Intervention studies in a rat model of bronchopulmonary dysplasia : effects on cardiopulmonary injury and lung development

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Chapter 1

General introduction and Outline of the thesis

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Background

Premature birth and bronchopulmonary dysplasia (BPD) are significant global health problems. Premature birth occurs in 5-10 % of all pregnancies and one of the major complications with preterm birth is immaturity of the lung. In the Netherlands, each year BPD affects an estimated 500 very premature infants, with a gestational age less than 28 weeks and a birth weight less than 1000 grams. The incidence of premature birth has risen over the past decades due to an increase in risk factors, including increased maternal age, more widespread application of fertility treatments and more multiple pregnancies. BPD is a chronic lung disease in very premature infants with underdeveloped and surfactant-deficient lungs with small gas exchange volumes and soon after birth these infants develop respiratory problems, respiratory distress syndrome (RDS). Their lungs are extremely susceptible to barotrauma and oxidant injury during the mechanical ventilation for respiratory failure and need postnatal surfactant instillation to open up their lungs. Due to airway injury, lung development fails to progress leading to alveolar hypoplasia and disturbed vascularization and ultimately lead to chronic lung disease, i.e. BPD, and at later stages by pulmonary hypertension. Until recently, preterm infants with BPD were weaned from the ventilator using glucocorticoids, which accelerate lung development, but inhibit alveolarization, thereby resulting in a permanent reduction of the gas-exchange surface area and lung function. In addition, despite improvements in neonatal and perinatal medicine, the incidence of BPD has not been reduced and most interventions applied to prevent or treat BPD are still not evidence-based. This thesis explores the therapeutic potential of phosphodiesterase inhibitors and apelin in the treatment and/or prevention of BPD and investigates the therapeutic potential of mesenchymal stem cells in pulmonary arterial hypertension.

Lung development

Lung development can be subdivided into five distinct stages, embryonic, pseudoglandular, canalicular, saccular and alveolar (Figure 1) [2,3]. The same stages are seen in other species but their duration varies, and the alveolar stage is entirely postnatal in some species (rat and mouse) [4]. Lung development begins as an endodermal outgrowth of the ventral foregut around the fourth week of human development. During the next two weeks this endodermal outgrowth grows caudally to form the early tracheobronchial tree and then bifurcates into

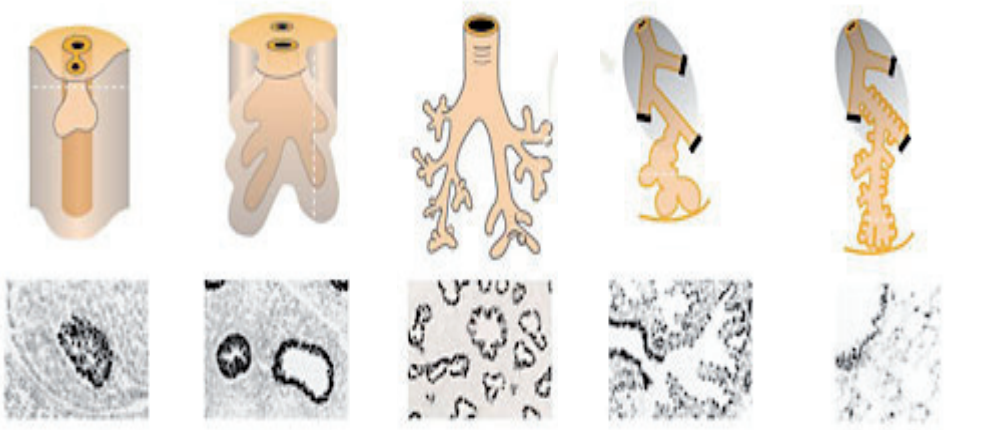
a right and a left primary lung bud. Around each lung bud is a capillary network which connects cranially to the aortic sac of the heart and caudally to the prospective left atrium. The left lung bud will give rise to two main stem bronchi, whereas the right lung bud gives rise to three mainstem bronchi. The primitive lung bud is lined with endodermally derived epithelium.

During the pseudo-glandular phase the conducting airways are formed, stimulated by the presence of the surrounding mesenchyme, by repeated dichotomous branching resulting in a tree of narrow, thick epithelial-lined tubules. The primitive airway epithelium starts to differentiate to form cartilage, connective tissue, blood vessels, lymphatics and smooth muscle cells [5]. The epithelial-mesenchyme interactions play a determining role in regulating the growth and branching patterns [6]. At the same time all pre-acinar pulmonary arteries and veins are formed.

In the subsequent canalicular phase, the airway branching pattern is completed and vascularized and the prospective gas-exchange region starts to develop. Thinning of the epithelium by underlying capillaries leads to the formation of a blood gas barrier which is sufficient to sustain life in extremely premature infants. During this period respiratory bronchioli appear, delineating the acinus, the gas-exchaning portion of the tracheobronchial tree, composed of respiratory bronchioles, alveolar ducts, sacs and alveoli. The initial differentiation of the cuboidal epithelium into type I and type II pneumocytes, of which the type I pneumocytes are responsible for gas exchange and the type II pneumocytes produce surfactant [7].

At the beginning of the saccular (terminal sac) phase airways terminate in large smooth-walled cylindrical structures subdivided by ridges called crests. The crests protrude into saccules, pulling a capillary network in close contact with them and creating subsaccules, which will eventually become alveoli. During this stage the growth of the pulmonary parenchyma, the thinning of the connective tissue between the airspaces, and the further maturation of the surfactant system are the most important steps towards *ex-utero* life.

At birth, although already functional, the lung is structurally still in an immature condition, because alveoli, the gas-exchange units of the adult lung, are practically missing. The airspaces present are smooth-walled transitory ducts and saccules with primitive septa that are thick and contain a double capillary network. During the alveolar stage, further thinning of the blood-gas barrier, increase in surfactant production and formation of alveoli through progressive branching of the respiratory airways greatly increases the gas exchange surface area. In addition, microvascular maturation takes place during the alveolarization stage between a few months to 3 years after birth. The double capillary network in the parenchymal septa is restructured to the mature aspect with a single capillary system. The phase of alveolarization is terminated at 2 weeks in the rat and at about 12–24 months in the human [8].



Embryonic (I)
Rat: day 13-14
Human: Wk 3-6

Pseudoglandular (II)
Rat: day 15-18
Human: Wk 6-17

Canalicular (III) `
Rat: day 18-20
Human: Wk 16-24

Saccular (IV)
Rat: day 19-birth
Human: Wk 24-36

Alveolar (V)
Rat: birth-28 days
Human: Wk 36-3 yrs

Figure 1. Stages of the developing lung in rats and humans. (adapted and modified from [1])

Bronchopulmonary dysplasia

Clinical presentation

BPD is a disease that affects preterm newborns weighing less than 1000g who are born at 24-26 weeks of gestation [9] and is a chronic lung disease of infancy that follows ventilator and oxygen therapy for acute respiratory failure after premature birth [10]. BPD has been defined by the presence of persistent respiratory symptoms, the need for supplemental oxygen to treat hypoxemia, and an abnormal chest radiograph at 36 weeks corrected age. The pathology of infants with BPD has changed over the last four decades from socalled "classical" to "new" BPD, reflecting differences in the patients and the therapies used. The classical BPD, a more mature population responded to the risk factors for BPD with fibrosis and smooth muscle augmentation of medium-sized airways, resulting in airway obstruction [10]. Surviving infants with "classical" BPD were born at 34-weeks of gestation, weighing around 2,200g and the mortality was around 67% [10]. The present population of BPD infants are often born very prematurely and lung fibrosis is replaced by abnormalities of lung growth, with less smooth muscle encircling larger airways, but markedly decreased numbers of alveoli [9], i.e. new BPD. The incidence of BPD is strongly correlated with birthweight, with 85% in neonates between 500-699g, 75% in neonates less than 1,000g and 5% in neonates with birthweights over 1,500g [11,12]. The use of surfactant, together with the advances in critical care management leading to less volutrauma and oxygen injury, has resulted in the pattern of injury, which reflects an extremely immature lung with impaired alveolar and capillary growth and development, with subsequent abnormal reparative processes. The lung injury is more uniform and is milder with less inflammation and fibrosis [13]. The new BPD is defined by inhibition of acinar and vascular growth during a vulnerable stage of lung development, whereas classis BPD was attributed primarily to oxygen injury and mechanical ventilation

in prematurity. As a result new diagnostic criteria of BPD were developed based on time of clinical assessment and severity and new BPD is now defined as the need for supplemental oxygen at 56 days postnatal age [11].

BPD infants have growth retardation and gastrointestinal problems due to decreased nutrient intake, hypoxia, concomitant dysfunction of other organ systems and increased requirements for energy [14]. Malnutrition can delay somatic growth and the development of new alveoli, and impair the response to oxidant-induced lung injury. Moreover, infants with BPD are at increased risk for neurodevelopmental delay affecting both cognitive (speech development, performance, IQ and receptive language) and motor function compared with premature control children matched for gestational age [15,16]. Preterm infants are also at risk for developing ophthalmological problems as they have incompletely vascularised retinas due to the fact that normal retinal vascular growth *in utero* ceases.

BPD is associated with long-term respiratory morbidity as long-term studies have demonstrated lung function abnormalities, airway obstruction, and airway hyperreactivity and hyperinflation persisting into adolescence [14,17]. Moreover, these children are at increased risk for asthma, infection, increased sensitivity to second hand cigarette smoke and other respiratory diseases, and are often re-hospitalized following respiratory infection [18,19]. In a mouse model of BPD, hyperoxia affected critical aspects of neonatal lung development, leading to longlasting changes in the innate response to respiratory viral infection [20], suggesting that neonatal hyperoxia disturbs key innate immunoregulatory pathways in lung contributing to the increased susceptibility to respiratory viral infections typically seen in people who had BPD.

