintenance of normal electrical signaling in the heart has profound implications for human cardiovascular health and disease. Furthermore, it is likely that insights into the developmental processes involved in building a cardiac conduction system will likely have implications for understanding fundamental biology applicable to other organ systems.

This part reviews and summarizes the current state of understanding of the development of spontaneous pacemaker activity and the cardiac conduction system. Christoffels' group reviews the signaling pathways and molecules involved in the

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development of the cardiac conduction system. The authors emphasize the importance of understanding the three-dimensional architecture of genomic loci to provide insight into the regulation (normal and abnormal) of gene expression. Mikawa and colleagues identify and characterize a previously unrecognized source of cells that are destined to become pacemaker cells, even before cardiac morphogenesis begins. These results will likely lead to better understanding of the mechanisms involved in pacemaker cell differentiation, which in turn may ultimately have therapeutic implications. Additional information on the mechanisms, molecular signals, and pathways involved in specification of cell fate are provided by Asai et al. and by Morikawa et al. Taken together, the papers presented in Part VIII provide an interesting, informative, and elegant overview of the current understanding of pacemaker and conduction system development. Moreover, they provide a road map for future investigations into this essential and fundamental biological process.

Regulation of Vertebrate Conduction System Development

38

Jan Hendrik van Weerd and Vincent M. Christoffels

Abstract

The cardiac conduction system (CCS) consists of distinctive components that initiate and conduct the electrical impulse required for the coordinated contraction of the cardiac chambers. The development of the CCS involves complex regulatory networks of transcription factors that act in stage, tissue and dose-dependent manners. As disrupted function or expression of these factors may lead to disorders in the development or function of components of the CCS associated with heart failure and sudden death, it is crucial to understand the molecular and cellular mechanisms underlying their complex regulation. Here, we discuss the regulation of genes driving CCS-specific gene expression and demonstrate the complexity of the mechanisms governing their regulatory networks. The three-dimensional conformation of chromatin has recently been recognized as an important regulatory layer, shaping the genome in regulatory domains and physically wiring gene promoters to their regulatory sequences. Knowledge of the mechanisms by which distal-acting regulatory sequences exert their function to drive tissue-specific gene expression and understanding how the three-dimensional chromatin landscape is involved in this regulation will increase our understanding of how disease-associated genomic variation affects the function of such sequences.

Keywords

Heart development • Conduction system • Sinus node • Atrioventricular node • Transcriptional regulation • Functional genomics • Patterning • Pacemaker

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38.1 Introduction

The cardiac conduction system (CCS) initiates and propagates the electrical impulse that is required for the rhythmic and synchronized contraction of the heart. The impulse initiates in the sinoatrial node (SAN) and is rapidly propagated through the atria, thereby activating the contraction of the atrial myocardium. The impulse is then propagated through the atrioventricular node (AVN), the only electrical connection between the atria and ventricles. The AVN delays conduction of the impulse, allowing for the atrial contraction and ventricular filling to complete before the ventricles contract. Further propagation of the impulse to the fast-conducting atrioventricular bundle (AVB), bundle branches (BBs) and Purkinje network causes the depolarization of the ventricular myocardium, leading to ventricular contraction. The development of the CCS is regulated by transcription factors that act in strictly stage, tissue and dose-dependent manners [1, 2]. A disruption in the function or expression of these factors could lead to disorders in the development or function of the CCS that can lead to lethal arrhythmias and heart failure. Knowledge of the mechanisms underlying regulation of genes involved in CCS development is therefore crucial.

