Chapter 10

Unique Phenotypes of Endothelial Cells in Developing Arteries: A Lesson from the Ductus Arteriosus

Norika Mengchia Liu and Susumu Minamisawa

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66501

Abstract

Endothelial cells (ECs) play a critical role in regulating vascular pathophysiology. Various growth factors and relaxation factors such as vascular endothelial growth factor (VEGF) and nitric oxide (NO), which are derived from ECs, are known to maintain homeostasis and regulate vessel remodeling. Although the inner lumens of all types of vessels are covered by an EC monolayer, the characteristics of ECs differ in each tissue and developing stage of a vessel. Previously, we identified the heterogeneity of ECs of the ductus arteriosus (DA) by analyzing its gene profiles. The DA is a fetal artery that closes immediately after birth due to the changes in concentrations of oxygen and vasoactive factors such as NO and prostaglandin E. Studying the unique gene profile of ECs in the DA can therefore uncover the novel key genes involved in developing vascular function and morphology such as O_2 sensitivity and physiological vascular remodeling. A comprehensive gene analysis identified a number of genes related to morphogenesis and development in the DA. In this chapter, we discuss the heterogeneity of vascular ECs in the developing vessel in the DA.

Keywords: vascular endothelial cells, ductus arteriosus, vascular remodeling, comprehensive gene analysis, oxygen, vitamin A

1. Introduction

The endothelial cells (ECs) in vessels control the vascular tone, permeability, attraction of blood cells, which exhibit both innate and adaptive immunity, and migration/proliferation of underlying cells such as pericytes and smooth muscle cells (SMCs). To accomplish these roles, vascular ECs exhibit phenotypic heterogeneity during development in a time- and tissue-specific manner. The most significant diversity of ECs involves the differences

between arteries and veins as well as between large and small vessels. ECs undergo constant changes in phenotype depending on different situations, both physiological and pathological. Physiological angiogenesis occurs during development and repair processes. Many events in vascular development during gestation are reciprocated in the adult neovascularization that takes place in wound healing and ischemic disease treatment. In these cases, ECs must express pro-angiogenic factors. Pathological angiogenesis is often implicated as the abnormal proliferation of ECs such as that seen in tumorigenesis. Accordingly, many cancer studies have focused on vascular endothelial growth factor (VEGF), a pro-angiogenic factor produced from ECs. Endothelial damage and dysfunction causes cardiovascular diseases. For example, endothelial dysfunction reduces nitric oxide (NO) production, which decreases vasodilatory effects on SMCs. In addition, a decrease in NO production is also involved in the attraction of leucocytes and the production of various growth factors that leads to unregulated intimal thickening (**Figure 1**). Therefore, ECs play a central role in modifying the phenotypes of vessels. ECs have different roles depending on where they are located. For instance, in a developing vessel, ECs become tip cells or other stalk cells to regulate different molecular signaling to guide vessel sprouting [1]. Endothelial tip cells coordinate to have less proliferative activity by repressing Notch activity, thus upregulating VEGFR-2 (Flk-1) and other downstream