Pathophysiology: Inflammation and coagulation

Lung inflammation is important in the pathogenesis of BPD and is defined by an increase in inflammatory cells in the airspaces and lung tissue producing pro-inflammatory mediators. Neutrophils and macrophages are central in mediating this inflammation and many pro-inflammatory cytokines, such as interleukin (IL)-1b, IL-6 and the neutrophil chemotactic factor IL-8, are increased in infants who develop BPD [21,22]. Preterm infants with BPD have much higher and persisting numbers of neutrophils and macrophages in the broncholaveolar lavage fluid compared to infants who have recovered from RDS [23]. Neutrophils invade airspaces within hours after birth and persist during the first weeks of life in the airways of these infants [24]. Activation of the inflammatory response in animal models of BPD shows increased pro-inflammatory cytokines and inflammatory cells, such as neutrophils, macrophages and monocytes, in lung tissue [25-27]. Animal studies have demonstrated that neutrophil-induced airway inflammation promotes an arrest of alveolarization, and that inhibiting the neutrophil influx preserves alveolar development in hyperoxia-exposed newborn rats [28]. In addition, antichemokine treatment with anti-MCP-1 attenuates alveolar macrophage accumulation in the lung and preserves alveolar development of neonatal hyperoxia-exposed rats [29]. The contribution of inflammation seems to be of crucial importance in the arrest in alveolarization.

Pro-inflammatory cytokines are important mediators of activation of coagulation. Several studies have shown the importance of IL-6, tumor necrosis factor α (TNF- α) and IL-1 in the regulation of anticoagulation. Inhibition of IL-6 attenuated the activation of coagulation in a model of endotoxaemia in chimpanzees [30] and infusion of TNF- α in healthy human

volunteers induced a systemic inflammatory response and activation of coagulation [31]. TNF- α and IL-1 reduce the levels of plasminogen activators and increase the antifibrinolytic mediator plasminogen activator inhibitor 1 (PAI-1) [32], resulting inadequate fibrin removal. This suggests that pro-inflammatory cytokines create a procougulant and antifibrinolytic state that may lead to fibrin deposition in the airspaces and microvasculature of the lungs.
Tissue factor (TF) plays a central role in the initiation of inflammation-induced coagulation. TF is the physiologic initiator of the coagulation pathway and activation of TF results in thrombin formation, which is converted into fibrin by fibrinogen. Fibrin is degraded by plasminogen activators, which are regulated by PAI-1. Blocking TF activity completely inhibits inflammation-induced thrombin generation in animal models of endotoxemia or bacteremia [33,34]. Disordered coagulation and fibrinolysis in the lung lead to fibrin deposition in alveoli, interstitium and capillaries [35,36]. Fibrin can increase the migration of inflammatory cells [37], disrupt the organization of endothelial cells and increase vascular permeability [38]. Thrombin increases pro-inflammatory cytokine expression, vascular permeability and chemotaxis of inflammatory cells [39]. Anti-activated protein C (APC), a natural anticoagulant, inhibits coagulation and expression of TNF- α, IL-1 and IL-6 and inactivates PAI-1 [40,41]. These data suggests that intra-alveolar fibrin deposition may function as a marker for the severity of experimental BPD with respect to coagulation, fibrinolysis and inflammation.

Pathophysiology: Alveolarization and angiogenesis

An arrest in both the formation of the alveolar and vascular system of the lung is the key characteristic of BPD. Infants susceptible to develop BPD are born in the early saccular phase, or even in the canalicular phase of lung development for the most premature of them [42], so the formation of alveoli by secondary septation is effectively an essentially postnatal event. Perinatal lung injury in neonates show alveolar simplification, loss of small arteries and decreased capillairy density. Alveologenesis is coordinated by multiple interactions through paracrine mechanisms between fibroblastic, epithelial, and microvascular lung components, and with extracellular matrix.
Elastogenesis is essential to alveolar septation, as elastin deposition in the tip of septa controls the budding and location of secondary septa via attracting myofibroblasts. Deletion of the elastin gene is associated with decreased alveolarization and emphysema [43], whereas increased elastin deposition is found in BPD infants [44] and ventilated preterm lambs [45], relating to the fibrotic repair process prominent in “classical” BPD. Myofibroblasts are essential in the normal process of septa formation but are also involved in the fibrotic process that often occurs in the reparative phase of lung injury. Migration of myofibroblasts to the tips is controlled by platelet-derived growth factor A (PDGFA), which is produced by epithelial cells [46] and myofibroblasts produced fibroblast growth factors, which stimulate alveolar septation and myofibroblasts growth. Both PDGFA and FGFs expression is reduced in lung of neonatal rats exposed to hyperoxia [25,47]. In addition, FGF7 is a potent proliferation stimulus of alveolar type II cells, which ensure adequate surfactant production and serve as stem cells of alveolar type I cells that line most of the alveolar surface and form air-blood barriers [48].
Interactions between airways and blood vessels are critical for normal lung development and contribute to maintenance of alveolar structures throughout life [49]. Maturation of pulmonary vasculature is a complex process that involves endothelial cell proliferation,

differentiation, migration, tube formation and stabilization. Vascular endothelial growth factor (VEGF) is crucial for normal blood vessel formation [50], is expressed by epithelial cells [51] and is a highly specific mitogen and survival factor for vascular endothelial cells. VEGF binds to transmembrane tyrosine kinase receptors, VEGFR1 and VEGFR2, which are expressed on the vascular endothelium [52]. Hyperoxia decreases lung levels of VEGF and its receptors [25,53] and is associated with alveolar enlargement and pulmonary vascular dysfunction [54,55]. VEGF or VEGFR2 inhibition during alveolar development decreases alveolarization and pulmonary arterial density [56-58]. In addition, increased VEGF expression enhances alveolarization and vessel growth and improves lung structure in hyperoxia-induced neonatal lung injury [50]. These data suggests that VEGF is required for the formation of the pulmonary vasculature and alveolar structures and inhibition of vascular growth results in pulmonary hypertension and may directly impair alveolarization and thereby contribute to the development of BPD.

Pathophysiology: Neonatal pulmonary hypertension and right ventricular hypertrophy

Pulmonary hypertension complicates the course of approximately 10% of infants with respiratory failure and is a source of mortality and morbidity in this population. Pulmonary hypertension is a disease of the small pulmonary arteries characterized by vascular narrowing due to structural remodeling, pulmonary vasoconstriction, impaired vascular growth and *in situ* thrombosis. Without therapy, high pulmonary vascular resistance contributes to right ventricular hypertrophy, low cardiac output and high mortality [59]. Pulmonary vasoconstriction is one of the earliest components of pulmonary hypertension, followed over time with vascular remodeling. Increased vasoconstriction is likely related to an imbalance between impaired production of endogenous vasodilators including nitric oxide (NO) and prostacyclin, and excessive production of vasoconstrictors, such as endothelin (Figure 2A). This imbalance reflects endothelial dysfunction, which results from injury due to several mechanisms including hyperoxia, inflammation and oxidative stress. Vascular remodeling of the pulmonary arteries involves all layers of the vessel wall and each cell type (endothelial, smooth muscle and fibroblast) and includes smooth muscle cells proliferation, abnormal matrix production and adventitial thickening [60]. In addition, a hallmark of severe pulmonary hypertension is the formation of a layer of myofibroblasts and extracellular matrix between the endothelium and the internal elastic lamina, i.e. neointima [61]. Finally, abnormalities of vascular growth, as related to impaired angiogenesis can cause pulmonary hypertension [56,62,63] and could play a role in the progression and severity on the setting of developmental lung diseases in children. In a baboon model of BPD, disruption of lung vascular growth was associated by abnormalities in microvascular development, angiogenic growth factors and endothelial cell receptors, which resulted in dysmorphic capillaries [64]. These abnormalities of lung vascular development, overgrowth of vascular smooth muscle and decreased number of small blood vessels, have been described in infants with severe BPD.

Drugs for managing pulmonary hypertension should influence vascular remodeling through actions of platelets, the coagulation cascade and smooth muscle and endothelial cell dysfunction, reverse vasoconstriction, prevent small vessel thrombosis and protect right ventricular function. Some of the drugs potentially affective in the treatment of pulmonary hypertension we shall discuss in the following sections, i.e. phosphodiesterase inhibitors (Figure 2B), apelin and endothelin receptor antagonists (Figure 2C).

