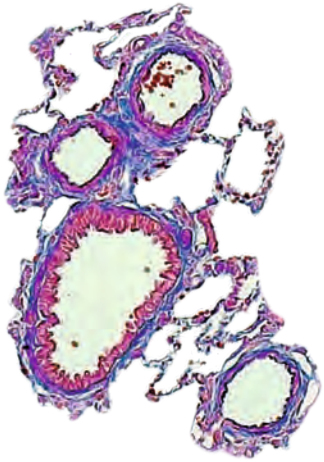
Nestin Expressing Progenitor Cells in Pulmonary Vasculature

FARHAN SABOOR



INAUGURAL DISSERTATION

submitted to the Faculty of Medicine
in partial fulfilment of the requirements for the PhD-Degree
of the Faculties of Veterinary Medicine and Medicine
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by

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Dedicated to

My Sister

List of abbreviations

5-HT	5-hydroxytryptamine
Ca	Calcium
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
eNOS	endothelial nitric oxide synthase
FGF	Fibroblast growth factor
GFP	Green fluorescence protein
GSK-3	Glycogen synthase kinase 3
HOX	Hypoxia
MCT	Monocrotaline
mRNA	Messenger ribonucleic acid
NG-2	Neural glial 2
NO	Nitric oxide
NOX	Normoxia
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PDGFR-β	Platelet derived growth factor receptor β
PGI-2	prostaglandin I-2
p-PDGFR-β	Phosphorylated platelet derived growth factor receptor β
PSA-NCAM	Polysialated nuclear cell adhesion molecule
siRNA	Small interfering ribonucleic acid
SMC	Smooth muscle cells
STRO-1	Stromal precursor antigen-1
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TRPC	transient receptor potential ion channels
Trx	Thioredoxin
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2
VSMC	Vascular smooth muscle cells
α-SMA	Alpha smooth muscle actin

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1 Introduction

1.1 Nestin discovery, classification and characteristics

Nestin is an intermediate filament protein. It was first discovered in 1985 as an antigen, identified by Rat-401 monoclonal antibody, which is transiently expressed in neuroepithelial stem cells during development of central nervous system in rats (Hockfield and Mckay, 1985). Upon differentiation, nestin is replaced by other intermediate filament proteins specific for mature cells: by glial fibrillary acidic protein (GFAP) in glial cells and by neurofilament proteins in neurons (Lendahl et al., 1990; Messam et al., 2002). The name nestin comes from the location where it was discovered: **ne**uroepithelial **st**em cell prote**in** (Lendahl et al., 1990).

After its discovery in neuroepithelial stem cells of rats, human (Dahlstrand et al., 1992) and mouse (Yang et al., 2001) nestin genes have also been characterized in detail. Intermediate filament family consists of around 60 – 70 proteins which are divided into six main types (type I – VI) on the basis of their molecular structure, nestin was classified as a type VI intermediate filament protein (Lendahl et al., 1990; Liem, 1993, Chang et al., 2004).

Nestin is a 240 kDa protein with a short N-terminus and a very long C-terminus. Unlike other intermediate filaments, nestin is unable to polymerize by itself. This is due to the short N-terminal head domain which is essential for polymerization and assembly of filament proteins (Fuchs and Weber, 1994; Hermann and Aebi, 2000). Instead of self-polymerization, nestin incorporates into the intermediate filament network by copolymerizing with various other intermediate proteins e.g. vimentin, desmin and α-internexin (Sjöberg et al., 1994; Marvin et al., 1998; Eliasson et al., 1999 and Steinert et al., 1999).

1.2 Nestin as marker of stem/progenitor cells

Nestin was initially described as marker of neural stem cells and neural progenitor cells (Cattaneo et al., 1990; Lendahl et al., 1990). In the meantime nestin was also observed in stem/progenitor cells of different tissues during embryonic development and repair or regeneration in adults (Wiese et al., 2004).

Apart from nervous system, during embryonic development, nestin expression was detected in other tissues and organs which include skeletal muscles (Sejersen and Lendahl, 1993), hepatic cells (Sun and An, 2004), testis (Fröjdman et al., 1997; Davidoff et al., 2004), kidney (Chen et al., 2006), pancreas (Aihara et al., 2004), heart (Kachinsky, 1995), teeth (About et al., 2000) and retina (Dore-Duffy et al., 2006).

In adults, nestin expressing cells are restricted to defined locations/niches where they may serve as a progenitor cell population capable of proliferation, differentiation and migration, when activated (Wiese et al., 2004). Nestin in adults is reported to be expressed in hair follicle cells (Li et al., 2003; Amoh et al., 2004), blood vessels between hair follicles (Aki et al., 2010), skeletal muscle cells (Birbrair et al., 2011), vascular smooth muscle cells and pericytes (Davidoff et al., 2004), cardiac progenitor cells (Calderone, 2011), skin (Toma et al., 2001) and retina (Mayer et al., 2003).

In recent years, nestin expression has also been found in tumors from different origins (see Krupkova et al., 2010) with suggested role in tumor angiogenesis (Shimizu et al., 2006; Teranishi et al., 2007; Gravdal et al., 2009; Chen et al., 2010; Ishiwata et al., 2011). Some studies have even described nestin as prognostic marker of progression of certain tumors and poorer survival probability for patients (Ehrmann et al., 2005; Ryuge et al., 2011).

1.3 Nestin in vascular wall

Some studies also show that nestin$^+$ cells are located in vascular walls representing the progenitor cell populations for different tissues. Different cell types in vascular wall e.g. endothelial cells, vascular smooth muscle cells and pericytes etc. were suggested to express nestin under certain conditions. In one previous study from our group, we have

already demonstrated nestin^{+} vascular smooth muscle cells and pericytes as progenitors of testosterone producing Leydig cells (Davidoff et al., 2004).

Vascular wall resident progenitor cells have been described in different cell populations of lung (Beers and Morrisey, 2011; Yeager et al., 2011) but there is virtually no data on expression and localization of nestin in the lung, during both pre- and postnatal development and repair.

1.4 Lung development

Lung consists of two highly branched, tree-like tubular systems, the airways and the vasculature which develop in a well-coordinated way starting from the primary lung bud to the generation of millions of alveoli. Both the airways and vascular systems allow lung to fulfill major function of gas exchange between air and tissue deep within an animal (Warburton, 2008; Morrisey and Hogan, 2010; Warburton et al., 2010).

1.4.1 Bronchial tree and branching system

Bronchial tree starts from trachea which bifurcates into segmental bronchi containing cartilage plates. Each bronchus further divides to form large and small sub-segmental bronchi. A small segmental bronchus is continuous with the bronchiole and the differences involve loss of cartilage plates and increase in number of elastic fibers. The terminal bronchiole give rise to further branches called respiratory bronchioles that end up into alveoli (Kierszenbaum, 2007). Branching morphogenesis of lung epithelium has been studied in different species. Morphometric analysis of mice lungs fixed at different embryonic time periods present a simple explanation of branching morphogenesis during development. The branching pattern can be divided into three geometrical modes that include domain branching, planer bifurcation and orthogonal bifurcation (Metzger et al., 2008).

1.4.2 Vascular tree and vascular system

In general the vascular tree is composed of three different types of blood vessels; arteries, capillaries and veins. The vascular system of lung consists of two circulations; bronchial circulation and pulmonary circulation. The bronchial circulation carries

systemic blood to walls of airways and large vessels and provides nutrients and oxygen while the pulmonary system is responsible for gas exchange at alveolo-capillary level. The pulmonary circulation is further classified into proximal and peripheral vasculature. Proximal vasculature consists of large, medium and small pulmonary arteries while peripheral vasculature is comprised of small non-muscular arteries and capillaries (Stenmark and Abman, 2005). Two different processes are involved in development of blood vessels; vasculogenesis and angiogenesis. Vasculogenesis is the process in which endothelial progenitor cells (angioblasts) are recruited that differentiates into mature endothelial cells that form blood vessels. On the other hand angiogenesis is defined as process where new vessels arise by sprouting from pre-existing vessels (Gao and Raj, 2010).

1.4.3 Different stages of lung development

In mammals, lung develops quite late during gestation. The earliest signs of primary lung buds appear at about 28 days in humans and at E9.5 in mouse (Morrisey and Hogan, 2010). Lung development can be divided into five stages on the basis of histological evidence;

1. Embryonic stage
2. Pseudoglandular stage
3. Canalicular stage
4. Saccular stage
5. Alveolar stage

The exact timing of different developmental stages varies in different species. The marsupials are born with the lungs in canalicular or saccular stage while, in sheep, lungs are already alveolarized at birth. In newborn humans, lungs contain only a fraction of adult alveoli and the capillary network is also immature. The rat lung is even less mature than human lung at birth (Burri, 2006).

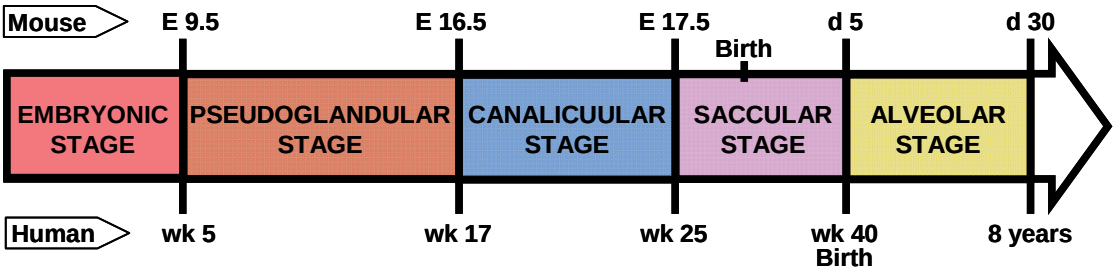


Fig. 1: Lung development**:** The developmental stages of lung are illustrated in chronological order showing comparison of time periods for each developmental stage between mouse lung and human lung (modified from Warburton et al., 2006).

The pulmonary vascular development and branching morphogenesis of airway system occur simultaneously. In embryonic stage the primary lung bud appears as ventral diverticulum of the foregut which divides to form two lung buds (Hislop, 2002). The most proximal part of pulmonary circulation is derived from the Tr. arteriosus which divides into the aorta and pulmonary trunk. At this stage, blood supply to the lung buds is through pulmonary artery coming from outflow tract of heart running alongside trachea and into the lung bud. On the ventral side of lung buds, there is a pulmonary vein that connects with prospective left atrium and between the arteries and veins; there is a plexus of capillaries around the lung buds. (Hall et al., 2000; Hall et al., 2002)

In pseudoglandular stage, the lung buds divide to form pre-acinar airways and at the same time pulmonary macrovasculature (arteries and veins) and microvasculature (capillaries) are formed independently and are connected to each other (deMello et al., 1997; deMello and Reid 2000).

During canalicular stage, further divisions of pre-acinar airways take place resulting in the formation of respiratory airways. This developmental stage is associated with marked increase of capillaries which are arranged in close apposition to the epithelium establishing the first air-blood barrier (Gao and Raj, 2010).

During saccular stage, the airways end up into clusters of thin walled air spaces called saccules. The saccules are separated from each other by primitive inter-saccular septae which contain a double layer of capillaries (Gao and Raj, 2010).

The last phase of lung development is the alveolar stage which is a post-natal event in most of the mammals. During the period of alveolarization, the inter-alveolar septae become thin, the microvasculature undergoes remodeling as the double layer capillary network regresses and transforms into a single layer adult type (Burri, 2006).

It is quite unclear when the alveolar stage ends. In classical view, the alveolar stage ends at 2 years in humans but recent studies in mice (Mund et al., 2008), rats (Schittny et al., 2008) and monkeys (Hyde et al., 2007) suggest that alveolarization does not stop until young adulthood.

1.5 Pulmonary hypertension

Pulmonary hypertension is a fatal disease characterized by pulmonary vascular remodeling resulting into progressive and sustained elevation of pulmonary arterial pressure and increased pulmonary vascular resistance eventually causing right heart failure and premature death (Humbert et al., 2004; Hoendermis, 2011).

Hemodynamically, pulmonary hypertension in man is defined as a mean pulmonary arterial pressure (PAP) greater than 25 mmHg at rest or greater than 30 mmHg with exercise by right heart catheterization (Gaine and Rubin, 1997).

Pulmonary hypertension was initially classified as primary or secondary pulmonary hypertension (Hatano and Strasser, 1975; Rubin, 1997) during first pulmonary hypertension meeting at world health organization symposium in 1973. In second world symposium on pulmonary hypertension held in Evian, France in 1998, a new classification was introduced. The Evian classification categorized pulmonary hypertension into five groups on the basis of shared clinical and pathological features as well as similar therapeutic options (Fishman, 2001).

1. Pulmonary arterial hypertension
2. Pulmonary venous hypertension
3. Pulmonary hypertension associated with disorders of the respiratory system or hypoxemia
4. Pulmonary hypertension caused by thrombotic or embolic diseases
5. Pulmonary hypertension caused by diseases affecting the pulmonary vasculature

This classification was reviewed in third world symposium on pulmonary hypertension in 2003 and modest changes were made. The current classification of pulmonary hypertension was formulated in 2008 during 4th world symposium on pulmonary hypertension held in Dana Point, California, USA. In this classification, the general philosophy and organization of Evian-Venice classifications is maintained but some modifications were made (Simonneau et al., 2009). According to Dana Point classification, the five major categories of pulmonary hypertension are;

1. Pulmonary arterial hypertension
2. Pulmonary hypertension owing to left heart disease
3. Pulmonary hypertension owing to lung diseases and/or hypoxia
4. Chronic thromboembolic pulmonary hypertension
5. Pulmonary hypertension with unclear multifactorial mechanisms

The pathogenesis of pulmonary hypertension is a very complex process. The main players in progression of pulmonary hypertension involve; pulmonary vasoconstriction, structural remodeling of pulmonary vessel wall and thrombosis (Mandegar et al., 2004). In recent years, the fact has been established that vascular remodeling is the hallmark of pulmonary hypertension pathogenesis (Yeager et al., 2011). Under physiological conditions, the thickness of vessel wall is maintained at an optimal level by establishing a balance between proliferation and apoptosis of resident cell types. When this balance is disturbed in favor of proliferation, the vascular wall thickens narrowing the vessel lumen and resulting in increased resistance. This structural change of vessels is referred to as vascular remodeling (Kato and Staub, 1966). Pulmonary vascular wall consists of three layers; intima containing endothelial cells, media harboring smooth muscle cells and the third is adventitia whose cellular components are fibroblasts. All the three cellular layers are involved in the complicated process of vascular remodeling (Mandegar et al., 2004; McGrath et al., 2005; Chan and Loscalzo, 2008; Yeager et al., 2011) by means of hypertrophy (cell growth) and/or hyperplasia (proliferation) of smooth muscle cells, signal transduction from neighboring fibroblasts and endothelial cells, as well as increased deposition of extracellular matrix components including collagen, elastin, and fibronectin (Jeffery and Wanstall, 2001).