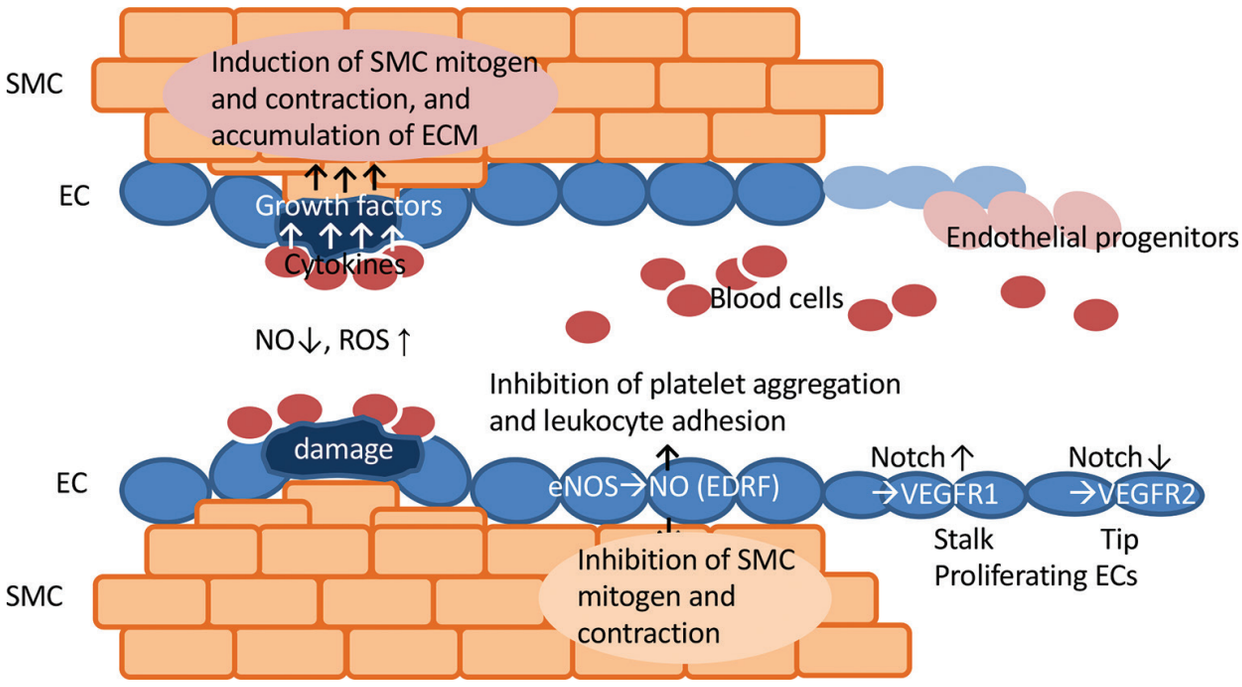

Figure 1. Summarized pathological and physiological vessel response. Damaged ECs are shown in dark blue. Due to the damage, there is a reduction of NO and an increase of ROS, which leads to platelet aggregation or leukocyte adhesion to the intima. Cytokines produced from platelets or leukocytes induce growth factor production and cause SMC hyperplasia and contraction. By contrast, healthy ECs constantly produce EDRF such as NO, so that SMC mitogen and contraction are absent. In developing vessels, ECs are proliferating or deriving from progenitor cells. Proliferating ECs can be distinguished into stalk cells and tip cells, which have different downstream VEGF pathways depending on Notch activity.

http://dx.doi.org/10.5772/66501

Notch transcription factors such as HASER1 [2]. By contrast, Notch signaling is more active and VEGFR-1 (Flt-1) expression is upregulated in stalk ECs. Although Notch and VEGF signals are greatly conserved in vessel sprouting among various tissues and species, how widespread it is in terms of tissue specificity remains to be elucidated (**Figure 1**). Increasing evidence shows that different signaling rules influence tissue-specific vessel sprouting—one study demonstrated that bone morphogenetic protein (BMP) signaling provides the cue for vein-specific angiogenesis during early development, and is independent from canonical VEGF-A signaling [3]. Casanello et al. reported that endothelial diversity is also present in the umbilico-placental vasculature, and emphasized that the heterogeneity of ECs is complicated and cannot be explained simply by comparing the differences between micro- and macro-vasculature, or artery versus vein [4]. Thus, EC shows great heterogeneity in health and disease, and studying the mechanisms of EC heterogeneity would contribute to the understanding of both vascular physiology and pathology.

We previously revealed the unique gene profile of ductus arteriosus (DA)-specific ECs. The DA, a fetal artery that connects the pulmonary artery (PA) and the aorta, is essential for fetuses to bypass the oxygenated blood delivered from the placenta directly to the descending aorta and not through the lung. The DA experiences a dramatic morphological change along with environmental factors after birth, though other connecting arteries remain unchanged. Therefore, even under similar physiological stresses underlying the DA and its connecting arteries, heterogeneity of ECs must exist. In this chapter, we focus on reviewing the unique identified gene profile of DA ECs, which should provide novel insights into heterogeneity in vascular ECs.

Moreover, investigating DA remodeling would potentially help the understanding of diseased vessels, just like other animal models in cardiovascular diseases. For instance, a wire injury model is used for studying pathology of endothelial injury/dysfunction [5]; low-density lipoprotein receptor-deficient mice [6] and apolipoprotein E-deficient mice [7] are commonly used as atherosclerosis models; calcium chloride [8], elastase [9], angiotensin II [10], or microRNA-21 [11] are infused to create an abdominal aortic aneurysms model. Developing a disease model occupies a great deal of scientific findings on pathophysiology, and so the existing models should always open to be refined. The DA can be an alternate model of an occluding vessel, an extracellular matrix (ECM)-enriched vessel, or an oxygen-sensitive vessel. Thus, studying DA ECs would be valuable for understanding an irregular angiogenic pathophysiology.

1.1. Embryonic vasculogenesis

Vasculogenesis and angiogenesis are nomenclaturally similar as they both refer to the genesis of blood vessels [12]. Vasculogenesis is the de novo formation of blood vessels differentiated from mesodermal cells. Angiogenesis is the sprouting of blood vessels that occurs as a result of the proliferation of existing vascular ECs. Despite the difference in these two processes, vasculogenesis and angiogenesis are often compared to further understand their underlying molecular mechanisms. Indeed, a significant amount of knowledge on

tumor angiogenesis was achieved by studying embryonic vasculogenesis [13]. Therefore, it is important to study developmental vascular biology and to understand vessel-specific heterogeneity. Moreover, determining the heterogenic diversity of ECs would help open up more options in clinical therapy, ultimately enabling individually designed therapeutic treatments.