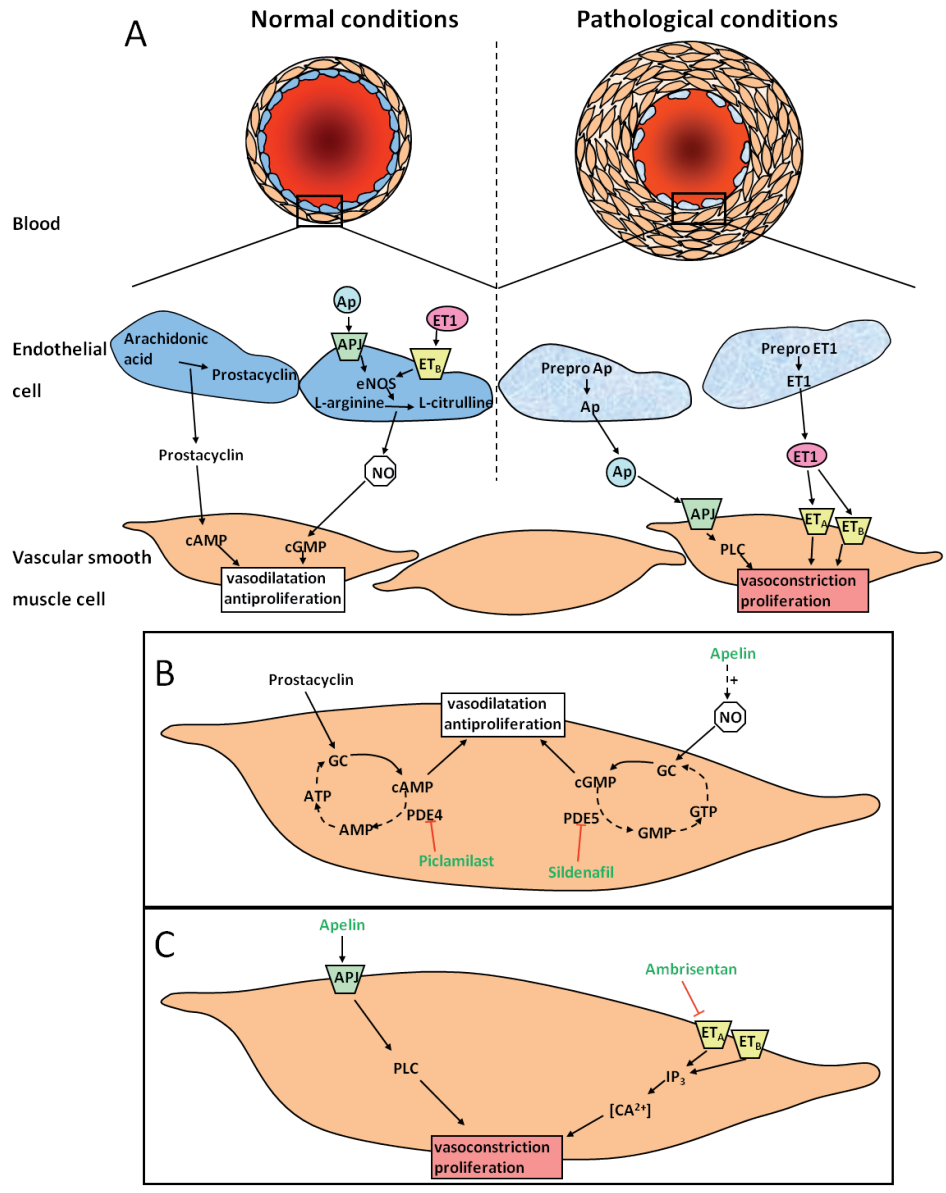


Figure 2. Targets for current or emerging therapies in pulmonary hypertension.
Four major pathways involved in the proliferation and contractility of smooth muscle cells of the pulmonary artery include the important therapeutic targets i.e. prostacyclin derivatives/cAMP-elevating drugs, phosphodiesterase type 5 inhibitors/cGMP-elevating drugs, apelin/APJ and endothelin receptor antagonists. Under normal conditions, the endothelial layer is intact, producing prostacyclin and nitric oxide keeping the arteries dilated. In pulmonary hypertension, the dysfunctional endothelial cells have decreased production of prostacyclin and nitric oxide and increased production of endothelin and apelin, thereby promoting vasoconstriction and proliferation of smooth muscle cells in the pulmonary arteries. Increased cAMP levels by piclamilast treatment and cGMP levels by sildenafil or apelin treatment and decreased activation of the endothelin A receptor by ambrisentan induce vasodilation and inhibit the proliferation of smooth muscle cells.

Animal models

Animal models have significantly improved the present understanding of the development and prevention of BPD, but positive effects in animal models do not necessarily translate into clinically meaningful outcomes in prematurely born infants. In rodents that present postnatal alveologenesis, neonatal exposure to hyperoxia that inhibits septal formation and affects alveologenesis [25,27,65] has been widely used for more than 20 years as a model to study associated cell and molecular alterations. The histological changes that occur during normal lung development are well described, but little is known about the signaling mechanisms that regulate saccular and alveolar development and understanding how aveoli and the underlying capillary network develop and how these mechanisms are disrupted in preterm infants with BPD is critical to develop efficient and effective therapies for lung diseases characterized by alveolar damage.

Intervention studies

BPD is characterized by an arrest in alveolar and vascular lung development, complicated by inflammation, abnormal coagulation and fibrinolysis, oxidative stress, and at later stages by pulmonary hypertension. Preventative strategies have been aimed at preventing or minimizing lung injury and, more recently, promoting lung growth. This suggests a potential therapeutic role for drugs with pro-angiogenic, anti-inflammatory, anticoagulant and vasodilative properties.

PDE4 inhibition by rolipram and piclamilast

In total, 11 PDE families have been identified, which vary in substrate affinity, selectivity and regulatory mechanism [66]. Among the 11 PDE enzymes, PDE4 is the major cAMP-metabolizing enzymes in all immunocompetent cells (figure 3) [67,68], encoding four genes (A, B, C and D). In addition, PDE4 inhibitors target pulmonary fibroblasts, vascular smooth muscle cells, airway epithelial and endothelial cells [69,70]. PDE4 inhibition prevents the release of pro-inflammatory mediators, inhibit adhesion molecule expression, chemotaxis, proliferation, migration and differentiation, and relax airway smooth muscle tone *in vitro* [71]. Similarly, numerous *in vivo* studies have shown that PDE4 inhibitors suppress characteristic features of BPD, namely cell recruitment, activation of inflammatory cells, proliferation of vascular smooth muscle cells and epithelial cell remodeling. PDE4 inhibition reduces neutrophil recruitment to the airways, release of chemokines and emphysematous changes to the lung in smoking, endotoxin, and LPS induced lung inflammation models of asthma, pulmonary fibrosis, acute lung injury and chronic obstructive pulmonary disease (COPD) [72-75]. Furthermore, PDE4 inhibition reduces pulmonary vascular remodeling and pulmonary hypertension in monocrotaline-, hypoxia- and bleomycin-induced pulmonary hypertension models [76,77]. Clinical investigation have shown that PDE4 inhibition improves lung function in COPD patients [78,79], an effect related in part to a reduction in the number of inflammatory cells and interleukin-8 and neutrophil elastase [80].

PDE4 deficient mice demonstrate arrhythmia and cardiomyopathy as well as accelerated heart failure after myocardial infarction [81], suggesting a role of PDE4 in myocyte and ventricular contractility, and myocyte viability. Rolipram significantly reduces inflammation and infarct size in a model of ischemic reperfusion injury in canine myocardium [82]. In addition,

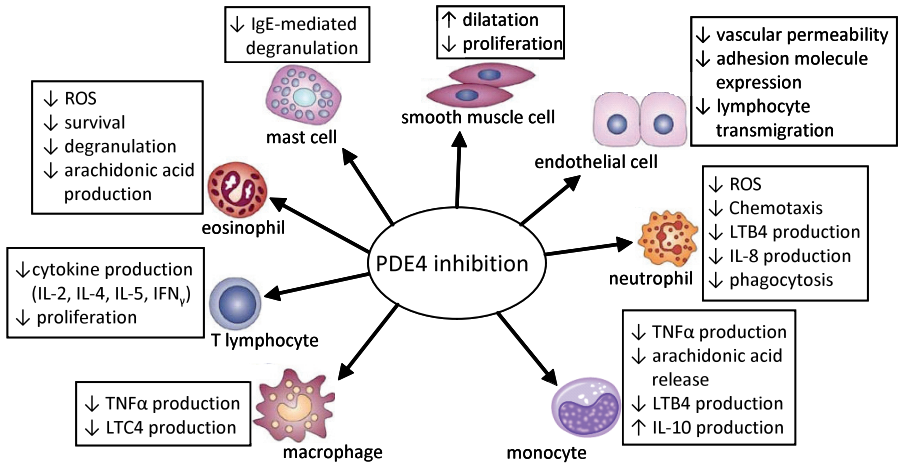


Figure 3. Targets of PDE4 inhibition
PDE4 inhibitors inhibit the recruitment and activation of key inflammatory cells, including mast cells, eosinophils, T lymphocytes, macrophages and neutrophils, as well as the hyperplasia and hypertrophy of structural cells, including airway smooth-muscle cells, epithelial cells and sensory and cholinergic nerves.

preclinical data indicate that PDE4 inhibition could improve memory function, suggesting a potential use of PDE4 inhibitors in neurological disorders [83,84]. The major disadvantage of PDE4 inhibitors are the mechanism-associated side effects, such as emesis, headache and nausea [79,85], which is linked to the gene PDE4D. Unlike the second generation PDE4 inhibitor piclamilast, the first generation PDE4 inhibitor rolipram shows some subtype selectivity for PDE4D. Taken together, these data indicate that PDE4 inhibition is a therapeutic target for the treatment of BPD.