1.6 Models to study pulmonary hypertension

Huge amount of research is going on in the field of pulmonary hypertension. There are a number of animal models used to study pulmonary hypertension. These animal models have provided, and will continue to provide, valuable insight into the numerous mechanisms and pathways that contribute to the pathogenesis and maintenance of pulmonary hypertension allowing us to develop novel therapeutic strategies. The best described and most frequently used models include hypoxia induced pulmonary hypertension in rodents (rat and mouse) and monocrotaline (MCT) induced pulmonary hypertension in rats (Pak et al., 2007; Stenmark et al., 2009).

1.6.1 Hypoxia induced pulmonary hypertension

Hypoxia is the most commonly used model to study vascular remodeling because it is very much predictable and reproducible within a specific species, however, response to hypoxia varies in different species. The effect of hypoxia has been studied in many animals but the most commonly used hypoxic animal systems are those of rat and mouse (Stenmark et al., 2009). Hypoxia is a more physiological model as compared to any other model as it occurs in nature as a pathological stimulus leading to development of pulmonary hypertension at high altitude or as consequence of hypoxic lung disease at sea level (Pak et al., 2007).

In this model, animals are exposed to hypoxic conditions ($\leq$ 10% O_2) for 3 weeks. During this time period they develop pulmonary hypertension which can be confirmed by hemodynamic measurements as well as histological observations. Hypoxia-induced pulmonary hypertension is associated with very similar structural changes in almost all mammals investigated so far; however, the magnitude of these changes may differ in different animals.

Thickening of media occurs consistently in the arteries at all levels of the pulmonary tree. There is extension of smooth muscle cells in small, normally nonmuscular arterioles that is obvious by increase in the appearance of cells expressing α-smooth muscle actin in the walls of previously nonmuscular arterioles. This process is called muscularization (Sakao et al., 2009; Stenmark et al., 2009). These changes were suggested to be due to differentiation of precursor pericytes and intermediate cells into

smooth muscle cells (Meyrick and Ried, 1980). Intermediate cells were defined as intermediates between pericytes and smooth muscle cells. Other suggestions include recruitment and differentiation of fibroblasts and circulating mononuclear progenitor cells into smooth muscle cells (Jones et al., 1999) and transdifferentiation of endothelial cells into mesenchymal cells (Sakao et al., 2009). Simultaneously, there is also increase in thickness of previously muscularized arteries which is the result of both smooth muscle cell proliferation and hypertrophy (Meyrick and Perkett, 1989; Stenmark et al., 2009).

After exposure to hypoxia, mice also show increase in pulmonary arterial pressure but pulmonary hypertension in mice is associated with less degree of vascular remodeling as compared to other animals e.g. rats. In mice, major changes include muscularization of previously non-muscularized arterioles and minimal medial thickening in muscular arteries where increased proliferation of smooth muscle cells is observed (Stenmark et al., 2009). The exact mechanisms and cell types involved in hypoxia induced vascular changes are still unclear.

On returning to normoxic atmosphere the experimental hypoxia induced pulmonary hypertension is reversed and after certain recovery periods, pulmonary arterial pressure comes to normal. In addition vessel walls regain their original thickness, whereas, the amount of collagen fibers increases (Meyrick and Ried, 1980; Marsboom & Janssens, 2004).

1.6.2 Monocrotaline model of pulmonary hypertension

Monocrotaline (MCT) is a plant derived toxin extracted from seeds of *Crotalaria spectabilis*. A single subcutaneous or intraperitonial injection of monocrotaline results in progressive pulmonary hypertension in different species. The preferred species to study monocrotaline induced pulmonary hypertension is rat, while mice show variable results after monocrotaline administration (Stenmark et al., 2009). In vivo, monocrotaline is activated by mixed function oxidases in liver to form bifunctional cross-linking compound MCT pyrrole which is transported to lungs where it causes vascular injury resulting into pulmonary hypertension (Marsboom & Janssens, 2004; Stenmark et al., 2009; Gomez-Arroyo et al., 2011).

Like hypoxia, monocrotaline induced pulmonary hypertension is also characterized by vascular remodeling. The exact mechanism by which monocrotaline causes vascular remodeling is not known. It is assumed by many investigators that monocrotaline causes endothelial damage which triggers smooth muscle cell proliferation and/or endothelial cell mesenchymal transition. Other investigators suggest that vascular remodeling and increase in pulmonary artery pressure is caused by early and dramatic accumulation of mononuclear inflammatory cells in adventitia of small intra-acinar vessels (Stenmark et al., 2009). The mechanism explaining the processes between early endothelial injury and medial hypertrophy is still missing. The monocrotaline rat model continues to be a frequently investigated model of pulmonary hypertension as it offers technical simplicity, reproducibility and low cost compared to other models of pulmonary hypertension (Gomez-Arroyo et al., 2011).

1.6.3 Su5416/hypoxia model for pulmonary hypertension

Hypoxia in combination with VEGF receptor-2 (VEGFR-2) inhibition by VEGFR-2 antagonist Sugen 5416 results in severe pulmonary hypertension (Taraseviciene-Stewart et al., 2001; Sakao et al., 2005) and is used as model to study experimental pulmonary hypertension (see also 1.7.2). In general, this animal model quite resembles the traditional monocrotaline model of pulmonary hypertension with prominent perivascular inflammatory infiltrates (Stenmark et al., 2009). The salient feature of this animal model is that animal treated with Su5416 and hypoxia develops certain characteristic vascular lesions found in human pulmonary hypertension (Abe et al., 2010). VEGF is an important survival factor and endothelial cells undergo apoptosis when deprived of VEGF (Gerber et al., 1998) or are exposed to VEGF-2 inhibitor (Kasahara et al., 2000; Taraseviciene-Stewart et al., 2001). In the Su5416/hypoxia model VEGFR-2 blockade is assumed to result in endothelial cell apoptosis which is followed by hypoxia induced proliferation of vascular smooth muscle cells and apoptosis-resistant endothelial cells (Taraseviciene-Stewart et al., 2001). It is still unclear whether proliferating endothelial cells contribute to formation of neointima or these are the vascular smooth muscle cells that proliferate and migrate into intima.

1.7 Molecular mediators of vascular remodeling in pulmonary hypertension

1.7.1 Vasoactive mediators

Pulmonary hypertension is characterized by imbalance in vasoactive mediators. In patients with pulmonary hypertension the production of vasodilators like nitric oxide (NO) and prostacyclin/prostaglandin I-2 (PGI2) is reduced (see Schermuly et al., 2011).

Nitric oxide is a potent vasodilator. It is synthesized by endothelial cells of the vessel wall, by endothelial NO synthase (eNOS). NO exerts its vasodilatory effect on smooth muscle cells with the help of guanylate cyclase/cyclic guanosine monophosphate (cGMP) pathway.

Prostacyclin is also a strong vasodilator produced by endothelial cells. Prostacyclin acts on adenylate cyclase that induces the synthesis of cyclic adenosine monophosphate (cAMP) and finally smooth muscle cell relaxation. PGI2 receptor knockout mice develop severe hypoxia induced pulmonary hypertension (Hoshikawa et al., 2001) while PGI2 over expressing mice do not develop pulmonary hypertension (Geraci et al., 1999) suggesting some significant role of prostacyclin in pathogenesis of pulmonary hypertension.

Both NO and prostacyclin possess anti-proliferative properties (Jeffery & Wanstall, 2001) and reduction in their generation may contribute to increased proliferation of smooth muscle cells or fibroblasts during pulmonary hypertension.

In contrast to vasodilators, the generation of vasoconstrictors like endothelin-1, thromboxane and serotonin (5-hydroxytryptamine 5-HT) is increased in patients with pulmonary hypertension (see Schermuly et al., 2011).

Endothelin-1 is a potent vasoconstrictor produced by vascular endothelial cells. Two different Endothelin-1 receptors, ETA and ETB, are present on smooth muscle cells which mediate vasoconstriction (Rabinovitch, 2008). Endothelin also stimulates proliferation of smooth muscle cells through downstream signaling pathways of ETA and ETB receptors (Davie et al., 2002).

Thromboxane is another vasoconstrictor produced by pulmonary endothelial cells and platelets. Along with vasoconstriction, it induces aggregation of platelets as well as proliferation of pulmonary artery smooth muscle cells (Fischer et al., 2000).

Serotonin, like ET-1 and thromboxane, also shows both vasoconstrictive and proliferative effects on smooth muscle cells. The vasoconstriction is mediated by 5-HT receptors 1B, 2A and 2B (MacLean et al., 2000) while serotonin stimulates smooth muscle cell proliferation via 5-HT transporter mediated uptake of serotonin (Eddahibi et al., 2006). The inhibition of 5-HT transporter inhibition reversed monocrotaline-induced PH in rats (Guignabert et al., 2005) suggesting the role of serotonin in proliferation of smooth muscle cells.

Variations in both K^+ and Ca^{2+} **ion channels** are associated with pathological vascular tone, disturbance of cellular homeostasis and induction of fibroproliferative action especially in smooth muscle cells.

The classic function of K^+ channels is regulation of vascular tone and membrane potential but in recent years, K^+ channels expressed by smooth muscle cells were suggested to take part in vascular remodeling by regulating proliferation and apoptosis (Moudgil et al., 2006; Burg et al., 2008). Sustained Ca^{2+} flux into the cells also exerts mitogenic effects on smooth muscle cells. This effect is produced via Ca^{2+}-calmodulin dependent stimulation of cell cycle and activation of mitogen induced protein kinase pathways upstream of several growth factors (Burg et al., 2008).

Ca^{2+} signaling via transient receptor potential ion channels (TRPC) has significant role in pulmonary hypertension. TRPC3 and TRPC6 were found to be up-regulated in patients of pulmonary hypertension while the inhibition of TRPC6 expression significantly reduced smooth muscle cell proliferation (Yu et al., 2004; Weissmann et al., 2006).

1.7.2 Pro-proliferative mediators

Apart from proliferative actions of certain vasoconstrictors, a number of pro-proliferative signaling pathways have been identified in the pathophysiology of pulmonary hypertension (see Schermuly et al., 2011).

Several growth factors are implicated in pulmonary hypertension and vascular remodeling. Growth factors act as potent mitogens and chemoattractants for vascular cells such as smooth muscle cells, fibroblasts, and endothelial cells by binding and activating cell surface tyrosine kinase receptors. Activation of the tyrosine kinase receptor initiates major intracellular signaling cascades, resulting in cellular proliferation, migration, and resistance to apoptosis (see Schermuly et al., 2011).

Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR-2) are expressed in the plexiform lesions of patients suffering from pulmonary hypertension suggesting a complex role for VEGF in the pathogenesis of pulmonary hypertension (Tuder & Yun, 2008). VEGF is up-regulated in rats with hypoxic treatment but down-regulated in MCT treated rats (Partovian et al., 1998a; Partovian et al., 1998b). Hypoxia, in combination with inhibition of VEGF receptor inhibition using VEGF receptor antagonist Su5416, results in severe pulmonary hypertension (Taraseviciene-Stewart et al., 2001).

Platelet derived growth factor (PDGF) signaling is implicated in many proliferative diseases. Many investigations suggest that platelet derived growth factor B promotes proliferation and migration of vascular smooth muscle cells and may play a role in the progression of pulmonary hypertension. The upregulated expressions of PDGF-BB homodimer and its receptor PDGF-Rβ is correlated with pulmonary hypertension in various animal models (Schermuly et al., 2005; Andrae et al., 2008; Li et al., 2010; Dahal et al., 2011) and in humans (Schermuly et al., 2005; Perros et al., 2008). Hypoxia is reported to regulate arterial smooth muscle cell proliferation in a paracrine manner via PDGFR-β as under hypoxic conditions, arterial endothelial cells are shown to secrete PDGF-BB that stimulates smooth muscle cell proliferation via PDGFR-β expression in vivo (Chanakira et al., 2012). PDGF receptors belong to the family of transmembrane receptor kinases. Treatment with the PDGF receptor antagonist Imatinib

which is an inhibitor of receptor tyrosine kinases (Hossein et al., 2006) resulted in reverse remodeling of vasculature in two animal models of pulmonary hypertension that include monocrotaline treated rat model and hypoxia treated mouse model (Schermuly et al., 2005). However, the activation of PDGF and its receptors during the course of pulmonary hypertension in association with nestin expression and vascular smooth muscle cell proliferation still needs to be investigated.

Fibroblast growth factor (FGF) is also up-regulated during vascular remodeling. Up-regulation of basic fibroblast growth factor (bFGF) occurs in vascular smooth muscle cells of rats exposed to hypoxia while FGF-2 is up-regulated in lambs with pulmonary hypertension (Li et al., 2003; Wedgwood et al., 2007; Schermuly et al., 2011). Inhibition of FGF-1 receptor causes reversal of established pulmonary hypertension (Izikki et al., 2009).

Transforming growth factor alpha (TGF-α) is a member of the epidermal growth factor (EGF) family. Over expression of TGF-α results in development of pulmonary hypertension and might be correlated with EGF-induced proliferation of smooth muscle cells in pulmonary vasculature (Le Cras et al., 2003).

TGF-β superfamily regulates many cellular functions including proliferation, migration, differentiation and secretion and deposition of extracellular matrix. TGF-β signaling is also implicated in the progression of pulmonary hypertension. Alterations in TGF-β signaling pathways, via bone morphogenic protein receptor 2 (BMPR-2) and TGF-β receptor 1, are associated with pathogenesis of pulmonary hypertension. In majority of patients suffering from heritable pulmonary hypertension, mutations have been found in BMPR2 gene suggesting a strong role of BMPR2/TGF-β signaling in pathogenesis of pulmonary hypertension (Machado et al., 2009).

Notch signaling is involved in vasculogenesis, angiogenesis and differentiation of vascular smooth muscle cells (Alva et al., 2004). Notch3 is an important mediator supposed to be involved in pulmonary hypertension by promoting proliferation of vascular smooth muscle cells (Campos et al., 2002). Notch 3 is also known to regulate the expression of PDGF receptor β in vascular smooth muscle cells (Jin et al., 2008).