38.2 Genetic Pathways Controlling SAN and AVC Development

The heart is the first organ to form during embryonic development and starts as a primitive, linear tube with the inflow region at the caudal side and the outflow region at the cranial side. The slow conductive properties of the embryonic muscle cells within the primitive tube at this stage and dominant pacemaker activity at the caudal end cause a slow peristaltic pattern of contraction along the tube to propagate the blood. At this stage, the entire sinus venosus acts as pacemaker, characterized by the expression of the pacemaker channel Hcn4, a member of the family of channels responsible for the hyperpolarization-activated current *if* that is crucial for the pacemaker potential [3, 4]. The heart tube elongates by the addition of rapidly proliferating progenitor cells that differentiate to cardiac muscle. This implies that cells added to the inflow tract will acquire dominant pacemaker activity. With further development, specific regions in the heart tube start to divide rapidly and activate a working myocardial gene program, resulting in the ballooning of the primitive atrium and ventricle. Concomitant with the ballooning of the primitive atria is the formation of the sinus venosus including the SAN. Dominant pacemaker activity will gradually be confined to the SAN at the junction of the sinus venosus and atrium.

The transcriptional activator Tbx5 is required for the sinus venosus expression of Shox2 [5], a homeobox transcription factor necessary for SAN formation and function [6, 7]. Shox2 represses cardiac homeobox transcription factor Nkx2-5 [6]. In the chambers, Nkx2-5 activates chamber-specific genes including *Nppa* and high-conductance gap junction subunit-encoding genes *Gja5* (Cx40) and *Gja1* (Cx43), whereas it represses SAN/CCS-specific genes *Hcn4* and T-box transcription

factor *Tbx3* [8]. Tbx3 is required for the formation of the SAN by directly repressing atrial myocardial genes *Gja5*, *Gja1* and *Nppa* to prevent atrialization of the SAN and indirectly activating *Hcn4* and other SAN genes [9, 10].

During ballooning of the primitive cardiac chambers, the region in between the atria and ventricles does not proliferate and forms a constriction, the atrioventricular canal (AVC). Bmp2 expression in the AVC activates the expression of Tbx3 and Tbx2 [11]. Together with Msx2, these T-box factors repress the working myocardial gene program in the AVC and AVC-derived AVN [12, 13] and stimulate the pacemaker gene program and the program required for the formation of the AV cushions. Within the AVC, Tbx2 and Tbx3 interact with Nkx2-5 to repress genes that are activated by Nkx2-5 and Tbx5 in the working myocardium of the atria and ventricles [14–16]. Tbx2 and Tbx3 thus suppress working myocardial differentiation of the AVC, thereby causing the retention of the primitive phenotype of slow conduction and low rates of proliferation, providing a primitive morphological and functional constriction in between the atrial and ventricular chambers. Other factors that regulate the formation of the AVC and its border with the chamber myocardium include Wnts, acting upstream of Bmp2; Hey1 and Hey2, Notch target genes expressed in the chambers that suppress Tbx2 [17]; Tbx20, which represses BMP-mediated activation of Tbx2 in the chambers [18]; and Gata4/6, which act in complex with Smads and histone acetyltransferases (HATs) to activate AVC-specific enhancers in the AVC and with histone deacetylases (HDACs) and Hey1/2 to suppress these enhancers in the chambers [19]. The resulting pattern of conduction—fast in the atria, slow in the AVC and fast in the ventricles—results in the alternating contraction pattern of the chambers and an ECG that resembles the adult ECG (Fig. 38.1a).

38.3 Transcriptional Regulation of CCS Genes

Although the expression patterns and functions of genes involved in the development of the cardiac conduction system are relatively well studied, little is known about the molecular mechanisms underlying their regulation of expression. Tissue-specific gene expression often involves long-range regulatory elements, such as enhancers, which dictate the strictly time-, tissue- and dosage-dependent expression of their target genes. The identification and function of such enhancers is therefore highly relevant to fully understand the complex regulatory networks in CCS formation. However, to date only few studies have been carried out investigating in depth the regulation of genes driving CCS development.