PDE5 inhibition by sildenafil

Sildenafil is a selective PDE5 inhibitor and has been used clinically in the treatment of pulmonary hypertension [86]. Of the 11 PDE enzymes, PDE5 is highly selective for cGMP and is widely expressed in human tissues, but is most abundant in the lung and in pulmonary vascular smooth muscle cells [87]. PDE5 is mainly responsible for modulating intracellular cGMP levels and protein kinase-dependent signaling produced by NO. Cyclic GMP regulates the pulmonary vascular tone and influences pulmonary vascular structure directly, through effects on vascular smooth muscle proliferation and survival [86]. Upregulation of PDE5 expression is occurring during pulmonary hypertension, thereby contributing to increased lung vascular resistance [88]. The vasodilative properties of sildenafil have been shown in monocrotaline-, bleomycin- and hyperoxia-induced pulmonary hypertension in rats [89-91]. PDE5 is also expressed in the coronary vasculature and only in myocytes in the right ventricle under pressure overload [92]. PDE5 inhibition enhances contractility of the myocardium *in vitro*, suggesting that PDE5 inhibition might directly improve right ventricular function in pulmonary hypertension [86]. In addition, cGMP signaling enhances endothelial cell migration,

growth and organization into capillary-like structures *in vitro* and angiogenesis *in vivo* [93,94]. Pyriochou and colleagues showed that sildenafil stimulates angiogenesis through the cGMP/protein kinase-dependent pathway [95]. These findings suggest that PDE5 inhibition may represent potential therapeutic target in reducing the major issues concerning the development of BPD, namely the arrest in lung development and pulmonary hypertension.

Apelin

Apelin is an endogenous bioactive peptide for the 7-transmembrane G protein-coupled APJ receptor (figure 4). It is derived from a 77 amino acid prepropeptide that is cleaved into a 12-36 amino acid fragments that are biologically active [96]. APJ shares a 30% homology with the angiotensin II type I receptor, but angiotension II does not bind to APJ [97]. APJ is also a coreceptor for the entry of HIV into host cells [98]. Apelin is produced and secreted by mature human and murine adipocytes [99] and endothelial cells [100], and its expression is induced by hypoxia in endothelial cells [101]. APJ and apelin mRNA have been detected in various human and rat tissues, including the lung, heart, artery, vein, skeletal muscle, kidney, brain and liver [96,102-104]. The presence of apelin receptors and apelin in the lungs, heart and blood vessels suggest that this peptide may have a cardiopulmonary role.

Apelin receptor activation leads to phosphorylation of ERK, Akt and phospholipase C (PLC) [105,106], which constitute the basis for a dual function of apelin signaling at the endothelial level. Activation of Akt and PLC induces the activation of endothelial nitric oxide synthase (eNOS) and NO release, which relaxes the smooth muscle and lowers blood pressure [104,107]. On the other hand, activation of the apelin receptor promotes phosphorylation of the ERKS and Akt proteins that can promote cell migration and proliferation of endothelial cells, leading to angiogenesis [106,108]. Apelin knockout mice shows reduced vascular development [109] and abrogated angiopoietin I-mediated vascular enlargement [110]. Overexpression of the apelin-APJ pathway promotes blood vessel and neointima formation in animal models of ischemia [111,112]. However, the molecular mechanisms by which the apelin/APJ pathway promotes angiogenesis is not clear. Inhibition of VEGF and FGF receptor activity failed to inhibit apelin-induced cell proliferation, suggesting that the effect of apelin on angiogenesis is independent of VEGF and FGF receptors [113]. It is well known that NO is a mediator of angiogenic processes. Apelin induces phospholyration of eNOS and NO release from endothelial cells, and thus NO could mediate stimulation of angiogenesis by apelin.

In the heart, APJ is expressed by myocardial cells, endothelial cells and smooth muscle cells [114]. Activation of the apelin receptor at the surface of cardiomyocytes results in a potent inotropic effect of apelin both in normal and diseased hearts [114-116]. Chronic treatment with apelin had cardioprotective effects by reducing cardiac loading without inducing ventricular hypertrophy [117,118] and reduced myocardial injury and improved right ventricular function in monocrotaline-induced pulmonary hypertension [119]. Apelin knockout mice demonstrate enhanced cardiac dysfunction and myocardial remodeling in aging and in response to pressure overload [120], which is supported by *in vitro* data that loss of apelin would reduce beneficial positive inotropic apelin actions [115]. Both apelin and APJ knockout mice have decreased basal cardiac contractility [121], normotensive baseline levels, but have increased vasopressor response to angiotensin II administration [122], suggesting a counter-measure against angiotension II-mediated pressor effects. Taken together, these data indicate

that the apelin/APJ system is a therapeutic target for the treatment of heart failure and ventricular overload.

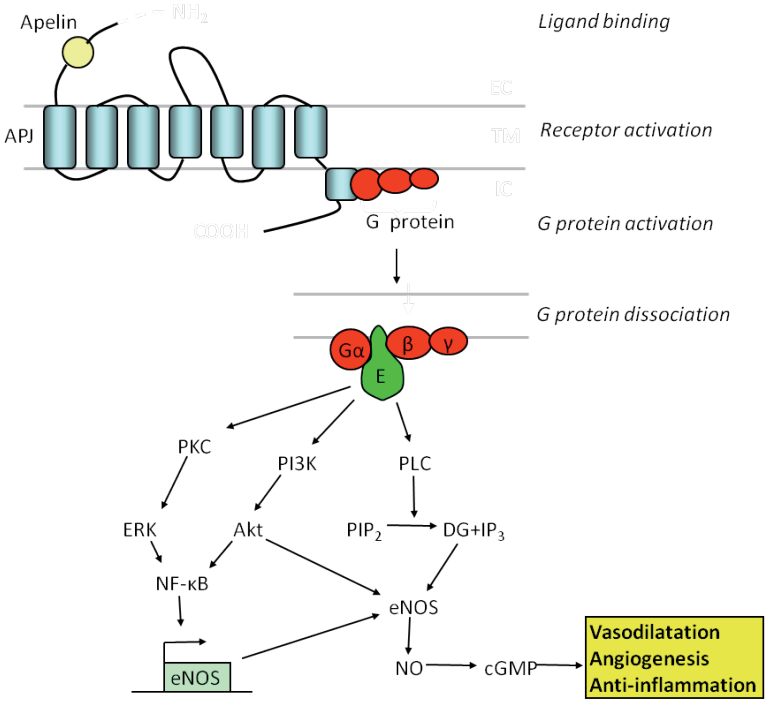


Figure 4. Schematic overview of the signalling cascade in endothelial cells after binding of apelin to its receptor APJ.

Binding of apelin to APJ results in the activation of G protein, which will increase endothelial NOS production via activation of ERK and Akt pathways leading to increased transcription of the eNOS gene or via activation of phospholipase C. Increased eNOS will induced NO production that activates guanylate cyclase, which induces smooth muscle cell relaxation, angiogenesis and anti-inflammation via increased cGMP levels.

Both murine monocytes and macrophages express APJ with highest expression in activated macrophages, suggesting a role for apelin during macrophage activation [123]. Apelin was found to have an direct anti-inflammatory effect in cultured cells by downregulating TNF alpha and MCP-1 and a trend toward less IL6, M-CSF and MIP-1alpha. Recently, Leeper and co-workers showed an anti-inflammatory role for apelin *in vivo*, by blocking the macrophage burden and inflammatory chemokine and cytokine production in the aneurysmal aorta [123]. Apelin is also involved in fluid balance, hormone release, water and food intake and circadian rythms [116,124,125]. In a study of both human and mouse adipocytes, and in models of obesity, apelin has been identified as a novel adipokine that is released from fat cells and is upregulated directly by insulin [99].

Ambrisentan

Ambrisentan is a endothelin (ET) A receptor antagonist. Endothelins are a family of three 21-amino acid peptides (ET-1, ET-2 and ET-3), each with distinct gene and tissue distributions, that are cleaved from preproproteins by ET converting enzyme (ECE) to form biological active ETs [126]. Among these, ET-1 is the most predominant isoform synthesized in the human vasculature and the most potent vasoconstrictor [127], which is primarily produced by endothelial cells and to a lesser extent by vascular smooth muscle cells or macrophages. The biological effects of ET-1 are mediated by two G protein-coupled receptors, ET A receptor and ET B receptor, which activated distinct signaling pathways [128]. The ET A receptors are expressed on pulmonary arterial smooth muscle cells, fibroblasts and cardiomyocytes, whereas ET B receptors are expressed by endothelial cells and to a lesser extent by pulmonary arterial smooth muscle cells and fibroblasts [129]. ET-1 has opposite vascular effects mediated through the different receptors. Activation of ET receptors on pulmonary arterial smooth muscle cells mediate a potent vasoconstrictive response, whereas ET B receptors on endothelial cells mediate vasodilation via increased production of NO and prostacyclin [130,131]. In addition, ET-1 is involved in several other processes, including endothelial dysfunction, extracellular matrix production, inflammation, cell proliferation and fibrosis [132].
Hyperoxia has been shown to elevate ET-1 levels in endothelial cells [133] and in experimental model of bronchopulmonary dysplasia [25] and circulating levels are raised in rats with hyperoxia-induced pulmonary hypertension [134]. Both selective ET A and mixed ET A/B have similar beneficial effects in *in vivo* models of pulmonary arterial hypertension [135,136], chronic heart failure [137,138], atherosclerosis [139,140] and hypertension [141,142]. Although the discovery of ET receptor antagonists is a milestone in the treatment of pulmonary hypertension, its role in experimental bronchopulmonary dysplasia is still unknown.