1.8 Progenitor cells in pulmonary vasculature

Repair, remodeling and regeneration of the respiratory system for maintenance of homeostasis of lung after injury have been one of the main focuses of pulmonary biologists in recent years. Like other externally exposed organs e.g. skin, lung is also confronted by various insults and injuries and the occurrence of a complex spatial organization and variety of cell types that form the mature organ, suggest that multiple pools of multipotent stem/progenitor cells capable of self-renewal are required for repair, remodeling or regeneration after injury (Beers and Morrisey, 2011).

Traditionally, the cell components of the vessel walls are thought to be mature and terminally differentiated (Psaltis et al., 2011). Until the last decade, angiogenesis, formation of new vessels from pre-existing vessels, was considered to be the only process for the formation of new blood vessels (Carmeliet, 2003).

In recent years, the traditional view has been revised due to discoveries of diverse stem/progenitor cells relevant to cell types in the vessel wall which include endothelial cells, vascular smooth muscle cells, pericytes and fibroblasts (Stenmark et al., 2006; Pearson, 2010; Crisan et al., 2011; Majesky et al., 2011). Significant amount of evidence indicate that these different stem/progenitor cells exist within the vessel wall, where they may reside permanently, or appear as the result of migration (Psaltis et al., 2011).

1.8.1 Circulating progenitor cells

Endothelial progenitor cells are the most studied adult progenitor cells in recent years. Endothelial progenitor cells were first discovered by Asahara et al. in 1997 where they were shown to be recruited to the sites of active angiogenesis in adults (Asahara et al., 1997). Endothelial progenitor cells are thought to derive from bone marrow and circulate in the peripheral blood. Most frequently used marker proteins to identify endothelial progenitor cells include vascular endothelial growth factor receptor-2 (VEGFR-2), mucosialin (CD34), prominin-1 (CD133) and c-kit (CD117; the receptor for stem cell factor) (Timmermans et al., 2009).

Circulating smooth muscle progenitor cells were also reported to contribute to vascular remodeling (Daniel and Sedding, 2011) but this progenitor cell population is not so intensively studied as endothelial progenitor cells. Circulating smooth muscle progenitor cells are also reported to originate from bone marrow and can be found in circulating blood or peripheral tissues. They can be identified by using certain marker proteins which include endoglin (CD105), α-smooth muscle actin (α-SMA), calponin, smooth muscle myosin heavy chain and platelet derived growth factor receptor-β (Simper et al., 2002; Sugiyama et al., 2006).

Another potential vascular progenitor cell type that has got attention in recent years is the fibrocyte. "Fibrocytes" are bone marrow derived mesenchymal progenitor cells which migrate to the region of injured tissue. These cells can be distinguished by the expression of hematopoietic and monocyte lineage markers and extracellular matrix proteins (Grieb et al., 2011). Fibrocytes are also implicated in pulmonary hypertension (Gambaryan et al., 2012).

1.8.2 Vessel wall resident progenitor cells

Along with the circulating progenitor cells, recent findings from studies on human tissues and in vivo studies in animal models confirm the presence of progenitor and stem cells in vessel wall. It is not investigated whether a putative stem/progenitor cell population exists in vessel wall which is capable of differentiating to all types of cells but there is increasing evidence of different types of stem/progenitors cells within the vessel wall capable of differentiating into mature vascular wall cells (Klein et al., 2010; Psaltis et al., 2011; Torsney and Xu, 2011; Yeager et al., 2011).

Although typically thought to originate from bone marrow, it is now reported that endothelial progenitor cells can also arise locally within various tissues, including the vascular wall (Tilki et al., 2009). The localization of endothelial progenitor cells in vessel wall was identified in sub-endothelial space and in the so-called vasculogenic zone in the tunica adventitia (Zengin et al., 2006).

Similarly, smooth muscle progenitor cells can also reside within the vessel wall. One possible niche is tunica intima, where certain mature endothelial cells can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation (Frid et al.,

2002). Another cell population capable of differentiating into smooth muscle cells identified by stem cell antigen-1 (Sca-1) was found to reside in the vasculogenic zone at the border of media and adventitia (Hu et al., 2004; Passman et al., 2008).

Mesenchymal stem cells are present in almost all organs and recent data suggest that mesenchymal stem cells also reside in certain niches within the vessel wall e.g. sub-endothelial space and adventitia (da Silva et al., 2006; Zengin et al., 2006). Mesenchymal stem cells are thought to be closely related with pericytes (Caplan, 2008; Kovacic et al., 2009). Both pericytes and mesenchymal stem cells have been assigned similar niches, predominantly perivascular region, in various tissues and both cell types also express common cell markers like CD44, CD90, CD105, stromal precursor antigen-1 (STRO-1), neural glial antigen-2 (NG2), alkaline phospatase, α-smooth muscle actin (α-SMA) and platelet derived growth factor receptor-β (PDGFR-β) (Psaltis et al., 2011). The presence of pericytes and/or mesenchymal stem cells throughout the vasculature suggests these cells as progenitors of a variety of cell types (Kovacic et al., 2009).

1.9 Nestin^{+} resident progenitor cells in the vessel wall

In a previous study from our group we found that nestin expressing vascular smooth muscle cells and pericytes in testicular blood vessels are the progenitors of testosterone producing Leydig cells (Davidoff et al., 2004). Here we demonstrated that vascular smooth muscle cells and pericytes in peri-tubular blood vessels express nestin. These nestin expressing cells proliferate and protrude out of blood vessel and transdifferentiate into Leydig cells.

The question was that whether these nestin expressing progenitor cells also occur in other organs? To answer this, our colleague Claudia Berndt investigated nestin expression in lungs using nestin-GFP transgenic mice and found nestin expression in pulmonary vessels. Nestin was predominantly found in arteries while veins and capillaries also showed nestin expression. Using cell specific markers (α-SMA for smooth muscle cells and CD31 for endothelial cells), she was able to characterize nestin expressing cells as vascular smooth muscle cells (Fig. 2). Nestin was only found in vascular smooth muscle cells while smooth muscle cells around the airways were negative for nestin which is clearly visible in staining of nestin-GFP sections with smooth muscle actin (Fig. 2A) where we found co-localization of nestin and SMA in vessels (Fig 2A-C) while only SMA staining was observed in smooth muscle layer around the airways (Fig 2A).

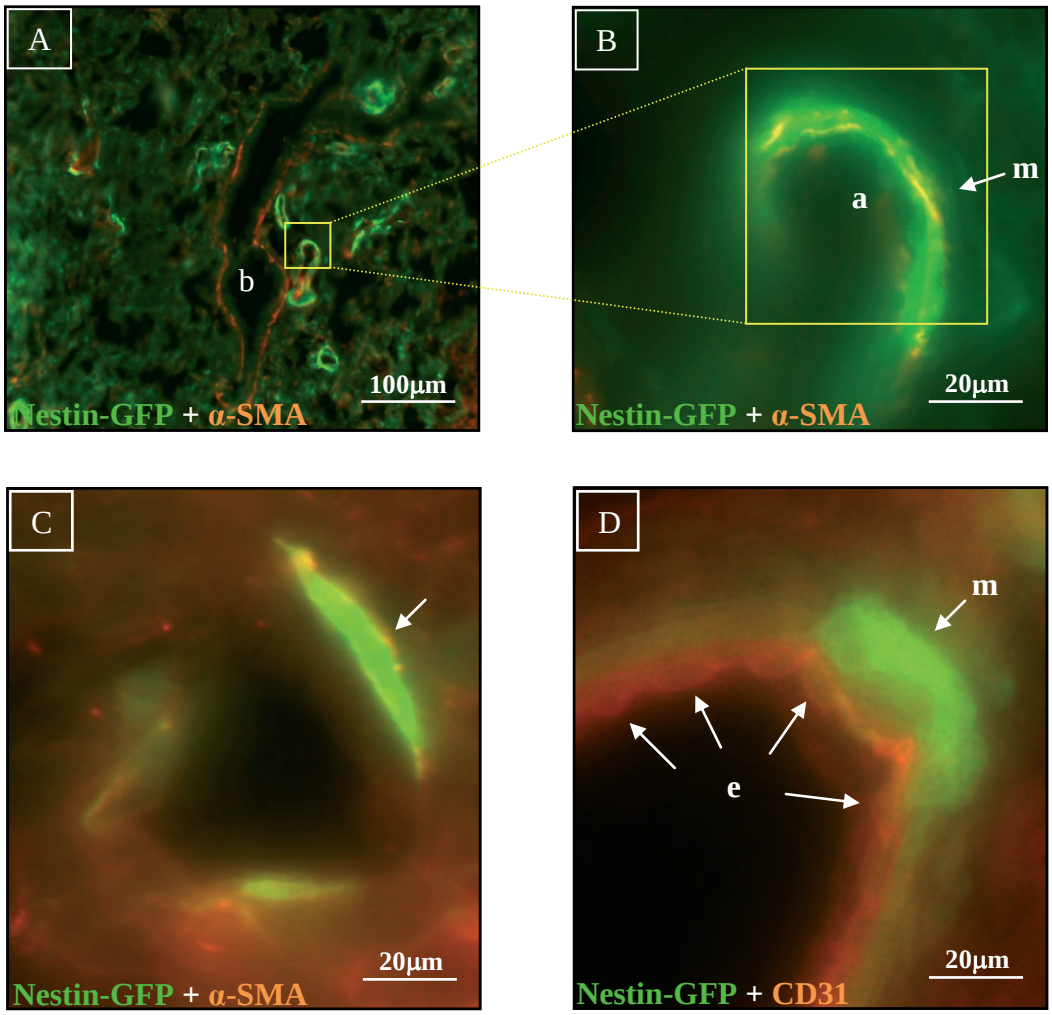


Fig. 2: Characterization of nestin expressing cells in pulmonary vasculature (From Claudia Berndt, dissertation). **A-C:** Immunofluorescence staining of lung sections from nestin-GFP transgenic mice with smooth muscle cells' marker α-SMA. Nestin^{+} smooth muscle cells are visible in vessels (yellow-green) and nestin- negative smooth muscle cells are present around a bronchiole (b, red) (A). Higher magnification of colocalization of nestin-GFP and α-SMA in the wall of an artery (a) confirming nestin in vascular smooth muscle cells (B-C). **D:** Immunofluorescence staining of lung sections from nestin-GFP transgenic mice with endothelial cells' marker CD31 showing nestin-GFP signal is absent in endothelial cells (e, endothelial cells; m, smooth muscle cell).

1.10 Aim of the study

The present study was performed to investigate the role of nestin expressing vascular smooth muscle cells as stem cell like progenitor cells in lungs. Since nestin expressing vascular progenitor cells are identified in various organs including our study revealing progenitors of Leydig cells (Davidoff et al., 2004) and knowing that nestin expressing cells in lungs are vascular smooth muscle cells, we aimed;

- To investigate a potential role of nestin expressing vascular smooth muscle cells in postnatal development of lung

- To investigate a potential role of nestin expressing vascular smooth muscle cells in adult lungs under pathological conditions.

In order to achieve the aims, lung samples from newborn mice and three different models of pulmonary hypertension (hypoxic mouse model, MCT and Su5416/hypoxia rat model) as well as human samples from patients of pulmonary hypertension and pulmonary fibrosis were used and a series of experiments were performed which are as follows;

- Evaluation of nestin expression in correlation to vascular smooth muscle cell proliferation in postnatal development

- Analysis of nestin expression in correlation to vascular smooth muscle cell proliferation in hypoxic mouse model of pulmonary hypertension

- Analysis of nestin expression in monocrotaline and Su5416/hypoxia treated rat lung samples

- Analysis of nestin expression in human lung samples from patients of pulmonary hypertension and pulmonary fibrosis

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Agarose gel electrophoresis apparatus	PeqLab, Erlangen, Germany
Cryostat, CM1900	Leica, Wetzlar, Germany
Fluorescence microscope, Axioskop 2 plus	Zeiss, Munich, Germany
Gel documentation system	Phase, Lübeck, Germany
High temperature Incubator	Heraeus, Hanau, Germany
iCycler IQ™ Realtime PCR detection system	Bio-Rad, Munich, Germany
MasterCycler Gradient	Eppendorf, Hamburg, Germany
Microscope Axiostar plus	Zeiss, Munich, Germany
Microtome, RM2255	Leica, Wetzlar, Germany
PALM Laser Microdissection System	Zeiss, Munich, Germany
Polyacrylamide gel electrophoresis apparatus (SE600)	Hoefer, San Fransisco, USA
Power supply	Hoefer, San Fransisco, USA
Safety Cabinet	Heraeus, Hanau, Germany
Safety Cabinet	Köttermann, Uetze, Germany
Elisa Reader	Dynatech, Denkendorf, Germany
Spectrophotometer, Biophotometer	Eppendorf, Hamburg, Germany
Ultra centrifuge (Sorvall ultra pro 80)	Sorvall, Hertfordshire, UK

2.1.2 Kits

Amersham™ ECL WB detection system	GE healthcare, Freiburg, Germany
Bio-Rad protein assay	Bio-Rad, Munich, Germany
Envision G|2 double staining kit	DAKO, Hamburg, Germany
GeneAmp® RNA PCR kit	Applied Biosystems, Darmstadt, Germany
Horseradish peroxidase labeled polymer	DAKO, Hamburg, Germany
iScript™ cDNA synthesis kit	Bio-Rad, Munich, Germany

Platinum® SYBR® green qPCR SuperMix-UGD	Invitrogen, Karlsruhe, Germany
RNeasy Micro kit	Qiagen, Hilden, Germany
RNeasy Mini kit	Qiagen, Hilden, Germany

2.1.3 Consumables

Eppendorf tubes (1.5 ml/2 ml)	Eppendorf, Hamburg, Germany
Filter tips	Eppendorf, Hamburg, Germany
PCR tubes (0.2 ml)	Applied Biosystems Darmstadt, Germany
Pipettors 10, 20, 100, 200, 1000 µl	Eppendorf, Hamburg, Germany
Serological pipette: 5, 10, 25, 50 ml	BD Falcon, Heidelberg, Germany
X-ray films	Fuji, Tokyo, Japan