The vascular network is the first functional system established in the embryo. A primitive vascular network is formed shortly after gastrulation by deriving endothelial progenitor cells from the mesoderm. This first process is called the formation of angioblasts. Angioblasts then differentiate into ECs by expressing various transcription factors and pan endothelial markers for tubular formation, which is called the primitive vascular plexus [13]. Some of the homeobox (Hox) transcription factors are known to be involved in this process. For instance, Hox A9 regulates the expressions of endothelial NO synthase (eNOS), VEGF-receptor 2 (VEGFR2), and vascular endothelial-cadherin (VE-cadherin), and is responsible for the tubulogenesis of mature ECs [14]. Hox B3 also plays a role in tubulogenesis [15]. Hox D3 induces the differentiation of ECs from angioblasts [16]. The primitive vascular plexus then undergoes complex remodeling accompanied by specification among arteries, veins, and capillaries to become the functional vascular system [13]. Sry-related HMG box (Soxs)-F subgroups Sox7, Sox17, and Sox18, along with vascular endothelial zinc finger-1 (Vezf-1), were found to be essential to the remodeling process [17, 18]. Thus, vasculogenesis in general consists of three steps: formation of angioblasts, formation of the primitive vascular plexus, and vascular remodeling. During these steps, the heterogeneity of vascular ECs is established.

1.2. Physiology of the DA

After the vascular system appears during embryonic development, the heart starts to function, and fetal circulation is established. Fetal circulation is different from adult circulation since the blood is oxygenated in the placenta instead of the lung. Prenatal lungs do not yet need to function so the DA bypasses the pulmonary artery and the descending aorta to send most blood to the body instead of the lungs. Patency of the DA is maintained due to the low oxygen level and high concentration of prostaglandin E_2 (PGE_2) in the blood circulated from the placenta, as well as the production of NO from ECs of the DA. Once the infant has been delivered and lung ventilation has begun, the DA must close properly to enable the transformation to adult circulation. Normal closure happens in two steps: functional closure and anatomical occlusion [19]. The first closure is triggered by an increase in pO_2 and a drop in PGE_2, as well as a drop in blood pressure within the DA caused by the reduction in pulmonary vascular resistance. This functional closure causes the loss of blood flow which therefore induces hypoxia and extensive intimal thickening, followed by fibrosis. The hypoxia on the vessel wall further inhibits endogenous prostaglandin and NO production, which leads to an irreversible closure. Two to three weeks later, the sealed DA eventually becomes a fibrous band called the ligamentum arteriosum (**Figure 2**) [19]. Failed DA closure after birth is a condition called patent DA (PDA), and occurs frequently in premature infants. Medical or surgical treatment of PDA is required when the left-to-right blood shunt is significant.

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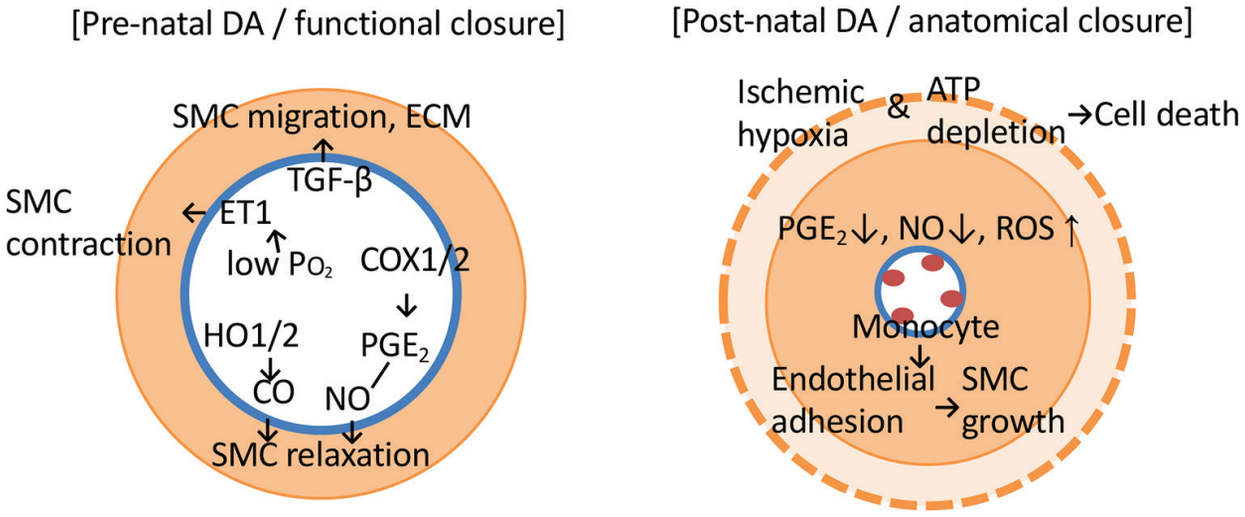

Figure 2. Representative pathways during DA remodeling. In early gestation, the DA remained open due to the high concentration of PGE_2 from placental circulation, and by producing EDHF (NO and CO in the figure). Low oxygen concentration induced ET-1 signaling and TGF-β expression in ECs, leading to functional closure. Postnatal DA is exposed to oxygenated blood that has reduced concentrations of PGE_2 and NO. Due to reduced NO production, ROS are produced and monocytes are attracted to the intima. Monocyte-endothelial interaction induces cytokine and growth factor upregulation, thus promoting SMC growth. Extensive neointimal formation at a later stage causes ischemic hypoxia and ATP depletion, and eventual cell death.