The T-box transcription factor Tbx5 plays indispensable roles in the early patterning of the heart and CCS and is involved in limb development [15, 20, 21]. Mutations in *TBX5* are associated with Holt-Oram syndrome, a developmental disorder characterized by hand-heart defects [22, 23]. Using modified bacterial artificial chromosomes (BACs), the regulatory landscape of the *TBX5* locus was determined, and within this landscape, multiple cardiac-specific enhancers were identified by utilizing multiple genome-wide ChIP-seq datasets and evolutionary

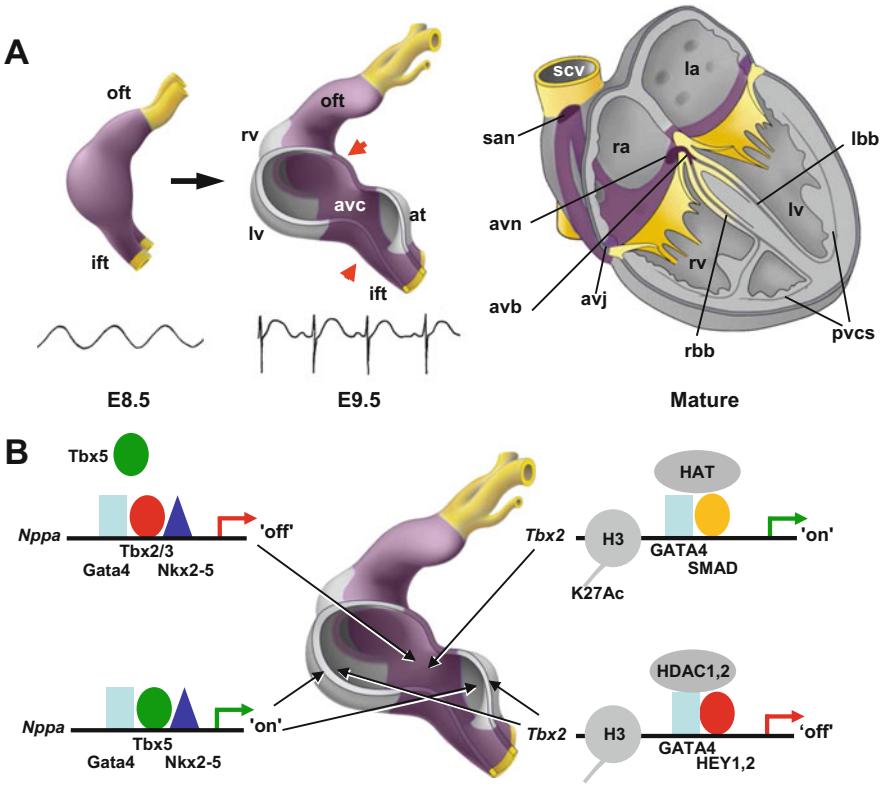

Fig. 38.1 Schematic overview of cardiac development. (**a**) The early heart tube has a primitive phenotype of slow conduction, represented by a sinusoidal ECG. With further development, regions at the outer curvatures of the primary heart tube expand and obtain a working myocardium phenotype of fast conduction (*grey*). The sinus venosus (sv), AVC, outflow tract (oft) and inner curvatures retain their primitive pacemaker-like phenotype and slow conductivity (*purple*). A more mature ECG can be derived from these hearts. Eventually, these non-chamber myocardial regions will give rise to the mature conduction system components. (**b**) The transcriptional repressors Tbx2 and Tbx3 compete with the transcriptional activator Tbx5 to regulate their target genes. In the AVC, expression of chamber myocardium genes like *Nppa* is actively repressed by Tbx2 and Tbx3, whereas in the developing chambers Tbx5 activates these genes. Transcriptional activation in the AVC is regulated by GATA binding site-dependent histone modifications which render the chromatin more (e.g. HATs) or less (e.g. HDACs) accessible for transcription factors to bind their target regulatory sequences, resulting in activation or repression of gene expression

conservation. These enhancers were shown to recapitulate part of the TBX5 expression pattern in the heart, but interestingly, none of these fragments drove reporter expression in the limbs, suggesting that the *cis*-regulation of TBX5 in the heart and limbs is compartmentalized [24]. Such knowledge can highly improve the understanding of the mechanisms underlying the development of congenital heart diseases by decoupling the heart and hand phenotypes seen with Holt-Oram syndrome, thereby presenting more compartmentalized phenotypes compared to disorders caused by protein-coding mutations.