Stem cells

Bone marrow stromal cells, also known as mesenchymal stem cells, marrow stromal cells and more recently mesenchymal stromal cells (MSC), have been the subject of intensive investigation over the past decade. These cells, critical to the support of hematopoiesis (), can differentiate *in vitro* along mesenchymal lineages, i.e. adipocytic, osteoblactic and chondrocytic lineages [143] and into parenchymal cells of various non-hematopoietic tissues including the lung [144] and can exhibit neuronal [145], hepatic [146], and cardiac [147] characteristics, suggesting a possible role in tissue repair. The International Society for Cellular Therapy (ISCT) have provided the following three criteria for defining multipotent MSCs [148] a) plastic-adherent inder standard culture conditions, b) express CD105, CD73, CD90 and lack the expression of CD45, CD34, CD14, CD79 and HLA-DR and c) must be able to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*.
MSC treatment can ameliorate bleomycin, monocrotaline, endotoxin, or hyperoxic-induced lung injury [149-153], by migrating and repairing tissue damage but also to deliver protection by secretion of specific growth, vasoprotective and immunoprotective factors. In addition, MSCs overexpressing the prosurvival protein Akt improved hemodynamic endpoints and cardiac function in rat models of experimental myocardial infarction [154,155], which is probably mediated by paracrine factors, including vascular endothelial growth factors, fibroblast growth factors and hepatocyte growth factor. These data indicate that stem cell treatment

of infants with bronchopulmonary dysplasia may be beneficial to improve alveologenesis and pulmonary hypertension.

Aim and outline of the thesis

The aim of the studies presented in this thesis is to test potential treatment options in an animal model of bronchopulmonary dysplasia. **Chapter 1** contains a general introduction to lung development and bronchopulmonary dysplasia in respect to relevant topics further studied in this thesis. The inflammatory process is one of the major players in experimental BPD and inhibition of this process by phospohodiesterase type 4 inhibition is explored in **chapter 2**. In **chapter 3** we investigated the effect of phophodiesterase type 4 inhibition on the cardiopulmonary aspect of experimental BPD, as hyperoxia exposure leads pulmonary hypertension and to right ventricular hypertrophy**.** BPD is characterized by arrest in alveolar development or loss of alveoli and currently lack effective therapy. In **chapters 4** and **5** we explored the therapeutic potential on alveologenesis of apelin and phosphodiesterase type 5 inhibitor sildenafil, both very potent pro-angiogenic and vasodilative agents. In **chapter 6** we investigated the therapeutic potential of stem cell therapy in an animal model of pulmonary hypertension by mimicking autologous MSC therapy. **Chapter 7** contains general conclusions and a discussion regarding these results.

References

1. Whitsett JA, Wert SE, Trapnell BC. Genetic disorders influencing lung formation and function at birth. Hum Mol Genet 2004;13 Spec No 2: R207-R215
2. Copland I, Post M. Lung development and fetal lung growth. Paediatr Respir 2004;Rev 5 Suppl A: S259-S264
3. Chinoy MR. Lung growth and development. Front Biosci 2003;8: d392-d415
4. Kauffman SL, Burri PH, Weibel ER. The postnatal growth of the rat lung. II. Autoradiography. Anat Rec 1974;180: 63-76
5. Yamada T, Suzuki E, Gejyo F, Ushiki T. Developmental changes in the structure of the rat fetal lung, with special reference to the airway smooth muscle and vasculature. Arch Histol Cytol 2002;65: 55-69
6. Shannon JM, Hyatt BA. Epithelial-mesenchymal interactions in the developing lung. Annu Rev Physiol 2004;66: 625-645
7. DiFiore JW, Wilson JM. Lung development. Semin Pediatr Surg 1994;3: 221-232
8. Burri PH. Structural aspects of postnatal lung development - alveolar formation and growth. Biol Neonate 2006;89: 313-322
9. Coalson JJ. Pathology of new bronchopulmonary dysplasia. Semin Neonatol 2003;8: 73-81
10. Northway WH, Jr., Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med 1967;276: 357-368
11. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med 2001;163: 1723-1729
12. Rojas MA, Gonzalez A, Bancalari E, Claure N, Poole C, Silva-Neto G. Changing trends in the epidemiology and pathogenesis of neonatal chronic lung disease. J Pediatr 1995;126: 605-610
13. Jobe AJ. The new BPD: an arrest of lung development. Pediatr Res 1999;46: 641-643
14. Allen J, Zwerdling R, Ehrenkranz R, Gaultier C, Geggel R, Greenough A, Kleinman R, Klijanowicz A, Martinez F, Ozdemir A, Panitch HB, Nickerson B, Stein MT, Tomezsko J, Van Der AJ. Statement on the care of the child with chronic lung disease of infancy and childhood. Am J Respir Crit Care Med 2003;168: 356-396
15. Short EJ, Klein NK, Lewis BA, Fulton S, Eisengart S, Kercsmar C, Baley J, Singer LT. Cognitive and academic consequences of bronchopulmonary dysplasia and very low birth weight: 8-year-old outcomes. Pediatrics 2003;112: e359
16. Lewis BA, Singer LT, Fulton S, Salvator A, Short EJ, Klein N, Baley J. Speech and language outcomes of children with bronchopulmonary dysplasia. J Commun Disord 2002;35: 393-406

17. Robin B, Kim YJ, Huth J, Klocksieben J, Torres M, Tepper RS, Castile RG, Solway J, Hershenson MB, Goldstein-Filbrun A. Pulmonary function in bronchopulmonary dysplasia. Pediatr Pulmonol 2004;37: 236-242
18. Smith VC, Zupancic JA, McCormick MC, Croen LA, Greene J, Escobar GJ, Richardson DK. Rehospitalization in the first year of life among infants with bronchopulmonary dysplasia. J Pediatr 2004;144: 799-803
19. Weisman LE. Populations at risk for developing respiratory syncytial virus and risk factors for respiratory syncytial virus severity: infants with predisposing conditions. Pediatr Infect Dis J 2003;22: S33-S37
20. O'Reilly MA, Marr SH, Yee M, McGrath-Morrow SA, Lawrence BP. Neonatal hyperoxia enhances the inflammatory response in adult mice infected with influenza A virus. Am J Respir Crit Care Med 2008;177: 1103-1110
21. Yoon BH, Romero R, Jun JK, Park KH, Park JD, Ghezzi F, Kim BI. Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. Am J Obstet Gynecol 1997;177: 825-830
22. Zanardo V, Savio V, Giacomin C, Rinaldi A, Marzari F, Chiarelli S. Relationship between neonatal leukemoid reaction and bronchopulmonary dysplasia in low-birth-weight infants: a cross-sectional study. Am J Perinatol 2002;19: 379-386
23. Groneck P, Gotze-Speer B, Oppermann M, Eiffert H, Speer CP. Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of high-risk preterm neonates. Pediatrics 1994;93: 712-718
24. Hsiao R, Omar SA. Outcome of extremely low birth weight infants with leukemoid reaction. Pediatrics 2005;116: e43-e51
25. Wagenaar GT, ter Horst SA, van Gastelen MA, Leijser LM, Mauad T, van der Velden PA, de Heer E, Hiemstra PS, Poorthuis BJ, Walther FJ. Gene expression profile and histopathology of experimental bronchopulmonary dysplasia induced by prolonged oxidative stress. Free Radic Biol Med 2004; 36: 782-801
26. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. Am J Respir Crit Care Med 1999;160: 1333-1346
27. Warner BB, Stuart LA, Papes RA, Wispe JR. Functional and pathological effects of prolonged hyperoxia in neonatal mice. Am J Physiol 1998;275: L110-L117
28. Auten RL, Jr., Mason SN, Tanaka DT, Welty-Wolf K, Whorton MH. Anti-neutrophil chemokine preserves alveolar development in hyperoxia-exposed newborn rats. Am J Physiol Lung Cell Mol Physiol 2001;281: L336-L344
29. Vozzelli MA, Mason SN, Whorton MH, Auten RL, Jr. Antimacrophage chemokine treatment prevents neutrophil and macrophage influx in hyperoxia-exposed newborn rat lung. Am J Physiol Lung Cell Mol Physiol 2004;286:L488-L493
30. Levi M, van der Poll T, ten Cate H, Kuipers B, Biemond BJ, Jansen HM, ten Cate JW. Differential effects of anti-cytokine treatment on bronchoalveolar hemostasis in endotoxemic chimpanzees. Am J Respir Crit Care Med 1998;158: 92-98
31. van der Poll T, Buller HR, ten Cate H, Wortel CH, Bauer KA, van Deventer SJ, Hack CE, Sauerwein HP, Rosenberg RD, ten Cate JW. Activation of coagulation after administration of tumor necrosis factor to normal subjects. N Engl J Med 1990;322: 1622-1627
32. Levi M, van der Poll T. Two-way interactions between inflammation and coagulation. Trends Cardiovasc Med 2005;15: 254-259
33. Levi M, ten CATE H, Bauer KA, van der Poll T, Edgington TS, Buller HR, van Deventer SJ, Hack CE, ten CATE JW, Rosenberg RD. Inhibition of endotoxin-induced activation of coagulation and fibrinolysis by pentoxifylline or by a monoclonal anti-tissue factor antibody in chimpanzees. J Clin Invest 1994;93: 114-120
34. Welty-Wolf KE, Carraway MS, Miller DL, Ortel TL, Ezban M, Ghio AJ, Idell S, Piantadosi CA. Coagulation blockade prevents sepsis-induced respiratory and renal failure in baboons. Am J Respir Crit Care Med 2001;164: 1988-1996
35. Idell S, Koenig KB, Fair DS, Martin TR, McLarty J, Maunder RJ. Serial abnormalities of fibrin turnover in evolving adult respiratory distress syndrome. Am J Physiol 1991;261: L240-L248