2. Endothelial heterogeneity in terms of the DA

2.1. Current methodologies to study the heterogeneity of DA-specific ECs

The DA is a small shunt vessel in fetuses or neonates. The size of the tissue has always been a study limitation in small mammals such as rodent models. Therefore, although many previous studies used larger mammals such as lamb or pig fetuses, they are inefficient for conducting primary-level research due to the difficulty in handling, low number of offspring, and long gestational period. Rodents overcome these disadvantages, and experimental tools of molecular biology are more available for rodents. Thanks to advancements in technology, there are now more options than ever to overcome the limitation of tissue size in rodents.

2.1.1. Isolation of ECs of the rat DA

DA researchers have used different species, including baboon, pig, sheep, rabbit, chicken, rat, and mouse [20]. These studies focused on a specific population of the cells in the DA and always faced limitations in using rodent animals due to the small size of the fetal tissue. To date, there are only three studies in which pure ECs were successfully isolated from rat DA, including ours. Weber et al. applied a magnetic-activated cell sorting (MACS) method to purify the ECs from collagenase-digested DA tissue [20]. They used von Willebrand factor (vWF) polyclonal antibody in MACS for the isolation. With their experimental method, they succeeded in passaging a pure population of ECs up to three times, which overcame the small number of primary-cultured ECs for further experiments. Following the isolation, they confirmed the purification by flow cytometry and immunohistochemistry analyses. In our

previous study, we used a florescence-activated cell sorter (FACS) to purify ECs from collagenase-digested DA tissues [21]. In this experiment, we incubated pooled fresh cells with fluorescein isothiocyanate (FITC)-conjugated anti-CD31 and APC/Cy7-conjugated anti-CD45 antibodies to separate EC and hematopoietic derivation cells, respectively. We confirmed the purity by performing quantitative reverse transcription polymerase chain reaction (RT-PCR) for Tie2 and gamma2-actin expressions, which are markers of ECs and SMCs, respectively. After the FACS sorting, we proceeded directly to RNA isolation from the collected ECs for application to a DNA microarray experiment, which minimized differentiation of the isolated ECs after purification. More recently, a study focused on the heterogeneity of tissue-specific cells that separated ECs and SMCs from the DA using laser-capture microdissection [22].

2.1.2. Comprehensive gene expression analysis of DA tissues

During the past decade, several groups, including ours, have studied comprehensive gene expression in the DA using DNA microarray analysis. One study used human DA specimens, with a broad range of ages [23]. Because of the difficulty involved with human samples of the DA, they could not group the samples with biologic replicates. They found a tendency of expressing more genes that relate to ECM synthesis, which implied the presence of active neointimal proliferation in PDA. Other microarray studies used only rat vessels. Costa et al. compared rat DA samples from embryonic day 19 (E19) and 3 h after birth, examining the effects of oxygen [24]. Our group examined the expression profiles of rat DA and aorta at E19 and E21, and reported that the growth hormone (GH)-receptor signal is predominant in the SMCs of the DA [25]. We also investigated the effect of vitamin A maternal administration on the gene expression pattern of the DA at E19, E21 (full term), and 3–6 h after birth [26]. Moreover, our group utilized the unique phenotype of the Brown-Norway (BN) rat—this strain has been characterized as a novel animal model for PDA possibly due to systemic elastin-related impairments—to compare with vessels of its control strain Fisher 344 [27]. Although all of these studies reported somewhat overlapping results, none could determine the EC-specific gene profiles. As discussed above, the EC layer is maintained to form a single layer; the majority of genes that appear on microarray analysis using whole tissue are therefore from SMC origin.

Because the analysis of the expression profiles of the vascular ECs of the DA is challenging, only two studies have been published to date, including ours [21, 22]. It is difficult to compare these two studies because we used pooled DA ECs purified by FACS, whereas Bokenkamp et al. used laser-capture microdissection to isolate DA ECs from a frozen sample. Accordingly, some of the study results are inconsistent. For example, Bokenkamp et al. demonstrated that the expression of Rgs5 mRNA was higher in the DA compared to the aorta [22], whereas we did not find a difference in Rgs5 expression between DA ECs and aortic ECs. In our study, we divided samples into four groups: the DA and aorta of E21 fetals (F group) and neonates 30 min after birth (N group) rats. We further categorized the microarray data with GeneGo MetaCore software to clarify the meaning of enriched gene expressions. Interestingly, the majority of the identified DA-dominant genes had not previously been reported in previous DA-related studies. We review the unique gene profiles of DA-specific ECs in the following sections.