Another example of how enhancer-mediated gene expression is involved in the tight regulation of the CCS is presented by the transcriptional repressor *Id2*. This factor was identified by serial analysis of gene expression (SAGE) as having CCS-specific expression. *Id2* is expressed throughout development in the AVB and BB and in non-CCS compartments such as the AV endocardial cushions and valves. The requirement of *Id2* for ventricular CCS structure and function was demonstrated as *Id2*-deficient mice exhibit structural and functional conduction system abnormalities, including left bundle branch block. *Id2* is cooperatively regulated by *Nkx2-5* and *Tbx5* in the developing ventricular conduction system by binding of both *Tbx5* and *Nkx2-5* to a 1052 bp fragment of the *Id2* promoter. Mutation of the *Tbx5* binding site within this promoter region completely abolished CCS expression whereas extracardiac expression was unaltered, illustrating the specificity of this transcriptional mechanism in the coordinated development of the ventricular conduction system [20].

The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. Using a transgenic BAC approach, it was shown that the regulatory regions sufficient to recapitulate the endogenous Hcn4 expression pattern in the SAN, AVN, His bundle, bundle branches and left ventricular Purkinje fibres reside within the region covered by one bacterial artificial chromosome (BAC) of 200 kbp [25]. Using transgenic mouse assays, multiple evolutionary conserved cis-acting regulatory sequences were identified to drive *Hcn4* expression in the AV conduction system. One of these regions drives reporter expression specifically in the non-chamber myocardium in a Mef2c-dependent manner. Furthermore, depletion of histone deacetylases resulted in ectopic expression of reporter activity in chamber myocardium, revealing a role for histone modifications in Mef2c-regulated enhancer-mediated expression of Hcn4 in components of the CCS [26].

More recently, Contactin-2 (*Cntn2*), a cell adhesion molecule critical for neuronal patterning and ion channel clustering, was described as a marker for the ventricular conduction system, with expression in the AVB, BBs and Purkinje fibres. Using a GFP-modified BAC, the boundaries of the regulatory domain involved in the control of *Cntn2* expression were identified, since reporter activity of the modified BAC completely recapitulates endogenous *Cntn2* expression [27]. Such knowledge facilitates in the identification of single, individual regulatory elements driving CCS development and will greatly add to our understanding of how genes involved in the complex development of CCS components are regulated.

Enhancer function is regulated by modifications of specific histone tails that mark active or poised enhancers. Active enhancers are associated with an open, accessible chromatin state, whereas poised enhancers are associated with dense, closed chromatin. Histone modifiers such as histone deactelyases (HDACs), histone methyltransferases (HMTs) and histone acetyltransferases (HATs) therefore regulate the accessibility of long-range regulatory sequences, allowing for the binding by cell type-specific transcription factors to activate transcription in a tissue-dependent manner. Specification of the AVC is regulated by Gata4, which activates AVC enhancers in synergy with Bmp2/Smad signaling to recruit HATs

such as p300 [19, 28]. This leads to H3K27 acetylation, a marker of active enhancers. In contrast, in chamber myocardium, Gata4 cooperates with HDACs and chamber-specific genes Hey1 and Hey2, leading to H3K27 deacetylation and repression (Fig. 38.1b) [19].

38.4 Common Genomic Variants Influence CCS Function

The importance of the strict regulation of the spatial and temporal expression of CCS genes is illustrated by findings from recent genome-wide association studies, which revealed common genomic variation to be associated with conduction parameters like PR interval and QRS duration. Such variation was identified in non-coding regions flanking genes encoding ion channels like *SCN5A/10A*, *KCNQ1* and *KCNH2* and cardiac transcription factors like *NKX2-5*, *MEIS1* and *TBX3/5*, indicating they might affect the function of enhancers controlling the precise regulation of these genes [29–31]. Tbx5 is broadly expressed and acts as transcriptional activator, inducing transcription of genes involved in cardiac differentiation [15, 20]. The activity domain of Tbx3 is much more restricted and confined to the developing and mature CCS, where it acts as a transcriptional repressor, thereby imposing the pacemaker phenotype on cells within its expression domain [10, 32]. Tbx3 and Tbx5 both recognize the same regulatory sequences [33], suggesting that these factors compete for binding and implicating a fine balance between activation and repression of CS genes by these factors. The precise regulation of transcription and activity of both factors is therefore crucial for proper CCS patterning, and minor changes in regulatory elements controlling the regulation of expression of these factors could thus potentially have large consequences for CCS function and development. Knowledge of the mechanisms by which such developmental genes are regulated to exert their spatio-temporal transcriptional activity is therefore crucial in the understanding of how variation identified by GWAS influences development.