36. Barazzone C, Belin D, Piguet PF, Vassalli JD, Sappino AP. Plasminogen activator inhibitor-1 in acute hyperoxic mouse lung injury. J Clin Invest 1996;98: 2666-2673
37. Idell S. Coagulation, fibrinolysis, and fibrin deposition in acute lung injury. Crit Care Med 2003;31: S213-S220
38. Dang CV, Bell WR, Kaiser D, Wong A. Disorganization of cultured vascular endothelial cell monolayers by fibrinogen fragment D. Science 1985;227: 1487-1490
39. Stevens T, Garcia JG, Shasby DM, Bhattacharya J, Malik AB. Mechanisms regulating endothelial cell barrier function. Am J Physiol Lung Cell Mol Physiol 2000;279: L419-L422
40. Joyce DE, Gelbert L, Ciaccia A, DeHoff B, Grinnell BW. Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. J Biol Chem 2001;276: 11199-11203
41. Esmon CT. Introduction: are natural anticoagulants candidates for modulating the inflammatory response to endotoxin? Blood 2000;95: 1113-1116
42. Thebaud B. Angiogenesis in lung development, injury and repair: implications for chronic lung disease of prematurity. Neonatology 2007;91: 291-297
43. Wendel DP, Taylor DG, Albertine KH, Keating MT, Li DY. Impaired distal airway development in mice lacking elastin. Am J Respir Cell Mol Biol 2000;23: 320-326
44. Thibeault DW, Mabry SM, Ekekezie II, Truog WE. Lung elastic tissue maturation and perturbations during the evolution of chronic lung disease. 2000. Pediatrics 106: 1452-1459
45. Pierce RA, Albertine KH, Starcher BC, Bohnsack JF, Carlton DP, Bland RD. Chronic lung injury in preterm lambs: disordered pulmonary elastin deposition. Am J Physiol 1997;272: L452-L460
46. Lindahl P, Karlsson L, Hellstrom M, Gebre-Medhin S, Willetts K, Heath JK, Betsholtz C. Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. Development 1997;124: 3943-3953
47. Buch S, Han RN, Cabacungan J, Wang J, Yuan S, Belcastro R, Deimling J, Jankov R, Luo X, Lye SJ, Post M, Tanswell AK. Changes in expression of platelet-derived growth factor and its receptors in the lungs of newborn rats exposed to air or 60% O(2). Pediatr Res 2000;48: 423-433
48. Kaza AK, Kron IL, Leuwerke SM, Tribble CG, Laubach VE. Keratinocyte growth factor enhances post-pneumonectomy lung growth by alveolar proliferation. Circulation 2002;106: I120-I124
49. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, Voelkel NF. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. J Clin Invest 2000;106: 1311-1319
50. Thebaud B, Ladha F, Michelakis ED, Sawicka M, Thurston G, Eaton F, Hashimoto K, Harry G, Haromy A, Korbutt G, Archer SL. Vascular endothelial growth factor gene therapy increases survival, promotes lung angiogenesis, and prevents alveolar damage in hyperoxia-induced lung injury: evidence that angiogenesis participates in alveolarization. Circulation 2005;112: 2477-2486
51. Acarregui MJ, Penisten ST, Goss KL, Ramirez K, Snyder JM. Vascular endothelial growth factor gene expression in human fetal lung in vitro. Am J Respir Cell Mol Biol 1999;20: 14-23
52. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999;13: 9-22
53. Maniscalco WM, Watkins RH, D'Angio CT, Ryan RM. Hyperoxic injury decreases alveolar epithelial cell expression of vascular endothelial growth factor (VEGF) in neonatal rabbit lung. Am J Respir Cell Mol Biol 1997;16: 557-567
54. Bhatt AJ, Pryhuber GS, Huyck H, Watkins RH, Metlay LA, Maniscalco WM. Disrupted pulmonary vasculature and decreased vascular endothelial growth factor, Flt-1, and TIE-2 in human infants dying with bronchopulmonary dysplasia. Am J Respir Crit Care Med 2001;164: 1971-1980
55. Klekamp JG, Jarzecka K, Perkett EA. Exposure to hyperoxia decreases the expression of vascular endothelial growth factor and its receptors in adult rat lungs. Am J Pathol 1999;154: 823-831
56. Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF, Abman SH. Inhibition of angiogenesis decreases alveolarization in the developing rat lung. Am J Physiol Lung Cell Mol Physiol 2000;279: L600-L607
57. Le Cras TD, Markham NE, Tuder RM, Voelkel NF, Abman SH. Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal lung structure. Am J Physiol Lung Cell Mol Physiol 2002;283: L555-L562

58. Gerber HP, Hillan KJ, Ryan AM, Kowalski J, Keller GA, Rangell L, Wright BD, Radtke F, Aguet M, Ferrara N. VEGF is required for growth and survival in neonatal mice. Development 1999;126: 1149-1159
59. Steinhorn RH. Neonatal pulmonary hypertension. Pediatr Crit Care Med 2010;11: S79-S84
60. Humbert M, Sitbon O, Simonneau G. Treatment of pulmonary arterial hypertension. N Engl J Med 2004;351: 1425-1436
61. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkel NF, Rabinovitch M. Cellular and molecular pathobiology of pulmonary arterial hypertension. J Am Coll Cardiol 2004;43: 13S-24S
62. Eddahibi S, Guignabert C, Barlier-Mur AM, Dewachter L, Fadel E, Dartevelle P, Humbert M, Simonneau G, Hanoun N, Saurini F, Hamon M, Adnot S. Cross talk between endothelial and smooth muscle cells in pulmonary hypertension: critical role for serotonin-induced smooth muscle hyperplasia. Circulation 2006;113: 1857-1864
63. Tuder RM, Groves B, Badesch DB, Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. Am J Pathol 1994;144: 275-285
64. Maniscalco WM, Watkins RH, Pryhuber GS, Bhatt A, Shea C, Huyck H. Angiogenic factors and alveolar vasculature: development and alterations by injury in very premature baboons. Am J Physiol Lung Cell Mol Physiol 2002;282: L811-L823
65. Randell SH, Mercer RR, Young SL. Postnatal growth of pulmonary acini and alveoli in normal and oxygen-exposed rats studied by serial section reconstructions. Am J Anat 1989;186: 55-68
66. Kass DA, Takimoto E, Nagayama T, Champion HC. Phosphodiesterase regulation of nitric oxide signaling. Cardiovasc Res 2007;75: 303-314
67. Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev 2006;58: 488-520
68. Sanz MJ, Cortijo J, Morcillo EJ. PDE4 inhibitors as new anti-inflammatory drugs: effects on cell trafficking and cell adhesion molecules expression. Pharmacol Ther 2005;106: 269-297
69. Rabe KF, Tenor H, Dent G, Schudt C, Nakashima M, Magnussen H. Identification of PDE isozymes in human pulmonary artery and effect of selective PDE inhibitors. Am J Physiol 1994;266: L536-L543
70. Torphy TJ. Phosphodiesterase isozymes: molecular targets for novel antiasthma agents. Am J Respir Crit Care Med 1998;157: 351-370
71. Press NJ, Banner KH. PDE4 inhibitors - a review of the current field. Prog Med Chem 2009;47: 37-74
72. Spina D. Phosphodiesterase-4 inhibitors in the treatment of inflammatory lung disease. Drugs 2003;63: 2575-2594
73. Ariga M, Neitzert B, Nakae S, Mottin G, Bertrand C, Pruniaux MP, Jin SL, Conti M. Nonredundant function of phosphodiesterases 4D and 4B in neutrophil recruitment to the site of inflammation. J Immunol 2004;173: 7531-7538
74. Leclerc O, Lagente V, Planquois JM, Berthelier C, Artola M, Eichholtz T, Bertrand CP, Schmidlin F. Involvement of MMP-12 and phosphodiesterase type 4 in cigarette smoke-induced inflammation in mice. Eur Respir J 2006;27: 1102-1109
75. Corbel M, Germain N, Lanchou J, Molet S, Silva PM, Martins MA, Boichot E, Lagente V. The selective phosphodiesterase 4 inhibitor RP 73-401 reduced matrix metalloproteinase 9 activity and transforming growth factor-beta release during acute lung injury in mice: the role of the balance between Tumor necrosis factor-alpha and interleukin-10. Pharmacol Exp Ther 2002;301: 258-265
76. Izikki M, Raffestin B, Klar J, Hatzelmann A, Marx D, Tenor H, Zadigue P, Adnot S, Eddahibi S. Effects of roflumilast, a phosphodiesterase-4 inhibitor, on hypoxia- and monocrotaline-induced pulmonary hypertension in rats. J Pharmacol Exp Ther 2009;330: 54-62
77. Cortijo J, Iranzo A, Milara X, Mata M, Cerda-Nicolas M, Ruiz-Sauri A, Tenor H, Hatzelmann A, Morcillo EJ. Roflumilast, a phosphodiesterase 4 inhibitor, alleviates bleomycin-induced lung injury. Br J Pharmacol 2009;156: 534-544
78. Rabe KF, Bateman ED, O'Donnell D, Witte S, Bredenbroker D, Bethke TD. Roflumilast--an oral anti-inflammatory treatment for chronic obstructive pulmonary disease: a randomised controlled trial. Lancet 2005;366: 563-571