2.1.4 Reagents

1-chloro-2,2,2-trifluoroethly difluoromethyl ether (Isoflurane)	Abbott, Wiesbaden, Germany
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, Steinheim, Germany
Acetic Acid	Merck, Darmstadt, Germany
Acetone	Roth, Karlsruhe, Germany
Acrylamide solution, Rotiphorese Gel 30	Roth, Karlsruhe, Germany
Agarose	PeqLab, Erlangen, Germany
Amersham™ Hybond™- nitrocellulose membranes	GE healthcare, Freiburg, Germany
Ammonium persulfate (APS)	Roth, Karlsruhe, Germany
AmpliTaq DNA polymerase	Applied Biosystems, Darmstadt, Germany
Aniline blue	Merck, Darmstadt, Germany
Azocarmine	Chroma, Stutgart, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Steinheim, Germany
Bromophenol blue	Sigma-Aldrich, Steinheim, Germany
Calcium chloride (CaCl2•2H20)	Merck, Darmstadt, Germany
3,3'-Diaminobenzidine (DAB)	Merck, Darmstadt, Germany
Di-Sodium hydrogen phosphate	Roth, Karlsruhe, Germany

Dithiothreitol (DTT)	Invitrogen, Karlsruhe, Germany
DNA low range marker (100 bp)	Fermentas, Maryland, USA
DNase-1	Applied Biosystems, Darmstadt, Germany
Eosin	Sigma-Aldrich, Steinheim, Germany
Ethanol	Riedel de haen, Seelze, Germany
Ethidium bromide solution	Roth, Karlsruhe, Germany
Ethylendinitrilo-N, N, N´, N´, -tetra-acetic acid disodium salt (EDTA)	Roth, Karlsruhe, Germany
Glucose oxidase	Sigma-Aldrich, Steinheim, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycine	Roth, Karlsruhe, Germany
Hepes	Sigma-Aldrich, Steinheim, Germany
Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Hydrogen peroxide (H2O2)	Roth, Karlsruhe, Germany
Isopropanol	Sigma-Aldrich, Steinheim, Germany
Magnesium chloride (MgCl2•6H2O)	Merck, Darmstadt, Germany
Mayer's Hemalum solution	Merck, Darmstadt, Germany
Methanol	Fluka, Buchs, Switzerland
Milk powder	Roth, Karlsruhe, Germany
Mul-V RT	Applied Biosystems, Darmstadt, Germany
TEMED	AppliChem, Darmstadt, Germany
Nickle sulfate	Merck, Darmstadt, Germany
Normal goat serum	Sigma-Aldrich, Steinheim, Germany
Orange G	Sigma-Aldrich, Steinheim, Germany
Paraformaldehyde	Roth, Karlsruhe, Germany
Phosphotungstic acid	Riedel de haen, Seelze, Germany
Picric acid	Fluka, Buchs, Switzerland
Ponceau S solution	Sigma-Aldrich, Steinheim, Germany
Potassium chloride (KCl)	Merck, Darmstadt, Germany
Random hexamers	Boehringer, Mannheim, Germany
SigmaMarker™ (Molecular weight marker)	Sigma-Aldrich, Steinheim, Germany
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany
Sodium dodecyl sulphate (SDS)	Roth, Karlsruhe, Germany

Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
Sodium azide (NaN3)	Sigma-Aldrich, Steinheim, Germany
Thimerosal	Sigma-Aldrich, Steinheim, Germany
Tissue Tek O.C.T compound	Sakura, Zoeterwoude, Netherlands
Tris	Roth, Karlsruhe, Germany
Tri-Sodium citrate dihydrate	Merck, Darmstadt, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
Tween 20	Sigma-Aldrich, Steinheim, Germany
Western blocking reagent	Roche, Mannheim, Germany
Xylene	Roth, Karlsruhe, Germany
β-mercaptoethanol	Sigma-Aldrich, Steinheim, Germany

2.1.5 Primary antibodies

Anti-nestin	Mouse monoclonal	Chemicon, Schwalbach, Germany
Anti-nestin	Mouse monoclonal	BD Transduction,Heidelberg, Germany
Anti-nestin	Mouse monoclonal	Santa Cruz, Germany
Anti-PDGFR-β	Rabbit polyclonal	Upstate, Virginia, USA
Anti-p-PDGFR- β	Rabbit polyclonal	Santa Cruz, Heidelberg, Germany
Anti-Calponin-1	Rabbit monoclonal	Epitomics, Hamburg, Germany
Anti-α-SMA	Mouse monoclonal	Serotec, Oxford, UK
Anti-PCNA	Mouse monoclonal	Chemicon, Schwalbach, Germany
Anti-Ki67	Rabbit polyclonal	Novocartra, Newcastle, UK
Anti-vinculin	Mouse monoclonal	Sigma-Aldrich, Steinheim, Germany
Anti-β-Actin	Mouse monoclonal	Sigma-Aldrich, Steinheim, Germany

2.1.6 Secondary antibodies

Goat-anti-mouse IgG	Pierce, Bonn, Germany
Goat-anti-rabbit IgG	Pierce, Bonn, Germany
Cy3-goat-anti-mouse IgG	Jackson immunoresearch, Hamburg, Germany
Cy3-goat-anti-rabbit IgG	Jackson immunoresearch, Hamburg, Germany
Alexa-goat-anti-mouse IgG	Roche, Mannheim, Germany
Alexa-goat-anti-rabbit IgG	Roche, Mannheim, Germany

2.2 Animals and tissues

C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). The animals had access to food and water ad libitum. The experiments were performed according to the guidelines for the use of laboratory animals using study protocols approved from local authorities (Federal Authorities for Animal Research of the Regierungspräsidium Giessen, Hessen, Germany).

2.2.1 Postnatal development

For postnatal studies, female pregnant C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). After the mice gave birth to pups, the newborns were sacrificed at different time points after birth and tissues were frozen in liquid nitrogen or fixed with Bouin's fixative and 4% PFA respectively for further analysis.

2.2.2 Hypoxic mouse model

For hypoxic experiments, male C57BL/6 mice (8 weeks) were purchased from Charles River Laboratories (Sulzfeld, Germany). Hypoxic pulmonary vascular remodeling was induced by exposure of mice to chronic hypoxia in ventilated chambers as described before (Weissmann et al., 2005). Animals were kept under hypoxic conditions (10% inspired O_2 fraction) in a normobaric chamber for different time periods starting from 1 day to 3 weeks. Constant level of hypoxia was maintained with the help of an autoregulatory control unit (model 4010, O_2 controller, Labotec, Göttingen, Germany) supplying either nitrogen or oxygen to the chambers. Excessive humidity in the system was prevented by condensation in a cooling system. CO_2 from the chambers was continuously removed by using soda lime. Hypoxic chambers were opened once a day for cleaning as well as to supply food and water. The chamber temperature was maintained at 22 - 24°C. Control animals were placed in similar condition in a normoxic chamber with normal oxygen environment (21% inspired O_2 fraction) (Weissmann al., 2005). Animals were sacrificed after normoxic/hypoxic treatment and tissues were frozen in liquid nitrogen or fixed with Bouin's fixative and 4% PFA respectively for further analysis.

2.2.3 MCT rat samples

Rat lungs from monocrotaline (MCT) treated animals (3 weeks, 4 weeks and 5 weeks after MCT) as well as control samples were obtained from the laboratory of Prof. Norbert Weissmann (Department of Internal Medicine, Medical Clinic II, Giessen, Germany).

2.2.4 Su5416/hypoxia treated rat samples

Rat lungs injected with Sugen 5416 (VEGF-2 receptor antagonist) followed by hypoxic treatment for 5 weeks and control samples were obtained from the laboratory of Prof. Ralph Schermuly (Department of Internal Medicine, Medical Clinic II, Giessen, Germany).

2.2.5 Human lung samples

Human samples from patients suffering from pulmonary hypertension, pulmonary fibrosis and from donor lungs as control were obtained from the laboratory of Prof. Ralph Schermuly (Department of Internal Medicine, Medical Clinic II, Giessen, Germany).

2.3 Methods

2.3.1 Western Blot

2.3.1.1 Protein isolation

Western blot analysis was performed as described before (Müller et al., 2004). In detail, frozen lung samples were crushed with the help of a hammer and then ground to powder in liquid nitrogen with the help of a mortar and pestle. Powdered tissue was suspended in ice cold homogenizing buffer (10ml/g of tissue) and homogenized in Potter-Elvehjem homogenizer. The homogenate was centrifuged at 3000 x g for 8 minutes at 4°C. The pellet was discarded while the supernatant contained the total isolated proteins.

To separate cytosolic and membrane associated proteins, total proteins were further centrifuged at 100,000 x g for 30 minutes at 4°C. The supernatant that contained

cytosolic proteins was picked up by pipetting while the pellet containing membrane associated proteins was resuspended in 50mM Tris buffer (pH 7.5).

Homogenization buffer components	**Final concentration**
Tris (pH 7.5)	50 mM
EDTA	1 mM
Dithiothreitol (DTT)	1 mM
Phenylmethanesulfonyl fluoride (PMSF)	0.1 mM

2.3.1.2 Estimation of protein concentration

The quantity of protein was measured by Bio-Rad Protein Assay (Bio-Rad, Munich, Germany) which is based on the method of Bradford. The method involves incorporation of Coomassie® Brilliant Blue G-250 dye to proteins. The absorbance maximum of acidic solution of the dye shifts from 465 nm to 595 nm when it binds to the protein. Protein concentration was detected at 595 nm by microplate reader. Series of bovine serum albumin (BSA) (Sigma, fraction V) was used as standard solution in range of 3 – 18 µg/µl to measure the exact concentration of proteins in the sample.

2.3.1.3 SDS polyacrylamide gel electrophoresis

Protein samples of same concentration were mixed with 3x SDS gel loading buffer at a ratio of 2:1 (v/v) and denatured at 100°C for 2 minutes. Protein samples (60 µg for nestin in postnatal samples, 120 µg for nestin in hypoxic samples, 40 µg for PDGFR-β and p-PDGFR-β) or protein molecular weight marker (Sigma, USA) were loaded in the wells of 7% gel. The gel was run in SE 600 vertical electrophoresis system (Hoefer Scientific Instruments, MA, USA) at 13W for one hour until the samples crossed the stacking gel and then at 17W for about 3 hours for separation.

3X gel loading buffer components	**Final concentration**
Tris	375 mM
Dithiothreitol (DTT)	200 mM
Sodium dodecyl sulfate (SDS)	15% (w/v)
Glycerin	20% (v/v)
Bromophenol blue	0.6 mg/dl

Gel running buffer components	**Final concentration**
Tris	25 mM
Glycine	192 mM
Sodium dodecyl sulfate (SDS)	0.1% (w/v)

Resolving gel buffer components	**Final concentration**
Tris	375 mM
Sodium dodecyl sulfate (SDS)	0.1% (w/v)

Resolving gel (7%) components	**Volume**
Resolving gel buffer (pH 8.8)	7.5 ml
Acrylamide solution (30%)	7.0 ml
Ammonium per sulphate (APS) 10%	0.25 ml
TEMED	0.05 ml
Distilled H_2O	14.75 ml
Total volume	30 ml

Stacking gel buffer components	**Final concentration**
Tris	500 mM
Sodium dodecyl sulfate (SDS)	0.1% (w/v)

Stacking gel (7%) components	**Volume**
Stacking gel buffer (pH 6.8)	5.0 ml
Acrylamide solution (30%)	2.7 ml
Ammonium per sulphate (APS) 10%	0.1 ml
TEMED	0.025 ml
Distilled H_2O	2.175 ml
Total volume	10 ml

2.3.1.4 Immunoblotting

After separation on SDS-PAGE under reducing conditions, proteins were transferred to Amersham™ Hybond™-ECL nitrocellulose membranes (GE healthcare, Freiburg, Germany) at 30 volts for 12 – 14 hours at 4°C. To estimate the efficiency of protein transfer after blotting, the membranes were incubated with Ponceau-S (Sigma, Munich, Germany) for 10 minutes at room temperature with constant shaking. The membranes were washed with distilled water and scanned. After incubating with blocking solution (Roche, Mannheim, Germany) for 2 hours at room temperature, the immunoblots were probed with primary antibody [anti-nestin (Chemicon, 1:1000, for rat tissues), anti-nestin (BD Transduction, 1:1000, for mouse tissues), anti- PDGFR-β (Upstate, 1:500) and anti-p-PDGFR-β (Santa Cruz, 1:250)] diluted in antibody incubation buffer (90% TBS-T, 10% blocking buffer and 0.005% Thimerosal). After washing with TBS-T, blots were incubated with respective horseradish peroxidase conjugated secondary antibody

(goat-anti-mouse IgG, 1:2000 or goat-anti-rabbit IgG, 1:2000 from Pierce). After washing, the blots were treated with Amersham™ enhanced chemiluminescence (ECL) reagents (GE healthcare, Freiburg, Germany) and the chemiluminescence signal was captured on X-ray film (Fuji, Tokyo, Japan).

Blotting buffer components	**Final concentration**
Tris	100 mM
Glycine	193 mM

TBS-T components	**Final concentration**
Tris	20 mM
Sodium Chloride (NaCl)	137 mM
Tween 20	0.05% (v/v)

2.3.1.5 Densitometry

Densitometric quantification of Western blots was performed using Image-J software (NIH, Bethesda, Maryland, USA). Expression of proteins was quantified using band intensity values which were normalized to β-Actin or vinculin. Statistical analysis was performed using Graphpad prism version 4 (Graphpad software, San Diego, California, USA).

2.3.2 Immunohistochemistry

For immunohistochemistry, 8 µm thick lung sections from Bouin fixed paraffin embedded tissues were used. After deparaffinization in xylene and rehydration in series of decreasing ethanol concentration, sections were subjected to microwave treatment in sodium citrate buffer (pH 6.0) for antigen retrieval or directly preceded to serum blocking step. After blocking of unspecific binding sites in 2% normal goat/rabbit serum (Sigma-Aldrich, Steinheim, Germany) for 1 hour at room temperature, sections were incubated overnight at 4°C with primary antibody [anti-nestin(BD Transduction, 1:50, for mouse tissues), anti nestin (Chemicon, 1:50, for rat tissues), anti nestin (Santa

Cruz, 1:50, for human tissues), anti-PCNA (Chemicon, 1:25), anti-Ki67 (Novocastra, 1:1000), anti-Calponin-1 (Epitomics, 1:500) and anti-α-SMA (Serotec, 1:500)] diluted in PBS with 0.2% BSA and 0.1% sodium azide. After washing with PBS, horseradish peroxidase labeled polymer (DAKO, Hamburg, Germany) was applied to the sections for 1 hour at room temperature. Peroxidase activity was detected using DAB by nickel-glucose oxidase approach (Davidoff et al., 1995).