38.5 3D Architecture Regulates Transcription

Physical enhancer-promoter contacts are a requirement for enhancer-mediated cell type-specific gene expression, and as such, the three-dimensional topology of chromatin plays an indispensable role in gene regulation by physically wiring long-range regulatory sequences with their target promoters [34]. Several protein complexes, including CTCF, cohesin and mediator, have been proposed to be involved in the organization of these contacts [35]. Furthermore, recent data suggest that such genomic structural organizers not only mediate single enhancer-promoter contacts but also mediate the organization of the genome in relatively cell-type invariant topologically associated domains (TADs) within which sequences particularly contact each other. Genes located within the same TAD exhibit greater expression correlation than genes located in distinct ones,

suggesting that such domains may act as a backbone for tissue-specific regulatory contacts [36]. The recent emergence of techniques aimed at capturing the 3D conformation of genomic loci [37] therefore provides valuable tools to elucidate regulatory mechanisms on the chromatin level.

38.6 Regulation of Tbx3 by a Large Regulatory Domain

The evolutionary conserved *Tbx3/5* genomic locus is one of the few genomic loci of which the 3D architecture has been studied and reveals an example of the complexity of gene regulation on the level of chromatin topology. *Tbx3* and *Tbx5* form an evolutionary conserved gene cluster derived from a primordial T-box gene [38] and, as mentioned above, play crucial roles in the formation and function of the cardiac conduction system. Using circular chromosome conformation capture sequencing (4C-seq), which captures all the genomic regions in close proximity to a chosen point of view [39], the 3D architecture of the Tbx3/5 locus was probed, and genomic regions contacting *Tbx3* or *Tbx5* were identified in different tissues (Fig. 38.2a). Interestingly, these data revealed that the regulatory landscape is in a preformed conformation that is similar in embryonic heart, brain and limb. Rather than the de novo formation of enhancer-promoter loops upon binding by cell-type relevant transcription factors to initiate transcription, the locus is in a fixed, permissive structure in which enhancer-promoter loops are pre-existing [40]. Such a permissive structure has previously been described for different loci, including the *Hox* and *Shh* gene loci. Long-range enhancer-promoter contacts in these loci were shown to be irrespective of cell type, revealing a preformed topology [36, 41]. The permissive, preformed nature of these loci was exemplified by the fact that even in the absence of a distal-acting *Shh* enhancer, contacts between the *Shh* promoter and the enhancer region still occur [41]. The benefit of such preformed regulatory landscapes is believed to lie in the ease by which tissue-specific transcription factors can utilize preformed contacts to target the gene of interest, involving only slight variations in internal contacts within an otherwise rigid and conserved structure. In agreement with this, small differences in contact profiles for the different tissue types were observed in the *Tbx3/5* locus despite the fact that the domain is largely preformed, most probably caused by cell type-specific transcription factor mediated enhancer activation. Among the multiple sites contacting *Tbx3* in the gene desert upstream of the gene, two evolutionary conserved enhancers have been identified that also contact each other. They are bound by cardiac-specific transcription factors Nkx2-5, Gata4, Tbx5 and Tbx3 and were shown to respond to a BMP-mediated signalling pathway to drive atrioventricular conduction system expression of *Tbx3* [40].

As mentioned before, Tbx3 and Tbx5 are expressed in overlapping patterns and have overlapping functions in CCS development. It could therefore be expected that both genes share common regulatory mechanisms on a genomic level. Studies on the transcriptional regulation of other clustered developmental genes, like the *Irx* and *Hox* clusters, revealed that regulatory sequences are not uniquely associated