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2.2. Characteristics of DA-specific ECs in DA remodeling

As mentioned in the earlier section, the DA has special remodeling processes that differ from other vessels. Most research on DA remodeling has been conducted using the whole tissue or its SMCs. The importance of signals generated from blood or ECs has, however, begun to be realized.

2.2.1. Extracellular matrix remodeling of the DA

In the late 1980s, Rabinovitch's group discovered that the intimal cushion formation of the DA is attributed to a special character in its cells [28, 29]. Using *in vitro* cells from lamb tissues, they demonstrated that there are 10-fold and five-fold increased incorporations of hyaluronan and heparansulfate in the ECM of DA ECs, respectively, compared to cells of the adjacent aorta or pulmonary artery (PA). They further found that this remodeling, which involves the increased hyaluronan accumulation in DA ECs, contributes to the migration of DA SMCs [30], and is transforming growth factor-beta (TGF-β)-dependent [31]. About a decade later, the same group reported that TGF-β1 expression in DA ECs was upregulated in the early gestation of fetal lambs compared to aortic ECs, but was downregulated to the same level as aortic ECs by late gestation [32]. This dynamic modification in the DA EC was explained to relate to stability in the translation and transcription of its mRNA. This second study provided some of the first evidence showing that there are tissue-specific and developmental patterns of expression in DA ECs.

The comprehensive gene analysis study identified significantly high expressions of N-deacetylase/N-sulfotransferase (Ndst3), Glipican 3(Gpc3), and heparan-sulfate 6-O-sulfotransferase 2 (Hs6st2), all of which are involved in heparasulfate synthesis, in DA ECs in both full-term fetal and neonatal periods [21]. Ndst3 is the most important heparin-sulfate synthase among the three members of the NDST family [33]. Other genes that are known to relate to ECM, especially collagen synthesis, were also found to show higher expression levels in DA ECs than aortic ECs: the glycosyltransferase25 domain containing 2 (Glt25d2), which is known to strengthen collagen activity [34]; growth differentiation factor (Gdf6), and microfibrillar-associated protein 5 (Mfap5), which promotes collagen production [35, 36]; Mfap4, which stabilizes collagen activity [37]; anthrax toxin receptor 1 (Antxr1), which provides a link between collagen I and actin cytoskeleton [38]; and prolyl 4-hydroxylase-alpha polypeptide (P4ha1), which is related to the procollagen process [39]. ADAM metallopeptidase with thrombospondin type 1 motif-17 preproprotein (Adamts17), plasminogen activator tissue (Plat), and fibrillin 1 (Fbn1), which are also categorized as related to ECM formation, were upregulated in DA ECs [21]. Interestingly, connective tissue growth factor (CTGF) was found to show higher expression in DA ECs than in aortic ECs in the postnatal period, whereas there was no difference in the fetal period [21]. CTGF is a well-known downstream mediator of TGF-β1 in various cells and it exhibits diverse functions, such as cell proliferation, apoptosis, cell adhesion, ECM or collagen production, and angiogenesis [40, 41]. Moreover, a recent study demonstrated that, via stimulation of TGF-β1, CTGF binds to VEGF, and that the complex inhibited VEGF-mediated angiogenesis in cardiac cells [42]. Although further studies are needed, these results imply that there are intricate regulations among TGF-β1, CTGF, and VEGF in the DA remodeling after birth.

2.2.2. PGE_2, endothelial-derived relaxation, and hyperpolarizing factors in the DA

PGE_2 is a potent vasodilator for the DA. It is generated by the enzyme cyclooxygenase (COX). There are two isoforms, COX-1 and COX-2. Although COX-2 is an inducible isoform that requires cytokine, both COX-1 and COX-2 are known to be involved in fetal development [43]. The expression levels of these two vary among species as well as the term of gestation. For instance, COX-2 is barely detected in the DA of fetal pig, but more dominantly regulates DA tone in fetal lamb by expressing it in ECs [43]. Another study found that there is a cooperative interaction between PGE_2 and NO, an endothelial-derived relaxation factor (EDRF) [44]. Several studies showed that NO is more potent than PGE_2 in the preterm DA, whereas the opposite relationship is seen at term [45–47]. Another EDRF that is found to be related to controlling DA tone is carbon monoxide (CO). CO is naturally formed in the body from the enzymatic activity of heme oxygenase (HO-1/2). Coceani et al. demonstrated that CO formed by HO (ECs of DA only express HO-1 in rat and pig fetuses) interfered with the reaction with the cytochrome P450-based monooxygenase and inhibited the synthesis of endothelin-1 (ET-1), which is a potent vasoconstrictor that is also critical in DA tone [48–50]. CO generated from HO-1, but not HO-2, is known to have a protective effect on ECs of various vessels [51], and induces angiogenesis [52]. Importantly, compensatory mechanisms among PGE_2, NO, and CO were elucidated by using eNOS, COX, or HO-2-mutant mice [53]. The study showed that there is no narrowing of the DA in each mutant, and that endothelial-derived hyperpolarizing factor (EDHF) additionally exhibits a large reciprocal effect [53]. In addition to bradykinin, which has been shown to have the same relaxation effect as EDHF, there could be more agents potentially qualified as EDHF. A more recent study reported that hydrogen sulfide (H_2S) also acts as EDHF by expressing its synthetic enzymes cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) in the intima, likely ECs of the DA [54].