For double staining of paraffin embedded lung sections, after initial processing (deparaffinization, antigen retrieval, blocking etc.), Envision G|2 double staining kit (Dako, Hamburg, Germany) was used for double staining according to manufacturer's instructions.

For immunofluorescence, 10 µm thick frozen sections from tissues fixed with 4% PFA or unfixed lungs were used. The sections from unfixed tissue were fixed with ice cold acetone at -20°C for 10 minutes followed by air drying step of 5 minutes and again fixation with acetone for 10 minutes. After cutting cryosections from prefixed tissue or fixation with acetone, antigen retrieval and blocking of unspecific binding sites, sections were incubated overnight at 4°C with primary antibody [anti-nestin (BD Transduction, 1:50), anti-Ki67 (Novocastra, 1:1000), anti- PDGFR-β (Upstate, 1:100) and anti-p-PDGFR-β (Santa Cruz, 1: 50)]. After washing with PBS, slides were subjected to Cy3 (Jackson immunoresearch, Hamburg, Germany) or Alexa Fluor (Roche, Mannheim, Germany) conjugated secondary antibody and 4´,6-diamidino-2-phenyl-indole (DAPI, 1µg/ml) for 1 hour at room temperature. Sections without primary antibody treatment served as controls.

2.3.3 Azan staining

Heidenhain's AZAN staining method was performed on 8 µm thick lung sections from bouin fixed paraffin embedded tissues. After deparaffinization in xylene and rehydration in series of decreasing ethanol concentration, slides were incubated with Azocarmine solution at 56°C for 15 minutes. After washing with distilled water, slides were placed in 0.1% aniline alcohol until the cytoplasm and connective tissue became pale pink and nuclei were well defined. After rinsing the slides in 1% acetic alcohol and washing with distilled water, the slides were incubated in 5% phosphotungstic acid for 2

hours. After washing the slides were treated with aniline blue/orange-G solution for about 1 hour.

The slides were dehydrated in ethanol and cleared by three changes in xylene and coverslips were mounted using Eukitt® mounting medium (Fluka, Buchs, Switzerland). Images were obtained by conventional light and fluorescence microscopy using Axioskop 2 plus microscope and Axiovision software (Zeiss, Munich, Germany).

2.3.4 Laser assisted microdissection

Laser assisted microdissection of lung vessels was done with the help of PALM Laser Microdissection System with auto-catapult and Robocut Software (Zeiss, Munich, Germany). 10 µm lung cryosections were transferred to PALM membrane coated slides (Zeiss, Munich, Germany). Sections were fixed with ethanol and stained with hematoxylin. After 10 minutes of air drying, pulmonary vessels were microdissected and catapulted into oil-coated caps of sterile 0.5 ml tubes. Minimum 50 – 70 vessels corresponding to minimum 300,000µm^2 of required samples were collected per tube.

2.3.4.1 RNA isolation and cDNA synthesis

Total RNA from microdissected samples was extracted using RNeasy mini or micro kit (Qiagen, Hilden, Germany). Microdissected vessels were dissolved in lysis buffer with β-mercepto-ethanol. RNA was isolated using DNase on column digestion. Eluted RNA concentration was measured was used to synthesize 1st strand cDNA.

Genomic DNA contaminations were removed by DNase I [Invitrogen, Karlsruhe, Germany (1 U/reaction)] digestion for 15 min at 37°C. Synthesis of cDNA was performed according to the manufacturer's instructions using iScript™ cDNA synthesis kit (Bio-Rad, Munich, Germany) or alternatively with the help of GeneAmp® RNA PCR kit (Applied Biosystems, Darmstadt, Germany).

Recipe for cDNA synthesis with iScript™ cDNA synthesis kit

Reagents	**Amount per reaction**
5X iScript reaction mix	4 µl
iScript reverse transcriptase	1 µl
RNA	1 µg
H_2O	Up to 20 µl

Conditions for the reaction were 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C.

Recipe for cDNA synthesis with GeneAmp® RNA PCR kit

Reagents	**Amount per reaction**
10X PCR buffer	2 µl
2mM $MgCl_2$	4 µl
10mM dNTPs	1 µl
Random Hexamers	1 µl
RNase inhibitor	0.5 µl
Mul-V RT	1 µl
H_2O	0.5 µl

Reaction conditions for cDNA synthesis were 10 min at 20°C, 75min at 43°C, and 5 min at 99°C.

2.3.4.2 RT PCR

The intron-spanning primer pairs for PCR were designed with the Primer3 program (http://frodo.wi.mit.edu/primer3/) and synthesized from Eurofins (MWG synthesis GmbH, Ebersberg, Germany). Primers were cross checked by blasting with the whole genome to ensure specificity.

Target	**Sequence**		**Length**
Nestin	For	5´- AGGCTGAGAACTCTCGCTTG - 3´	152 bp
	Rev	5´- TGAGAAGGATGTTGGGCTGA - 3´	
SMA	For	5´- CTGACAGAGGCACCACTGAA - 3´	160 bp
	Rev	5´- CATCTCCAGAGTCCAGCACA - 3´	
β-Actin	For	5´- GTGGGAATGGGTCAGAAGG - 3´	300 bp
	Rev	5´- GGCATACAGGGACAGCACA - 3´	

The cDNA was amplified with target specific primer pairs according to manufacturer's instructions.

Recipe for PCR mix

Reagents	**Amount per reaction**
10x PCR buffer	2.5 µl
2mM $MgCl_2$	2.5 µl
10mM dNTPs	0.5 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
ATG polymerase	0.2 µl
cDNA	1 µl
H_2O	Up to 20 µl

Reaction conditions for PCR included initial denaturation of 15 min at 94°C followed by 40 cycles of 30 sec at 94°C, 20 sec at 60°C, 20 sec at 72°C and the final extension of 10 min at 72°C.

2.3.4.3 Agarose gel electrophoresis

PCR products were separated by electrophoresis on 2% agarose gels prepared in Tris-acetate-EDTA (TAE) buffer. 0.5 mg/ml ethidium bromide was added to gel to visualize DNA bands under UV light afterwards. Amplified product along with DNA ladder was loaded on to the gel and electrophoresis was done at a constant voltage of 130V for 30 min. PCR products were visualized under UV light and documented using a gel documentation system (Phase, Lübeck, Germany).

Positive controls for primers were run by using reversely transcribed total RNA from tissues abundantly expressing the respective target sequences (brain for nestin and lung for SMA). Negative controls were performed by omitting the reverse transcriptase during cDNA synthesis.

TAE buffer components	**Final concentration**
Tris	40 mM
Glacial acetic acid	20 mM
EDTA	1 mM

2.3.4.4 Real-time PCR

Quantitative real time PCR was performed on iCycler iQ™ real time PCR system (Bio-Rad, Munich, Germany) using Platinum® SYBR® green qPCR SuperMix-UGD (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions.

Recipe for Real time PCR mix

Reagents	**Amount per reaction**
SYBR green master mix	10 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
H_2O	Up to 20 µl

Reaction conditions for real time PCR

Step	**Time**	**Temperature**	**Cycle**
Initial denaturation	8.5 min	95°C	1
Denaturation	20 sec	95°C	40
Primer annealing	20 sec	60°C	
Extension	20 sec	72°C	
Denaturation	1 min	95°C	1
Dissociation curve	Indefinite	55-95°C	1
Final hold	Indefinite	4°C	1

Each reaction was run in triplicate and repeated three times independently. Negative controls were run omitting the cDNA template. In the end, PCR product was separated on 2% agarose gel as quality control to confirm product size and avoid false positive bands.

Real time PCR data was analyzed using iQ™ 5 Optical system software (Bio-Rad, Munich, Germany). Using the software, a dissociation curve was generated for each gene to ensure single product amplification and the threshold cycle (C_t values) for each gene was determined. The samples showing false positive bands or melting curve with more than one peak were not included in data analysis. The comparative $2^{-\Delta\Delta C_t}$ method

was used to analysis mRNA fold changes between normoxic and hypoxic lung samples, which was calculated as;

$$\text{Ratio} = 2^{-(\Delta C_t \text{ normoxic} - \Delta C_t \text{ hypoxic})}$$

where C_t is the threshold cycle and ΔC_t (C_t target-C_t reference) is the C_t value normalized to the reference/housekeeping gene β-Actin obtained for the same cDNA sample for both normoxic and hypoxic samples. The calculated $2^{-\Delta\Delta C_t}$ value was transformed into a percentage using the normoxic as 100% to show the difference in expression of mRNA.

3 Results

As shown before in a recent study by our group, nestin expression is present in lung vasculature. Nestin was found predominantly in arteries while veins and capillaries also showed nestin expression. Nestin$^+$ cells in vasculature were characterized as vascular smooth muscle cells and pericytes (Fig. 2, Dissertation, Claudia Berndt).

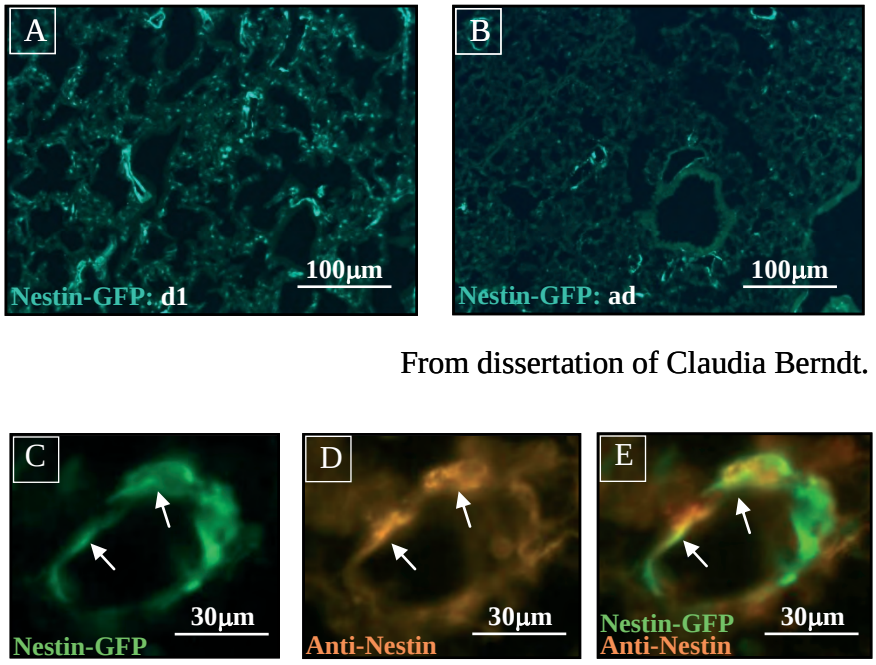
In the present study, it was intended to investigate whether nestin$^+$ progenitor cells are involved in development and remodeling. For this purpose we investigated, in detail, postnatal development and pulmonary hypertension. The reason of choosing postnatal development and pulmonary hypertension as models for our study was that both processes are associated with fundamental changes in vasculature of lungs and according to our hypothesis, that nestin$^+$ progenitor cells might be involved in these vascular changes.

3.1 Postnatal Development

During postnatal development, along with alveolarization, vasculature of lung also undergoes remodeling (Burri, 2006; Ni et al, 2010). Lung samples from postnatal mice were used to evaluate nestin expression and proliferation in vasculature of lungs.

3.1.1 Comparison of nestin expression and VSMC proliferation between postnatal d1 and adult

Using nestin GFP mice our colleague Claudia Berndt showed that significantly higher amounts of nestin are expressed in lungs of postnatal d1 mice as compared to adults (Fig. 3A, 3B). Nestin was found only in cells of vasculature while the smooth muscle cells around the bronchioli were negative for nestin (Fig. 2C). Anti nestin antibodies were used to confirm whether the GFP signal really represents the nestin staining and a co-localization of nestin and GFP staining was observed in vasculature of lungs.



From dissertation of Claudia Berndt.

Fig. 3: Comparison of nestin-GFP expression in lungs from post natal d1 and adult mouse. **A-B:** High amount of Nestin-GFP staining at postnatal d1 (A) compared to adult lung sample (B). **C-E:** Colocalization of nestin and nestin-GFP (arrows). **C:** GFP signal. **D:** Anti-nestin immunofluorescence. **E:** Merged photo from vessel with anti-nestin and GFP staining.

The observation of significantly higher nestin expression in postnatal d1 lungs as compared to adults was confirmed by Western blot analysis (Fig. 4A). 240 kDa protein bands in this Western blot analysis clearly correspond to nestin-GFP data. Brain samples from postnatal day 1 were routinely used as positive control for nestin in Western blots. Vinculin was used as loading control.

Next it was of interest whether nestin expression at day 1 correlates with higher proliferation rates of vascular smooth muscle cells, shown to express nestin (Fig. 2B-D). For this purpose, vascular smooth muscle cell proliferation was compared between lungs from postnatal d1 and adults using proliferation marker PCNA. Lungs from postnatal d1 showed high vascular smooth muscle cell proliferation (Fig. 4B) while there was no proliferation of vascular smooth muscle cells in adult samples (Fig. 4C). Epithelial cells of bronchioli which proliferate regularly, served as internal positive controls (arrowheads, Fig. 4C). Mouse testes that showed positive PCNA staining in nuclei of proliferating germ cells were used as further positive controls and to optimize immunohistochemistry protocol (Fig. 4D-E).