79. Calverley PM, Sanchez-Toril F, McIvor A, Teichmann P, Bredenbroeker D, Fabbri LM. Effect of 1-year treatment with roflumilast in severe chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2007;176: 154-161
80. Grootendorst DC, Gauw SA, Verhoosel RM, Sterk PJ, Hospers JJ, Bredenbroker D, Bethke TD, Hiemstra PS, Rabe KF. Reduction in sputum neutrophil and eosinophil numbers by the PDE4 inhibitor roflumilast in patients with COPD. Thorax 2007;62: 1081-1087
81. Lehnart SE, Wehrens XH, Reiken S, Warrier S, Belevych AE, Harvey RD, Richter W, Jin SL, Conti M, Marks AR. Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. Cell 2005;123: 25-35
82. Glover DK, Riou LM, Ruiz M, Sullivan GW, Linden J, Rieger JM, Macdonald TL, Watson DD, Beller GA. Reduction of infarct size and postischemic inflammation from ATL-146e, a highly selective adenosine A2A receptor agonist, in reperfused canine myocardium. Am J Physiol Heart Circ Physiol 2005;288: H1851-H1858
83. Rose GM, Hopper A, De Vivo M, Tehim A. Phosphodiesterase inhibitors for cognitive enhancement. Curr Pharm Des 2005;11: 3329-3334
84. Ghavami A, Hirst WD, Novak TJ. Selective phosphodiesterase (PDE)-4 inhibitors: a novel approach to treating memory deficit? Drugs R D 2006;7: 63-71
85. Rennard SI, Schachter N, Strek M, Rickard K, Amit O. Cilomilast for COPD: results of a 6-month, placebo-controlled study of a potent, selective inhibitor of phosphodiesterase 4. Chest 2006;129: 56-66
86. Wilkins MR, Wharton J, Grimminger F, Ghofrani HA. Phosphodiesterase inhibitors for the treatment of pulmonary hypertension. Eur Respir J 2008;32: 198-209
87. Corbin JD, Beasley A, Blount MA, Francis SH. High lung PDE5: a strong basis for treating pulmonary hypertension with PDE5 inhibitors. Biochem Biophys Res Commun 2005;334: 930-938
88. Black SM, Sanchez LS, Mata-Greenwood E, Bekker JM, Steinhorn RH, Fineman JR. sGC and PDE5 are elevated in lambs with increased pulmonary blood flow and pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 2001;281: L1051-L1057
89. Schermuly RT, Kreisselmeier KP, Ghofrani HA, Yilmaz H, Butrous G, Ermert L, Ermert M, Weissmann N, Rose F, Guenther A, Walmrath D, Seeger W, Grimminger F. Chronic sildenafil treatment inhibits monocrotaline-induced pulmonary hypertension in rats. Am J Respir Crit Care Med 2004;169: 39-45
90. Hemnes AR, Zaiman A, Champion HC. PDE5A inhibition attenuates bleomycin-induced pulmonary fibrosis and pulmonary hypertension through inhibition of ROS generation and RhoA/Rho kinase activation. Am J Physiol Lung Cell Mol Physiol 2008;294: L24-L33
91. Ladha F, Bonnet S, Eaton F, Hashimoto K, Korbutt G, Thebaud B. Sildenafil improves alveolar growth and pulmonary hypertension in hyperoxia-induced lung injury. Am J Respir Crit Care Med 2005;172: 750-756
92. Nagendran J, Archer SL, Soliman D, Gurtu V, Moudgil R, Haromy A, St Aubin C, Webster L, Rebeyka IM, Ross DB, Light PE, Dyck JR, Michelakis ED. Phosphodiesterase type 5 is highly expressed in the hypertrophied human right ventricle, and acute inhibition of phosphodiesterase type 5 improves contractility. Circulation 2007116: 238-248
93. Fukumura D, Gohongi T, Kadambi A, Izumi Y, Ang J, Yun CO, Buerk DG, Huang PL, Jain RK. Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability. Proc Natl Acad Sci U S A 2001.;98: 2604-2609
94. Zhang R, Wang L, Zhang L, Chen J, Zhu Z, Zhang Z, Chopp M. Nitric oxide enhances angiogenesis via the synthesis of vascular endothelial growth factor and cGMP after stroke in the rat. Circ Res 2003;92: 308-313
95. Pyriochou A, Zhou Z, Koika V, Petrou C, Cordopatis P, Sessa WC, Papapetropoulos A. The phosphodiesterase 5 inhibitor sildenafil stimulates angiogenesis through a protein kinase G/MAPK pathway. J Cell Physiol 2007;211: 197-204
96. Hosoya M, Kawamata Y, Fukusumi S, Fujii R, Habata Y, Hinuma S, Kitada C, Honda S, Kurokawa T, Onda H, Nishimura O, Fujino M. Molecular and functional characteristics of APJ. Tissue distribution of mRNA and interaction with the endogenous ligand apelin. J Biol Chem 2000;275: 21061-21067
97. Medhurst AD, Jennings CA, Robbins MJ, Davis RP, Ellis C, Winborn KY, Lawrie KW, Hervieu G, Riley G, Bolaky JE, Herrity NC, Murdock P, Darker JG. Pharmacological and immunohistochemical characterization of the APJ receptor and its endogenous ligand apelin. J Neurochem 2003;84: 1162-1172

98. Edinger AL, Hoffman TL, Sharron M, Lee B, Yi Y, Choe W, Kolson DL, Mitrovic B, Zhou Y, Faulds D, Collman RG, Hesselgesser J, Horuk R, Doms RW. An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. J Virol 1998;72: 7934-7940
99. Boucher J, Masri B, Daviaud D, Gesta S, Guigne C, Mazzucotelli A, Castan-Laurell I, Tack I, Knibiehler B, Carpene C, Audigier Y, Saulnier-Blache JS, Valet P. Apelin, a newly identified adipokine up-regulated by insulin and obesity. Endocrinology 2005;146: 1764-1771
100. Masri B, Knibiehler B, Audigier Y. Apelin signalling: a promising pathway from cloning to pharmacology. Cell Signal 2005;17: 415-426
101. Eyries M, Siegfried G, Ciumas M, Montagne K, Agrapart M, Lebrin F, Soubrier F. Hypoxia-induced apelin expression regulates endothelial cell proliferation and regenerative angiogenesis. Circ Res 2008;103: 432-440
102. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou MX, Kawamata Y, Fukusumi S, Hinuma S, Kitada C, Kurokawa T, Onda H, Fujino M. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. Biochem Biophys Res Commun 1998;251: 471-476
103. De Mota N, Lenkei Z, Llorens-Cortes C. Cloning, pharmacological characterization and brain distribution of the rat apelin receptor. Neuroendocrinology 2000;72: 400-407
104. Lee DK, Cheng R, Nguyen T, Fan T, Kariyawasam AP, Liu Y, Osmond DH, George SR, O'Dowd BF. Characterization of apelin, the ligand for the APJ receptor. J Neurochem 2000;74: 34-41
105. Masri B, Morin N, Pedebernade L, Knibiehler B, Audigier Y. The apelin receptor is coupled to Gi1 or Gi2 protein and is differentially desensitized by apelin fragments. J Biol Chem 2006;281: 18317-18326
106. Masri B, Morin N, Cornu M, Knibiehler B, Audigier Y. Apelin (65-77) activates p70 S6 kinase and is mitogenic for umbilical endothelial cells. FASEB J 2004;18: 1909-1911
107. Tatemoto K, Takayama K, Zou MX, Kumaki I, Zhang W, Kumano K, Fujimiya M. The novel peptide apelin lowers blood pressure via a nitric oxide-dependent mechanism. Regul Pept 2001;99: 87-92
108. Kasai A, Shintani N, Oda M, Kakuda M, Hashimoto H, Matsuda T, Hinuma S, Baba A. Apelin is a novel angiogenic factor in retinal endothelial cells. Biochem Biophys Res Commun 2004;325: 395-400
109. Kasai A, Shintani N, Kato H, Matsuda S, Gomi F, Haba R, Hashimoto H, Kakuda M, Tano Y, Baba A. Retardation of retinal vascular development in apelin-deficient mice. Arterioscler Thromb Vasc Biol 2008;28: 1717-1722
110. Kidoya H, Ueno M, Yamada Y, Mochizuki N, Nakata M, Yano T, Fujii R, Takakura N. Spatial and temporal role of the apelin/APJ system in the caliber size regulation of blood vessels during angiogenesis. EMBO J 2008;27: 522-534
111. Kidoya H, Naito H, Takakura N. Apelin induces enlarged and nonleaky blood vessels for functional recovery from ischemia. Blood 2010;115: 3166-3174
112. Kojima Y, Kundu RK, Cox CM, Leeper NJ, Anderson JA, Chun HJ, Ali ZA, Ashley EA, Krieg PA, Quertermous T. Upregulation of the apelin-APJ pathway promotes neointima formation in the carotid ligation model in mouse. Cardiovasc Res 2010;87: 156-165
113. Cox CM, D'Agostino SL, Miller MK, Heimark RL, Krieg PA. Apelin, the ligand for the endothelial G-protein-coupled receptor, APJ, is a potent angiogenic factor required for normal vascular development of the frog embryo. Dev Biol 2006;296: 177-189
114. Kleinz MJ, Skepper JN, Davenport AP. Immunocytochemical localisation of the apelin receptor, APJ, to human cardiomyocytes, vascular smooth muscle and endothelial cells. Regul Pept 2005;126: 233-240
115. Maguire JJ, Kleinz MJ, Pitkin SL, Davenport AP. [Pyr1]apelin-13 identified as the predominant apelin isoform in the human heart: vasoactive mechanisms and inotropic action in disease. Hypertension 2009;54: 598-604
116. Szokodi I, Tavi P, Foldes G, Voutilainen-Myllyla S, Ilves M, Tokola H, Pikkarainen S, Piuhola J, Rysa J, Toth M, Ruskoaho H. Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. Circ Res 2002;91: 434-440
117. Ashley EA, Powers J, Chen M, Kundu R, Finsterbach T, Caffarelli A, Deng A, Eichhorn J, Mahajan R, Agrawal R, Greve J, Robbins R, Patterson AJ, Bernstein D, Quertermous T. The endogenous peptide apelin potently improves cardiac contractility and reduces cardiac loading in vivo. Cardiovasc Res 2005;65: 73-82