2.2.3. Inflammatory response in DA ECs during anatomic remodeling

Anatomic remodeling of the full-term DA shares similar features of inflammatory vascular disorders such as atherosclerosis. As a consequence of the functional closure of the DA, ischemic hypoxia of the muscle media is induced due to the loss of luminal and vasa vasorum blood flow. Therefore, ATP depletion causes cell death [55, 56] and VEGF induction increases the penetration of vasa vasorum into the DA muscle media [57]. Clyman's group examined the inflammatory processes involved in the postnatal constriction of the DA [58]. They found that VLA4 integrin expressing mononuclear cells ($CD14^+/CD163^+$ cells [59]), in which the ligand is vascular cell adhesion molecule-1 (VCAM-1) in ECs, increased to adhere to the lumen of the DA after birth. Along with the increased monocytes recruitment, VCAM-1 and E-selectin expressions were also elevated in DA ECs after birth [58]. Unlike the pathophysiology of atherosclerosis, the upregulation of P-selectin and intracellular adhesion molecule-1 (ICAM-1) induced by monocytes adhesion was not seen in the DA. Interestingly, $VLA4^+$ mononuclear cell adhesion was found to predominantly regulate the extent of neointimal remodeling of the DA after birth, with T-lymphocyte adhesion to a lesser extent, but no neutrophil or platelet adhesion [58]. VCAM-1 and E-selectin were also induced by VEGF and several cytokines, such as TNF-α, IFN-γ, and CD154, likely due to the profound hypoxia in the DA wall after birth. These responses are also seen in atherosclerotic remodeling, but the pattern of gene

expression modification seemed less in DA closure since IL-1 and MCP-1 were not expressed in the closing DA [58]. Some researchers argue, however, that the inflammatory response during DA closure may cause a failure in constriction after birth [58], because TNF-α and IL-6 are known to have potent vasodilatory effects [60–63].

The monocyte-endothelial cell interaction has been implicated to play a critical role in vascular pathogenesis by inducing platelet-derived growth factor (PDGF) secretion that promotes the migration of SMCs into neointima [64]. Indeed, PDGF-B chain expression upregulation was confirmed in DA tissues after birth, and was inhibited by blocking monocyte adhesion using anti-VLA-4 monoclonal antibody treatment [59]. Moreover, the regulator of G-protein signaling 5 (Rgs5) that was found to be enriched in both ECs and SMCs of DA at full-term gestation compared to adjacent aortic cells [22] was suggested to be negatively regulated by PDGF [65]. PDGF-dependent repression of Rgs5 leads to SMC migration and G protein-coupled receptor-mediated-signaling pathways, such as mitogen-activated protein kinase activation, thus contributing to vessel contraction and remodeling [65]. The Rgs5 expression level in DA tissue after birth has not been studied, but it is reasonable to hypothesize that it would be decreased, likely due to increased PDGF secretion after birth. Further studies are required to elucidate the intricate effects of DA remodeling.

2.2.4. Epithelial/endothelial-to-mesenchymal transition-related gene expressions in the DA

Recent studies have suggested that epithelial/endothelial-to-mesenchymal transition (EMT/EndMT)-related genes play an important role in DA closure [21, 66]. Our microarray study on FACS isolated ECs from rat DA revealed that Tgfb2, actin alpha 2 smooth muscle aorta (ACTA2), N-cadherin (cadherin 2 or Cdh2), and met proto-oncogene (hepatocyte growth factor receptor or Met), which are known to be related to the EMT process, are significantly expressed compared to the aortic ECs [21]. In accordance with this finding, ACTA2 mutation is well characterized in PDA [67]. Another study that showed the importance of BMP9 and BMP10 as circulating growth factors in DA postnatal closure also found that they induced expressions of EMT/EndMT-initiating transcription factors SNAI1, SNAI2, ZEB2, TWIST1, and FOXC2 in ECs [66]. The study found that treatment with a neutralizing anti-BMP10 antibody on BMP9 knockout mice led to reopening of the DA. BMP9 and BMP10 are members of the TGF-β family, and are known to be elevated in mice around birth [68]. They have high affinity to bind to activin receptor-like kinase 1(ALK1), which is an EC-specific receptor [69], and additionally upregulate the expressions of BMPR2, ActR2A, and the co-receptor endoglin as well in the DA [66]. Moreover, BMP9 is reported to upregulate COX-2 and hyaluronic acid synthase 2 (HAS2) expressions, but not COX-1 [66, 70, 71]. Therefore, EMT or EndMT induced by BMP9 and BMP10 is thought to be a necessary process for anatomical closure of the DA.