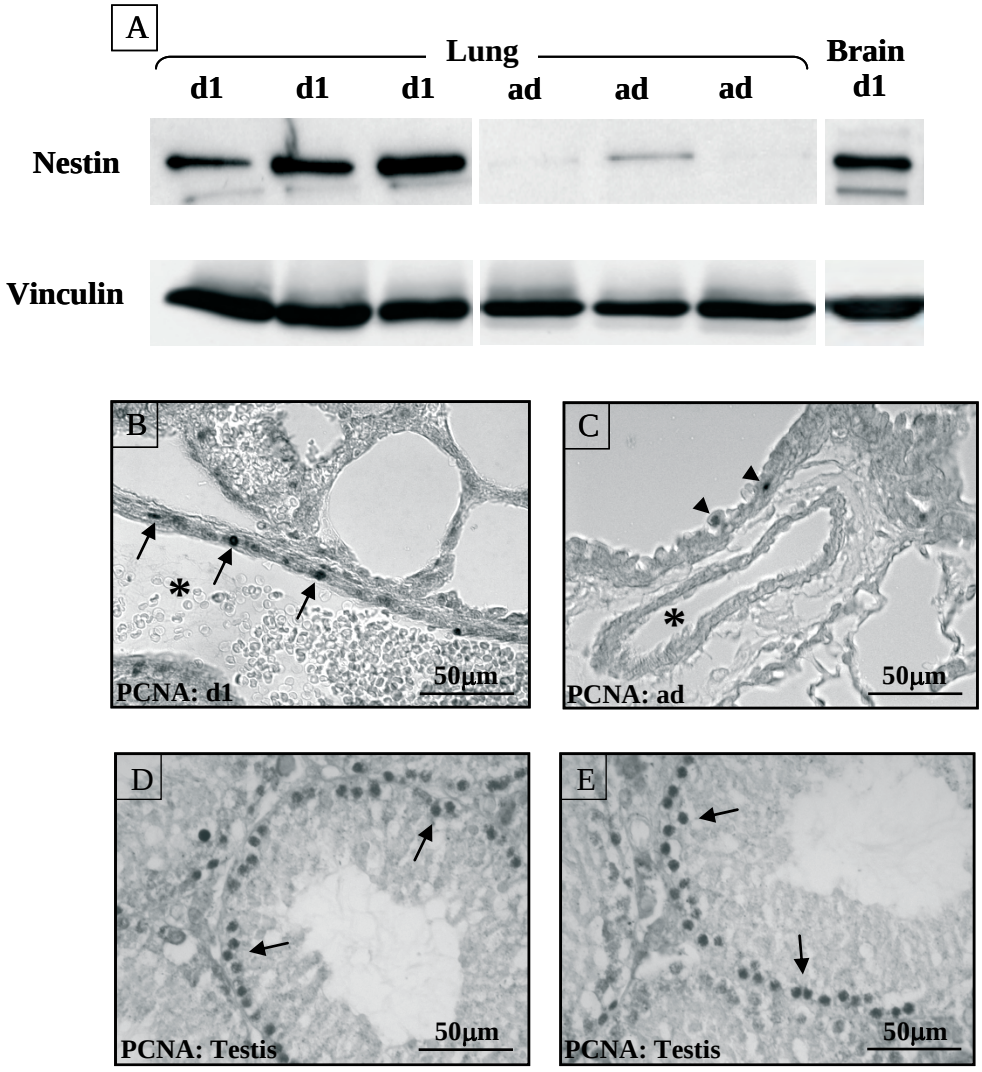


Fig. 4: Comparison of nestin expression and smooth muscle cell proliferation in mouse postnatal development; day 1 (d1) vs. adult (ad). **A:** Western blot analysis showed high amount of nestin in d1 lung samples. In adults, nestin was barely detectable. (60 µg protein was used for each sample) Brain sample from postnatal d1 mouse was used as positive control for nestin and vinculin was used as loading control. **B-E:** PCNA immunohistochemistry to reveal cell proliferation. **B-C:** Comparison of vascular smooth muscle cell proliferation between d1 and adult using PCNA antibodies. VSMCs in vessels (*) of d1 lungs showed marked proliferation as compared to adult (arrows). Proliferating epithelium serves as internal positive control (arrowheads). **D-E:** PCNA immunohistochemistry in mouse testis sections, used as further positive control, showed positive staining in spermatogonia.

3.1.2 Nestin expression and VSMC proliferation at different time points of postnatal development

Since postnatal changes in structure of mouse lung are not restricted to time immediately after birth (Burri, 2006), a series of further time points was used to investigate nestin expression and cell proliferation

Using Western blot analysis it was found that during postnatal development, lungs showed maximum nestin expression from postnatal d3 till d10 that reduced gradually during the course of development (Fig. 5A). Interestingly, nestin expression during the period between d3 and d10 was even higher than on d1 (Figs. 3 and 4) which was found to be much higher than in adults.

In agreement, proliferation of vascular smooth muscle cells also increased during early postnatal development with maximum proliferation between postnatal d3 and d10 (Fig. 5B-5E) that reduces gradually as the development proceeds (Fig. 5F) with adult samples showing no VSMC proliferation (Fig. 5G).

Expression pattern of nestin showing peak of nestin at d3-d10 is quite similar to proliferation of vascular smooth muscle cells with maximum proliferation in d3-d10 samples which strongly suggests that there is a definite correlation between nestin expression and proliferation of vascular smooth muscle cells in lungs.

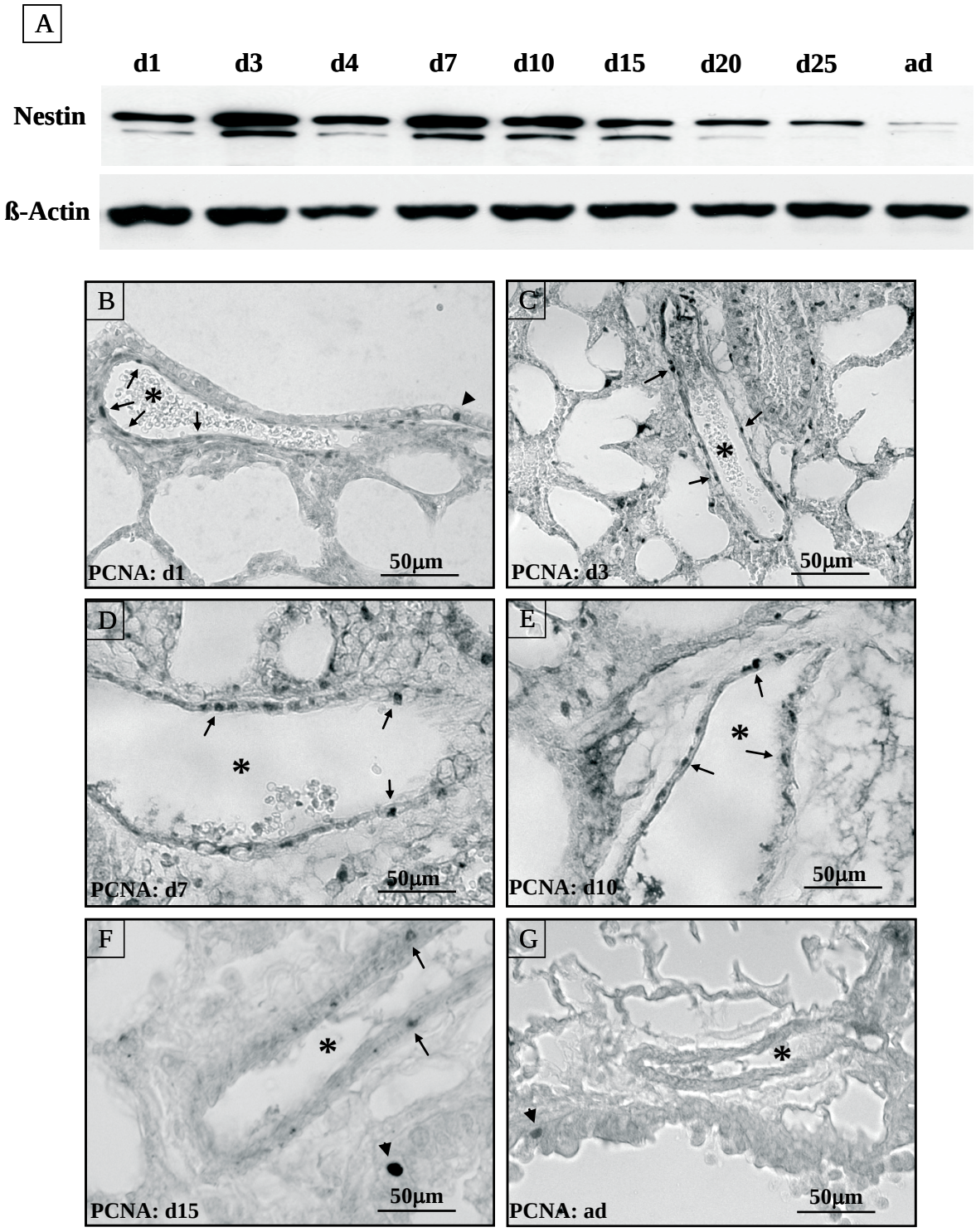


Fig. 5: Analysis of nestin expression and vascular smooth muscle proliferation in postnatal development. **A:** Western blot showing increased nestin expression between d3 and d10 (60 µg protein per lane). β-Actin was used as loading control. **B-G:** PCNA immunohistochemistry. Proliferating smooth muscle cells are clearly visible (arrows) in lung samples from postnatal d1, d3, d7, d10 and d15 (B-F). Lung section from adult mouse showing no proliferation of vascular smooth muscle cells (G). Proliferating epithelial cells serve as internal positive control (arrowheads). Arteries are marked by asterisks (*).

3.2 Mouse model of chronic hypoxia

Chronic hypoxia results in pulmonary hypertension and is characterized by vascular remodeling (Pak et al 2007) that is known to be visible after three weeks of hypoxic exposure. To investigate nestin expression and role of nestin^{+} cells in the process of vascular remodeling, mice were exposed to hypoxic conditions for different time periods up to 3w and series of experiments were performed including measurement of nestin mRNA level in micro-dissected arteries, analysis of nestin protein expression and proliferation of vascular smooth muscle cells in hypoxic lung samples.

3.2.1 Nestin mRNA levels in microdissected arteries from hypoxic lung samples

Since nestin was found to be localized predominantly in pulmonary arteries (Fig. 2), nestin expression was investigated in laser assisted microdissected arteries located in close proximity to bronchioli (Fig. 6A, 6B). Using RT-PCR, nestin was detected in pulmonary arteries (Fig. 6C).

3.2.2 Nestin protein expression in hypoxic lung samples

Western blot analysis of hypoxic samples taken after different time periods showed that exposure to hypoxia resulted in a characteristic pattern of nestin expression. Nestin expression in lungs increased significantly after three days of hypoxic exposure and remained up-regulated until one week under hypoxia and eventually came to normoxic level after three weeks of hypoxic treatment (Fig. 7).

It was an interesting observation that realtime PCR analysis from laser assisted microdissected arteries (data not shown) provides evidence for a peak of nestin mRNA expression immediately before day 3 after hypoxia and decrease to normoxic levels immediately thereafter.

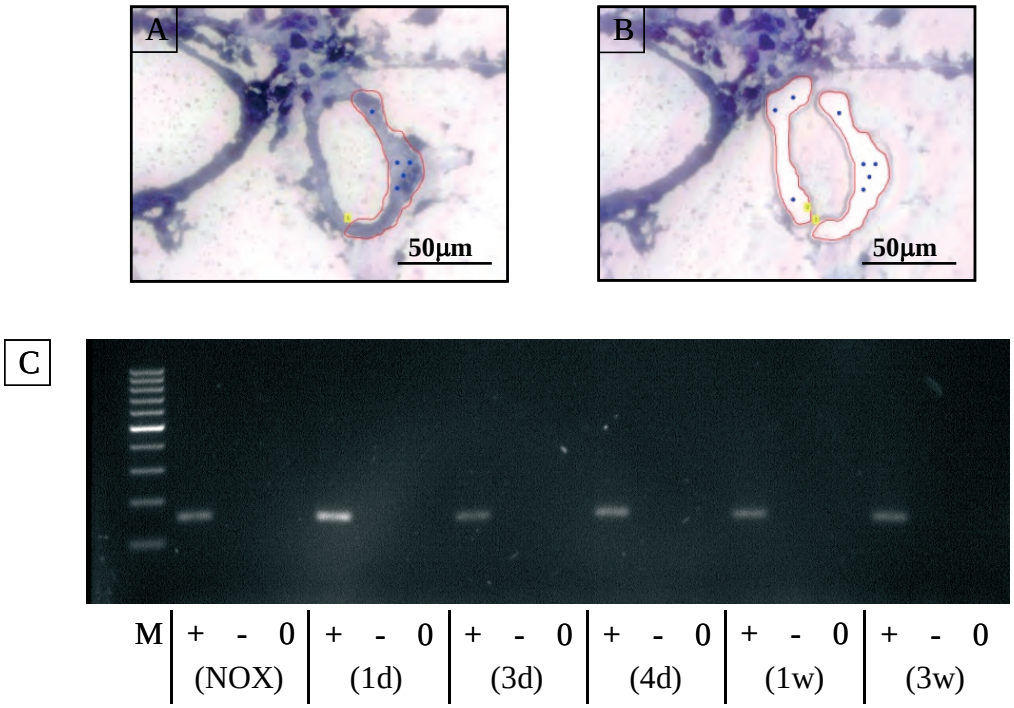


Fig. 6: RT PCR analysis for nestin expression in laser-assisted microdissected arteries from hypoxic lung samples. **A-B:** Photos showing arteries before and after microdissection. **C:** Agarose gel electrophoresis showing nestin expression in microdissected vessels. M= DNA ladder, "+" represents the normal PCR reaction, "-" represents the negative control with the same ingredients excluding reverse transcriptase during reverse transcription step and "0" indicates the water negative control.