118. Berry MF, Pirolli TJ, Jayasankar V, Burdick J, Morine KJ, Gardner TJ, Woo YJ. Apelin has in vivo inotropic effects on normal and failing hearts. Circulation 2004;110: II187-II193
119. Falcao-Pires I, Goncalves N, Henriques-Coelho T, Moreira-Goncalves D, Roncon-Albuquerque R, Jr., Leite-Moreira AF. Apelin decreases myocardial injury and improves right ventricular function in monocrotaline-induced pulmonary hypertension. Am J Physiol Heart Circ Physiol 2009;296: H2007-H2014
120. Kuba K, Zhang L, Imai Y, Arab S, Chen M, Maekawa Y, Leschnik M, Leibbrandt A, Markovic M, Schwaighofer J, Beetz N, Musialek R, Neely GG, Komnenovic V, Kolm U, Metzler B, Ricci R, Hara H, Meixner A, Nghiem M, Chen X, Dawood F, Wong KM, Sarao R, Cukerman E, Kimura A, Hein L, Thalhammer J, Liu PP, Penninger JM. Impaired heart contractility in Apelin gene-deficient mice associated with aging and pressure overload. Circ Res 2007;101: e32-e42
121. Charo DN, Ho M, Fajardo G, Kawana M, Kundu RK, Sheikh AY, Finsterbach TP, Leeper NJ, Ernst KV, Chen MM, Ho YD, Chun HJ, Bernstein D, Ashley EA, Quertermous T. Endogenous regulation of cardiovascular function by apelin-APJ. Am J Physiol Heart Circ Physiol 2009;297: H1904-H1913
122. Ishida J, Hashimoto T, Hashimoto Y, Nishiwaki S, Iguchi T, Harada S, Sugaya T, Matsuzaki H, Yamamoto R, Shiota N, Okunishi H, Kihara M, Umemura S, Sugiyama F, Yagami K, Kasuya Y, Mochizuki N, Fukamizu A. Regulatory roles for APJ, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure in vivo. J Biol Chem 2004;279: 26274-26279
123. Leeper NJ, Tedesco MM, Kojima Y, Schultz GM, Kundu RK, Ashley EA, Tsao PS, Dalman RL, Quertermous T. Apelin prevents aortic aneurysm formation by inhibiting macrophage inflammation. Am J Physiol Heart Circ Physiol 2009;296: H1329-H1335
124. O'Shea M, Hansen MJ, Tatemoto K, Morris MJ. Inhibitory effect of apelin-12 on nocturnal food intake in the rat. Nutr Neurosci 2003;6: 163-167
125. O'Carroll AM, Selby TL, Palkovits M, Lolait SJ. Distribution of mRNA encoding B78/apj, the rat homologue of the human APJ receptor, and its endogenous ligand apelin in brain and peripheral tissues. Biochim Biophys Acta 2000;1492: 72-80
126. Yanagisawa M, Inoue A, Ishikawa T, Kasuya Y, Kimura S, Kumagaye S, Nakajima K, Watanabe TX, Sakakibara S, Goto K. Primary structure, synthesis, and biological activity of rat endothelin, an endothelium-derived vasoconstrictor peptide. Proc Natl Acad Sci U S A 1988;85: 6964-6967
127. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988;332: 411-415
128. Opitz CF, Ewert R. Dual ET(A)/ET(B) vs. selective ET(A) endothelin receptor antagonism in patients with pulmonary hypertension. Eur J Clin Invest 2006;36 Suppl 3: 1-9
129. Davenport AP, O'Reilly G, Molenaar P, Maguire JJ, Kuc RE, Sharkey A, Bacon CR, Ferro A. Human endothelin receptors characterized using reverse transcriptase-polymerase chain reaction, in situ hybridization, and subtype-selective ligands BQ123 and BQ3020: evidence for expression of ETB receptors in human vascular smooth muscle. J Cardiovasc Pharmacol 1993;22 Suppl 8: S22-S25
130. Michael JR, Markewitz BA. Endothelins and the lung. Am J Respir Crit Care Med 1996;154: 555-581
131. Shetty SS, Okada T, Webb RL, DelGrande D, Lappe RW. Functionally distinct endothelin B receptors in vascular endothelium and smooth muscle. Biochem Biophys Res Commun 1993;191: 459-464
132. Dhaun N, Goddard J, Webb DJ. The endothelin system and its antagonism in chronic kidney disease. J Am Soc Nephrol 2006;17: 943-955
133. Higgins RD, Hendricks-Munoz KD, Caines VV, Gerrets RP, Rifkin DB. Hyperoxia stimulates endothelin-1 secretion from endothelial cells; modulation by captopril and nifedipine. Curr Eye Res 1998;17: 487-493
134. Habre W, Petak F, Ruchonnet-Metrailler I, Donati Y, Tolsa JF, Lele E, Albu G, Beghetti M, Barazzone-Argiroffo C. The role of endothelin-1 in hyperoxia-induced lung injury in mice. Respir Res 2006;7: 45
135. Eddahibi S, Raffestin B, Clozel M, Levame M, Adnot S. Protection from pulmonary hypertension with an orally active endothelin receptor antagonist in hypoxic rats. Am J Physiol 1995;268: H828-H835
136. Chen SJ, Chen YF, Opgenorth TJ, Wessale JL, Meng QC, Durand J, DiCarlo VS, Oparil S. The orally active nonpeptide endothelin A-receptor antagonist A-127722 prevents and reverses hypoxia-induced pulmonary hypertension and pulmonary vascular remodeling in Sprague-Dawley rats. J Cardiovasc Pharmacol 1997;29: 713-725
137. Mulder P, Boujedaini H, Richard V, Derumeaux G, Henry JP, Renet S, Wessale J, Opgenorth T, Thuillez C. Selective endothelin-A versus combined endothelin-A/endothelin-B receptor blockade in rat chronic heart failure. Circulation 2000;102: 491-493

138. Ohnishi M, Wada A, Tsutamoto T, Fukai D, Kinoshita M. Comparison of the acute effects of a selective endothelin ETA and a mixed ETA/ETB receptor antagonist in heart failure. Cardiovasc Res 1998;39: 617-624
139. Babaei S, Picard P, Ravandi A, Monge JC, Lee TC, Cernacek P, Stewart DJ. Blockade of endothelin receptors markedly reduces atherosclerosis in LDL receptor deficient mice: role of endothelin in macrophage foam cell formation. Cardiovasc Res 2000;48: 158-167
140. d'Uscio LV, Barton M, Shaw S, Luscher TF. Chronic ET(A) receptor blockade prevents endothelial dysfunction of small arteries in apolipoprotein E-deficient mice. Cardiovasc Res 2002;53: 487-495
141. Krum H, Viskoper RJ, Lacourciere Y, Budde M, Charlon V. The effect of an endothelin-receptor antagonist, bosentan, on blood pressure in patients with essential hypertension. Bosentan Hypertension Investigators. N Engl J Med 1998;338: 784-790
142. Nakov R, Pfarr E, Eberle S. Darusentan: an effective endothelinA receptor antagonist for treatment of hypertension. Am J Hypertens 2002;15: 583-589
143. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284: 143-147
144. Phinney DG, Prockop D. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. Stem Cells 2007;25: 2896-2902
145. Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 2000;164: 247-256
146. Weng YS, Lin HY, Hsiang YJ, Hsieh CT, Li WT. The effects of different growth factors on human bone marrow stromal cells differentiating into hepatocyte-like cells. Adv Exp Med Biol 2003;534: 119-128
147. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler P. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 2002;105: 93-98
148. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8: 315-317
149. Rojas M, Xu J, Woods CR, Mora AL, Spears W, Roman J, Brigham KL. Bone marrow-derived mesenchymal stem cells in repair of the injured lung. Am J Respir Cell Mol Biol 2005;33: 145-152
150. Ortiz LA, Gambelli F, McBride C, Gaupp D, Baddoo M, Kaminski N, Phinney DG. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci U S A 2003;100: 8407-8411
151. Spees JL, Whitney MJ, Sullivan DE, Lasky JA, Laboy M, Ylostalo J, Prockop DJ. Bone marrow progenitor cells contribute to repair and remodeling of the lung and heart in a rat model of progressive pulmonary hypertension. FASEB J 2008;22: 1226-1236
152. van Haaften T, Byrne R, Bonnet S, Rochefort GY, Akabutu J, Bouchentouf M, Rey-Parra GJ, Galipeau J, Haromy A, Eaton F, Chen M, Hashimoto K, Abley D, Korbutt G, Archer SL, Thebaud B. Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. Am J Respir Crit Care Med 2009;180: 1131-1142
153. Aslam M, Baveja R, Liang OD, Fernandez-Gonzalez A, Lee C, Mitsialis SA, Kourembanas S. Bone marrow stromal cells attenuate lung injury in a murine model of neonatal chronic lung disease. Am J Respir Crit Care Med 2009;180: 1122-1130
154. Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, Dzau VJ. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med 2003;9: 1195-1201
155. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS, Dzau VJ. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 2006;20: 661-669