Although it remains to be proved whether ECs at the lumen of closing DA would differentiate into mesenchymal cells, Levet et al. observed that there is a loss of EC-specific marker (PECAM or CD31)-positive cells at the lumen [66]. Since those cells at the lumen had an autophagic appearance, the authors speculated that the loss of ECs is at least partially due to cell death. However, it is also reasonable to assume that the EC loss is attributed to EndMT which resulted in loss of the EC characteristics.

2.3. Genetic responses to external stimuli on the DA and other vessels

The DA encounters great environmental changes during the perinatal period. Interestingly, the DA dramatically changes its morphology despite other neighboring arteries remaining unchanged. Therefore, it is reasonable to assume that the DA is sensitive to external or internal stimuli, which are primarily received by cells at the lumen, more than other neighboring arteries.

2.3.1. Response to oxygen

In fetal life when the lungs are not yet ventilated, the resistance of pulmonary vessels is high. Therefore, most of the blood that is oxygenated from the placenta passes to the descending aorta through the DA. At birth, in accordance with lung expansion, the blood passing via the DA is reduced, since the resistance of the pulmonary arteries is lower than that of the systemic arteries. In addition, an increase in oxygen concentration of the blood and a decrease in PGE_2 levels trigger the contraction of the DA. Our previous study demonstrated that α1G, a T-type voltage-dependent Ca^{2+} channel, mediates oxygenation-induced closure of the DA after birth [72].

Furthermore, as the neonatal period progresses, the DA constricts more and the vascular cells undergo hypoxic changes. As a result of hypoxia, reactive oxygen species (ROS) are generated by converting O_2 to $O_2^{\bullet-}$ by NADPH oxidase in ECs. Further activated redox-signaling pathways increase the tyrosine and serine/threonine phosphorylation of proteins, and result in various physiological and pathophysiological responses that are reviewed elsewhere [73]. VEGF is one of the best known genes that are elevated in response to hypoxia in the DA, which contributes to the ingrowth of vasa vasorum and neointimal proliferation [57].

Our microarray analysis identified a significant number of genes that more closely relate to oxygen in DA ECs than in aortic ECs. Aldehyde dehydrogenase 1 family-member A1 (Aldh1a1), aldolase C-fructose-bisphosphate (Aldoc), and CD38 are oxygen-related enzymes, and Vegfa, Tgfb2, and Ctgf are oxygen-related receptor ligands [21]. CD38 has been recently implicated to regulate Ca^{2+} signaling in response to ROS generation in pulmonary arterial SMCs [74]. Therefore, it would be interesting to examine the importance of CD38 in the DA.

2.3.2. Response to retinoic acid

Retinoic acid (RA), a metabolite of vitamin A, plays a critical role in organogenesis, such as the formation of the face, heart, eyes, limbs, and nervous systems [75]. Vitamin A maternal administration has been proven to increase the activities of vessel-contractile proteins and to accelerate the development of the O_2-sensing mechanism in the DA [76]. Yokoyama et al. compared gene expression profiles by microarray in the DA in the presence or absence of maternal vitamin A administration at different developmental stages, and found that 91 genes in total responded to the treatment [26]. In addition to the genes that were previously demonstrated to be induced by RA, such as fibronectin-1 and HAS2, the study also found that vitamin A treatment promoted the maturation of functions and structure of the DA. They also identified that VEGFA was increased by vitamin A administration.

Our microarray study on ECs from the DA versus the aorta also revealed the response to vitamin A to be one of the most dominant biological processes that worked in DA ECs [21]. TGF-beta 2, CD38, Ald1a1, Sp100, paired-like homeodomain 2-transcript variant 2 (Pitx2),

fatty acid desaturase 1 (Fads1), and dickkopf homolog 1 (Dkk1) were listed in the category. Although the MetaCore system did not mention it, lecithin-retinol acyltransferase (Lrat) was also increased in DA ECs. Indeed, Lrat was identified as one of the most significant expressions in DA ECs, as it had a more than five-fold increase compared to aortic ECs. Given the fact that Lrat is the predominant enzyme in retinoid absorption [77], it is reasonable to think that this gene could play a great role in the DA having higher sensitivity to RA.

2.4. Other genes uniquely expressed in DA ECs

Our previous study identified more than 80 genes that were expressed more than two-fold or greater in ECs of the DA compared to those of the aorta, in both terms (F and N) [21]. In this section, DA EC-unique genes that were not mentioned in the earlier section will be summarized.