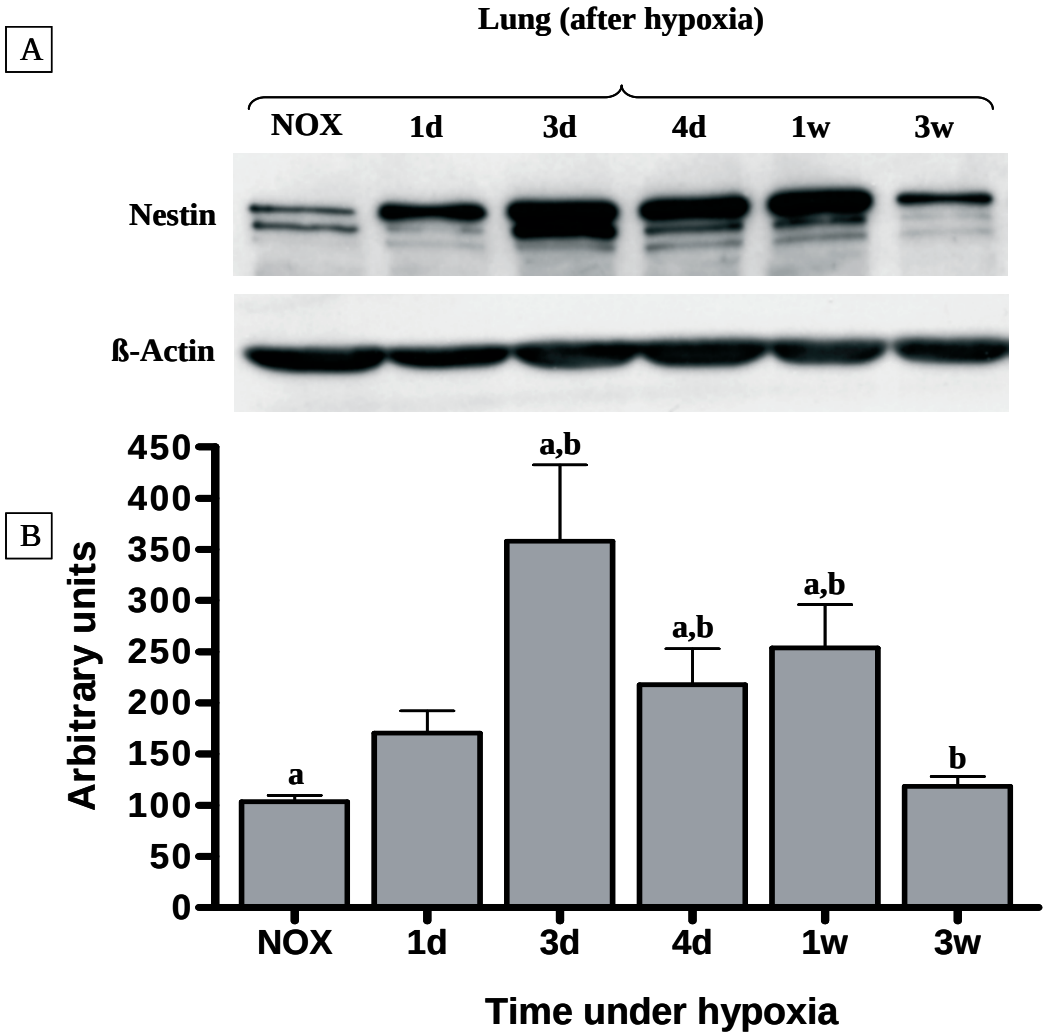


Fig. 7: Western blot analysis of nestin expression in cytosolic protein extracts from hypoxic lung samples (120 µg protein per sample). **A:** Nestin Western blot showing increase in nestin expression between 3d and 1w of hypoxic treatment that decreases and comes to normoxic level after 3w. Vinculin was used as loading control. **B:** Densitometric quantification of Western blots showing peak of nestin between 3d and 1w of hypoxic exposure. Bars show mean scores (+/- SD) from 3 assessments. ^{a,b} $P < 0.05$; a indicates that there was a significant difference when NOX values were compared with 1d, 3d, 4d, and 1w values each; b indicates that there was a significant difference when 3w values were compared with the 1d, 3d, 4d and 1w values each.

3.2.3 Proliferation of vascular smooth muscle cells in hypoxic lung samples

Immunohistochemistry of hypoxic samples with the proliferation markers PCNA revealed that there was no proliferation of vascular smooth muscle cells until one day after hypoxia (Fig. 8A-B). At three days after hypoxic treatment a significant number of proliferating vascular smooth muscle cells could be observed (Fig. 8C). Maximum number of proliferating vascular smooth muscle cells was observed after four days of hypoxic treatment (Fig. 8D). Amount of proliferating vascular smooth muscle cells decreased after 1 week under hypoxia (Fig. 8E) and eventually in 3 weeks hypoxic samples there were no proliferating vascular smooth muscle cells (Fig. 8F).

The same pattern of proliferating cells was visible when analyzing expression of another proliferation marker (Ki67) at the same time points under hypoxia. Again, there was no proliferation of vascular smooth muscle cells in normoxic and 1 day hypoxic samples (Fig. 9A-B) with significant increase of vascular smooth muscle cell proliferation at 3 days (Fig. 9C) and maximum proliferation at 4 days under hypoxia (Fig. 9D) followed by decrease in number of proliferating vascular smooth muscle cells after 1 week under hypoxia (Fig. 9E) and finally no proliferation of vascular smooth muscle cells after 3 weeks of hypoxic exposure (Fig. 9 F).

Specific pattern of nestin expression in hypoxic samples showing increase in nestin between 3 days and 1 week again correlates to increased proliferation of vascular smooth muscle cells during the same time period under hypoxia. This data is consistent with data of nestin expression and vascular smooth muscle cell proliferation in postnatal development suggesting again a strong correlation between nestin expression and proliferation.

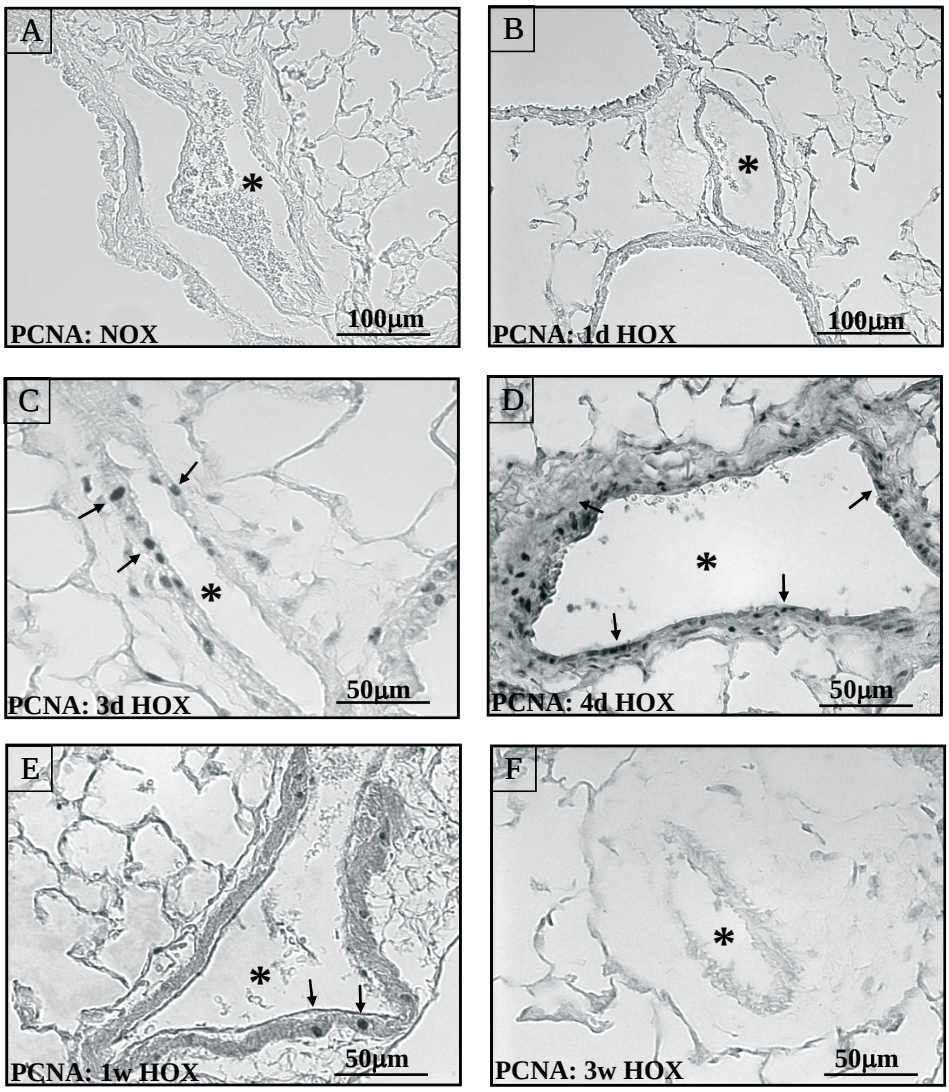


Fig. 8: Immunohistochemical analysis of vascular smooth muscle proliferation using proliferation marker PCNA. **A-B**: Vascular smooth muscle cells showing no proliferation in normoxic lung sample as well as in hypoxic lung sample 1d after hypoxic exposure. **C-D**: Vascular smooth muscle cells showing maximum proliferation in lungs isolated 3d and 4d after hypoxic treatment. **E**: After 1w of hypoxic exposure, lung samples show significantly reduced proliferation of smooth muscle cells. **F**: 3w hypoxic samples show no smooth muscle cell proliferation. Arteries are indicated by asterisks (*).

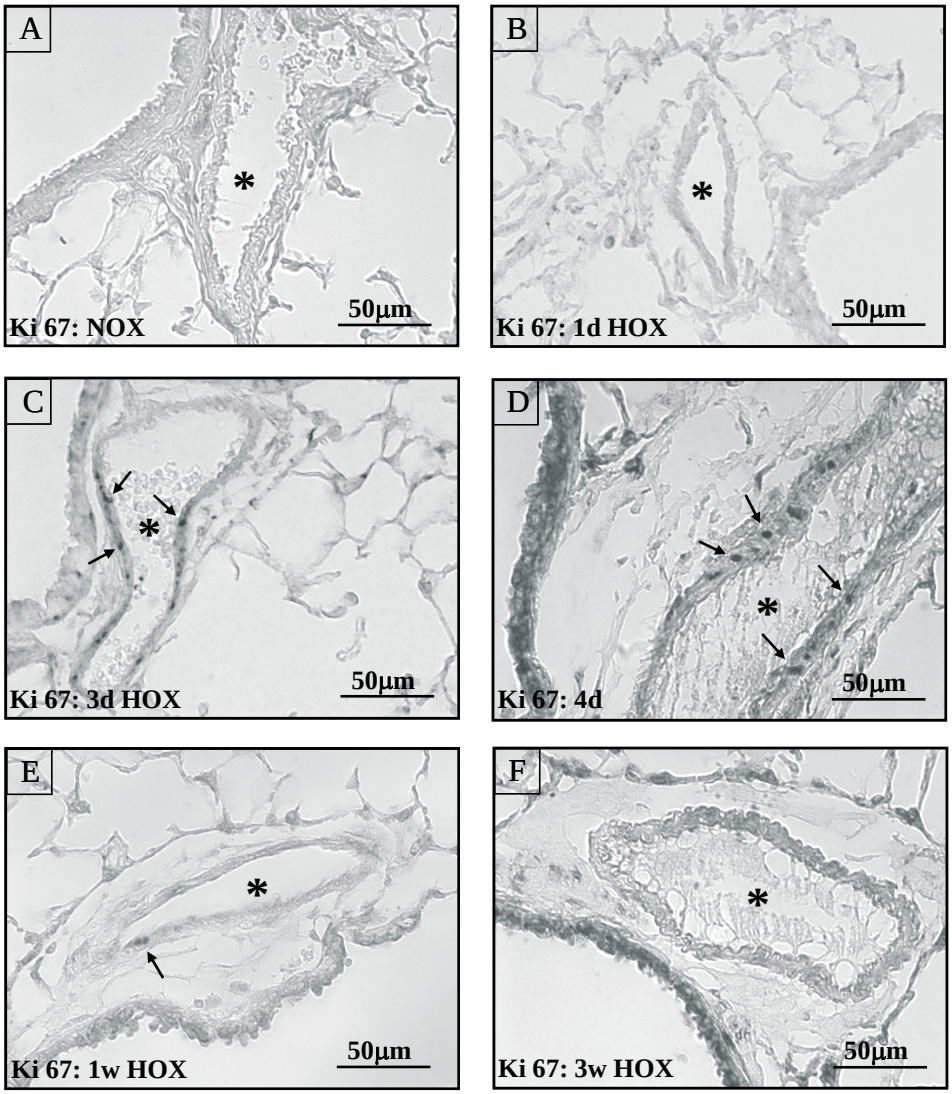


Fig. 9: Immunohistochemical analysis of vascular smooth muscle proliferation using proliferation marker Ki67. **A-B**: Vascular smooth muscle cells showing no proliferation in normoxic lung sample as well as in hypoxic lung sample 1d after hypoxic exposure. **C-D**: Vascular smooth muscle cells showing maximum proliferation in lungs isolated 3d and 4d after hypoxic treatment. **E**: After 1w of hypoxic exposure, lung samples show significantly reduced proliferation of smooth muscle cells. **F**: 3w hypoxic samples show no smooth muscle cell proliferation. Arteries are indicated by asterisks (*).

3.2.4 VSMCs represent the proliferating cell types in hypoxic vascular remodeling

Hypoxic lung samples were analyzed using double staining with smooth muscle marker α-SMA and proliferation marker PCNA (Fig. 10) to prove whether the proliferating cells are really vascular smooth muscle cells. In normoxic and 3 weeks hypoxic samples staining of smooth muscle cells is visible only while in 3 days and 4 days hypoxic samples, along with staining of α-smooth muscle actin in smooth muscle cells (red color), PCNA marked nuclei (stained in brown color) are clearly visible (Fig. 10A-B). This data from dual color immunohistochemistry confirms that in hypoxic lung samples, the proliferating cells in pulmonary vasculature are the vascular smooth muscle cells. It is worth mentioning here that we did not find any proliferation of endothelial cells (or adventitial cells) in hypoxic mouse samples.

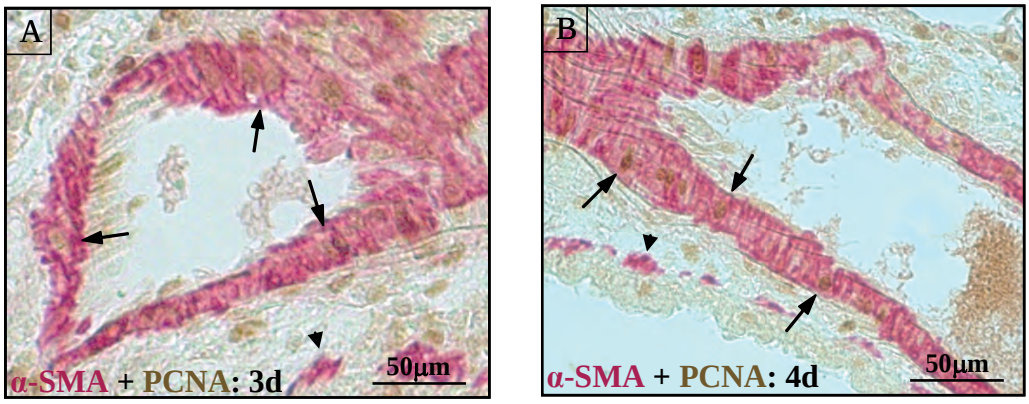


Fig. 10: Dual color immunohistochemical staining of hypoxic lung samples. Red color represents α-SMA highlighting the smooth muscle cells in vessels as well as around the airways (arrowheads) while $PCNA^+$ nuclei in brown color show proliferating cells. In arteries of 3d (A) and 4d (B) hypoxic samples, along with α-SMA, $PCNA^+$ nuclei are also visible representing $\alpha\text{-}SMA^+$ proliferating vascular smooth muscle cells (arrows).