2.4.1. Neural crest cell-related genes during development

The DA derives from neural crest cells that are located in the sixth pharyngeal arch artery [78, 79], which is one of the progenitors of the second heart field [78, 79]. We identified that Tbx1, a major transcriptional factor in the second heart field, was expressed approximately four-fold more in DA ECs compared to aortic ECs. Pitx2 and Fgf10, which are known to co-express with Tbx1 [80, 81], also showed more than two-fold expressions in DA ECs than in aortic ECs. Indeed, Momma suggested that the deletion of human chromosome 22q11.2, where Tbx1 is, increased DA anomalies [82]. Moreover, cadherin 2 (Cdh2), which is known to work downstream of Pitx2 [83], and Ephrin B1 (Efnb1), Hs6st2, and Isl1, which are known to be in the Fgf10 signaling pathway [84–86], were also expressed dominantly in DA ECs [21].

2.4.2. Solute carrier family 38, member 1 (Slc38a1)

Slc38a1 is a highly homologous protein subtype of placental system A, a Na^+-dependent amino acid transporter that contributes to nutrient fetal growth, by expressing in the placenta [87]. Placental system A activity increases along with the progression of pregnancy and therefore coincides with demands of fetal nutrient [88]. Slc38a1 was found to be one of the most dominant genes in DA ECs compared to aortic ECs [21]. Slc38a1 itself has not been fully characterized yet and has not been implicated in studies in the DA. Recently, using siRNA technology on cytotrophoblast cells, Slc38a1 was revealed to be a key contributor to total system A activity in term placenta [87]. Hence, the fact that Slc38a1 expressed approximately seven-fold more in DA ECs than in aortic ECs at full-term gestation implies its involvement in the vascular remodeling of the DA. Further study is needed to identify the role of Slc38a1 in the DA during development.

2.4.3. Calpain-6

The calpain family is a calcium-dependent cysteine protease that is ubiquitously expressed in human tissues. Calpain-6 was identified about two decades ago; it has special features that make it stand out from other family members [89]. Calpain-6 is the only family member that lacks a calmodulin-like domain; it therefore has no protease active site [89]. Calpain-6 was exclusively but highly expressed during embryogenesis [90] and in placenta in 50 adult tissues [89] (no DA examination). Our microarray study identified that calpain-6 was also one

of the most strongly expressed genes in DA ECs compared to aortic ECs, especially in fetal tissue [21]. Calpain-6 was recently implicated in tumor angiogenesis. Specifically, calpain-6 is suggested to play an important role in bone tumorigenesis and metastasis [91]. In the study, calpain-6 was found to be upregulated by ET-1, and to provide a protective effect against cell apoptosis and promote cell proliferation [91]. As mentioned earlier, ET-1 is increased in the DA to regulate its vasoconstriction [48–50]. Therefore, calpain-6 might be a newly identified gene in ET-1 signaling generated in DA ECs.

3. Conclusion

Studying EC heterogeneity aids our understanding of the physiology and pathophysiology of angiogenesis. It also has great potential to identify novel ways to regulate angiogenesis for treatment purposes. Comprehensive gene analysis using a microarray made it possible to reveal many genes that were previously functionally unidentified in tissue or disease. Molecular analyses using whole tissues hinder the data on specific cell types. ECs are the key cells responsible for primarily generating signaling pathways to modulate the functions or structure of a vessel. Vessels mainly consist of a medial layer (the majority of which is composed of SMCs), and a single layer of ECs. The separation of ECs would therefore be the first hurdle to overcome in order to acquire data on ECs.

This chapter focused on reviewing the current knowledge of DA ECs, since we believe that the DA could be utilized as a vessel model for studying the mechanisms of both neointimal formation and apoptosis in addition to embryonic vasculogenesis. DA-specific ECs are highly unique compared to aortic ECs in terms of their heterogeneity. DA ECs have a great number of specific genes related to ECM formation, inflammatory response, EMT or EndMT, and oxygen and retinoic acid response. DA ECs also have more genes that are conserved from embryogenesis compared to adjacent aortic ECs. In our previous study, Slac38a1, Capn6, and Lrat were found to be the most significantly expressed genes in DA ECs. Although much more research is required to validate the importance of these newly identified dominant genes in DA ECs, we expect that these findings will promote further studies on PDA, therapeutic angiogenesis, and cancer treatment.

Acknowledgements

We would like to thank Dr. Hua Cai for supporting N.M. Liu's continuing research at the University of California, Los Angeles, as well as the fellowship support from the American Heart Association, Western States Affiliate Winter 2014 Predoctoral Fellowship (Award#14PRE20380184), and the American Association of Japanese University Women 2016. This work was also supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (S.M.), the Vehicle Racing Commemorative Foundation (S.M.), The Jikei University Graduate Research Fund (SM) and the Miyata Cardiology Research Promotion Foundation (S.M.).

Author details

Norika Mengchia Liu[1] and Susumu Minamisawa[2]*

*Address all correspondence to: sminamis@jikei.ac.jp

1 University of California, Los Angeles, CA, USA

2 The Jikei Medical University, Tokyo, Japan

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