3.2.5 Nestin$^+$ subpopulation of VSMCs shows proliferation

Multicolor immunofluorescence technique was also applied on hypoxic lung samples using antibodies against nestin and proliferation marker Ki67 as well as DAPI. In normoxic and 3 weeks hypoxic samples, only DAPI$^+$ nuclei could be observed while nestin staining was barely detectable (Fig. 11A, D). In samples where increased nestin expression and proliferation of vascular smooth muscle cells was found, nestin localization could be observed in cytoplasm of a subpopulation of vascular smooth muscle cells and these nestin$^+$ cells also showed proliferation which is visible by localization of Ki67 in nuclei (marked by DAPI) of nestin$^+$ cells in 3 days and 4 days hypoxic samples (Fig. 11B-C).

This experiment showing proliferation of nestin$^+$ VSMCs proved the correlation between nestin expression and proliferation suggesting the involvement of a sub-population of nestin$^+$ vascular smooth muscle cells in postnatal development and vascular remodeling.

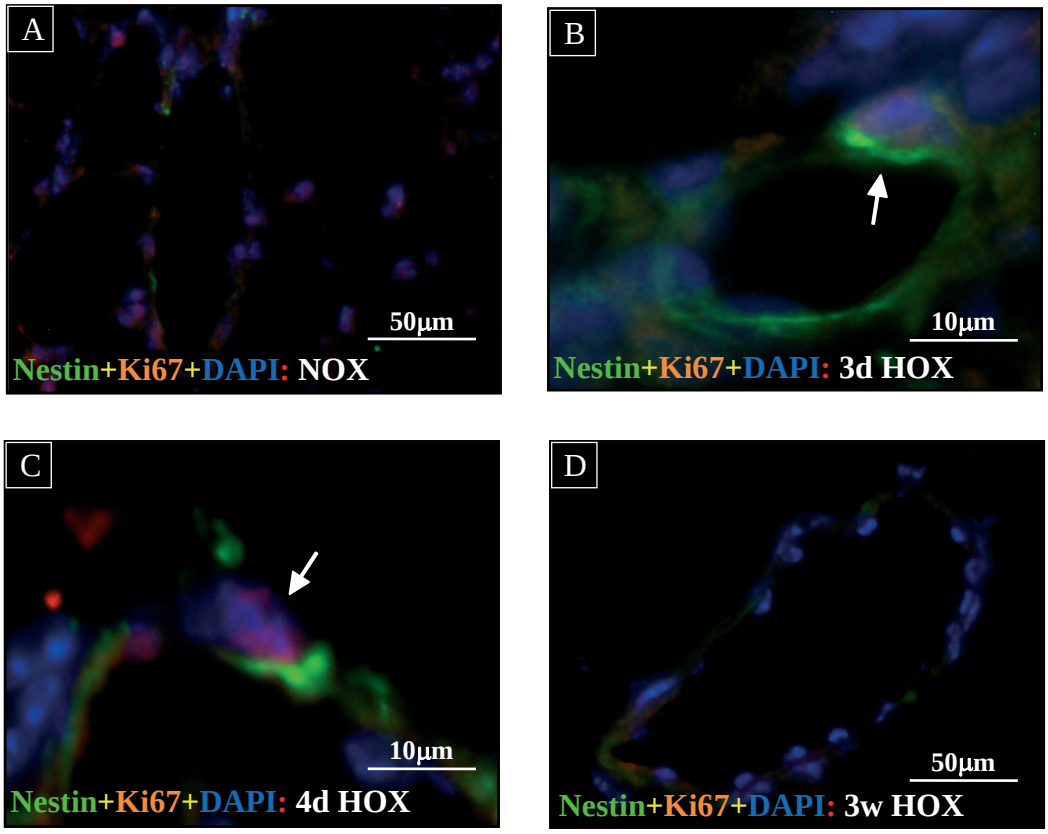


Fig. 11: Multicolor immunofluorescent staining of hypoxic lung samples. Green color identifies nestin immunoreactivity while $Ki67^+$ nuclei in red color represent proliferating cells. Nuclei are stained with DAPI. **A:** In normoxic sample, only $DAPI^+$ nuclei are visible without additional red staining (Ki67). **B, C:** In 3d and 4d hypoxic samples, $nestin^+$ vascular smooth muscle cells are also positive for the proliferation marker Ki67 (arrows). **D:** In 3w hypoxic sample, there are $DAPI^+$ but no $Ki67^+$ nuclei.

3.2.6 Different sub-types of vascular smooth muscle cells in media

Since we were able to demonstrate that only a sub-population of vascular smooth muscle cells expressed nestin and showed proliferation, we wanted to investigate whether different sub-types of smooth muscle cells exist in vessel wall. Therefore, we used calponin to mark smooth muscle cells of contractile phenotype (Owens, 1995; Sobue et al., 1999). After 4 days of hypoxic exposure, where we found maximum proliferation of vascular smooth muscle cells, it appears that two distinct sub-populations of vascular smooth muscle cells could be distinguished, one contractile phenotype showing strong calponin staining (arrows) and another with down-regulated calponin signal (arrowheads) indicating change of contractile phenotype to synthetic phenotype in a subpopulation of vascular smooth muscle cells. In opposite, all smooth muscle cells around the airways showed strong calponin staining suggesting a contractile phenotype only (Fig. 12A-B). Different to 4 days, in normoxic and 3 weeks hypoxic samples where no nestin expression and proliferation of smooth muscle cells was observed, there was strong calponin staining in vessels as well as around the airways (Fig. 12C-D).

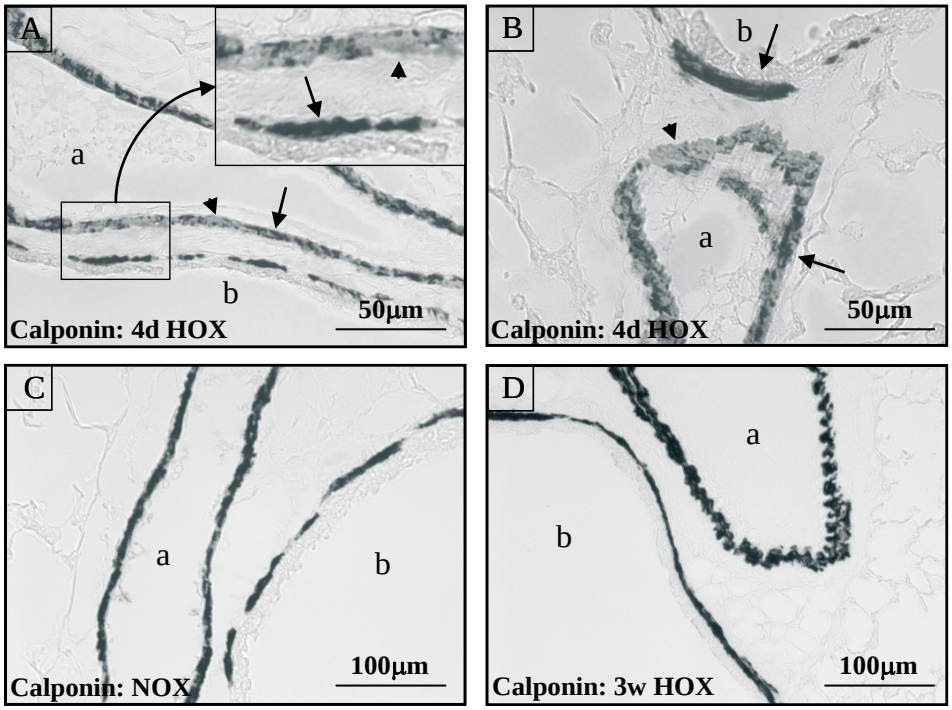


Fig. 12: Calponin immunohistochemistry of hypoxic lung samples to differentiate between a more contractile and a more synthetic phenotype of smooth muscle cells. **A-B:** After 4d of hypoxia, contractile SMCs around the bronchiole (b) showing strong calponin staining (arrows) while in blood vessels two populations of SMCs are clearly visible where cells with stronger (arrows) and low (arrowheads) calponin staining could be discriminated. **C-D:** Under normoxia and 3w hypoxia, strong calponin staining is visible in SMC of both airways and vessels (a, artery; b, bronchiole).

3.2.7 PDGFR-β expression during the course of vascular remodeling

PDGF-B/PDGFR-β signaling is known to be involved in vascular remodeling mediating the proliferation and migration of vascular smooth muscle cells (Schermuly et al, 2005). Western blot analysis was performed to investigate expression pattern of PDGFR-β and phosphorylated/activated PDGFR-β. There is slight but gradual increase in expression of PDGFR-β during the progression of pulmonary hypertension (Fig. 13A). On the other hand Western blot analysis and densitometric quantification clearly showed that its active form p-PDGFR-β showed a peak between 4 days and 1 week of hypoxic treatment with a tendency of decrease of p-PDGFR-β thereafter (Fig. 13B-C). This pattern of PDGFR-β phosphorylation could be correlated to nestin expression and proliferation of vascular smooth muscle cells.

Summarizing the results from hypoxic mouse model, it could be concluded that there was significant increase in nestin mRNA prior to day 3 after hypoxia which is followed by up-regulation of nestin protein and increase in proliferation of vascular smooth muscle cells at 3 days after hypoxia and in the end peak of the p-PDGFR-β from 4 days till 1 week after hypoxia. Interestingly, nestin expression and proliferation of vascular smooth muscle cells were down-regulated and came to normoxic levels after 3 weeks of hypoxic treatment when pulmonary hypertension is known to be established and is clearly visible clinically and histologically.

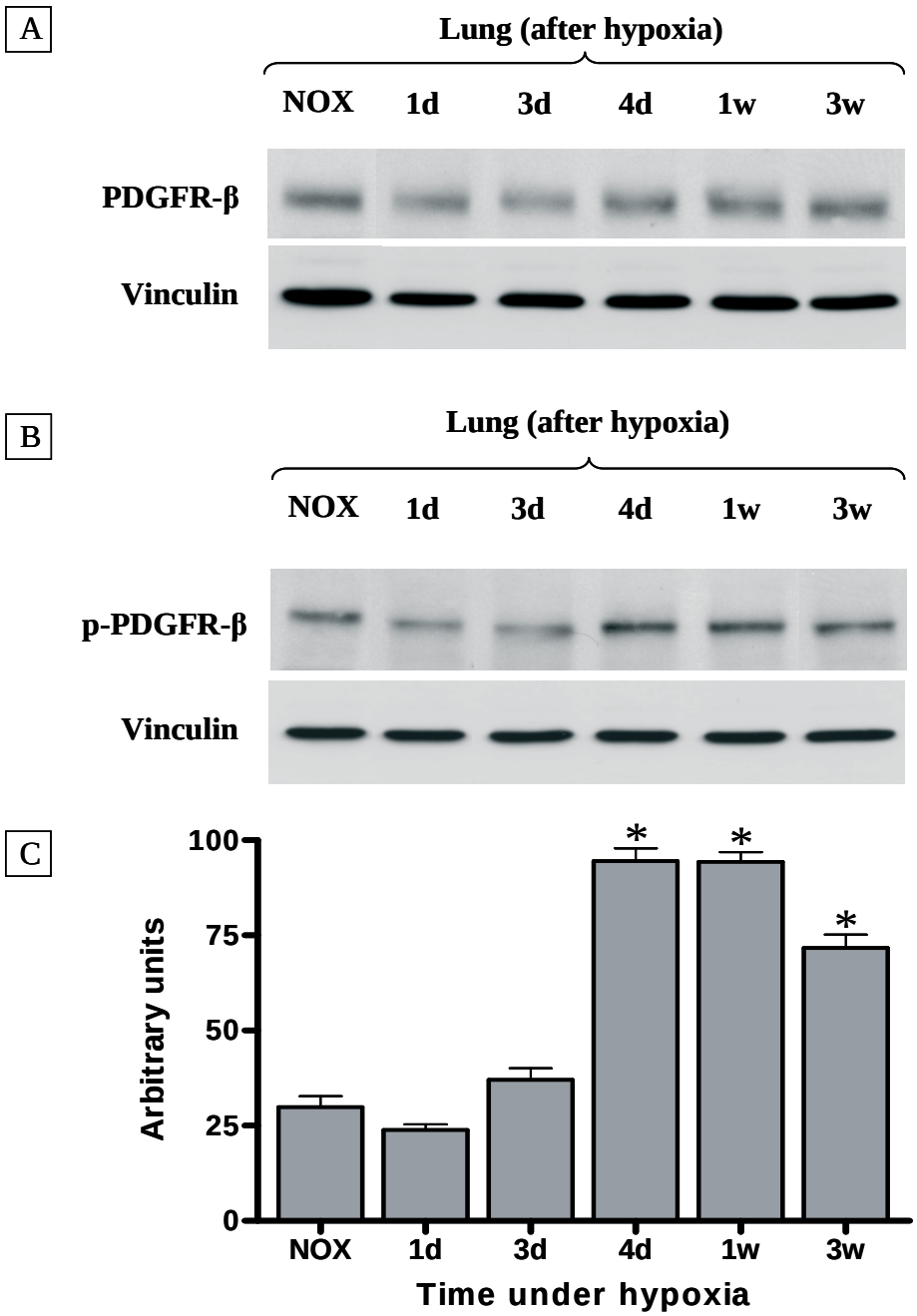


Fig. 13: Western blot analysis of PDGFR-β and p-PDGFR-β. **A:** Blot showing gradual increase of PDGFR-β (190 kDa) expression along with the progression of pulmonary hypertension (40 µg membrane protein per sample). **B:** Immunoblot showing peak of p-PDGFR-β (190 kDa) expression at 4d – 1w after hypoxia (40 µg membrane protein per lane). Vinculin served as loading control. **C:** Densitometric quantification of p-PDGFR-β expression. Bars show mean scores (+/- SD) from 3 assessments. Quantified values of each time point under hypoxia were compared with values of normoxic controls (*P < 0.05).

3.3 Nestin in MCT rat samples

To investigate whether nestin$^+$ vascular smooth muscle cells are also involved in monocrotaline induced vascular remodeling, nestin immunohistochemistry was performed on MCT treated rat samples. Nestin was found in vascular smooth muscle cells in rat lung samples after 3 weeks and 4 weeks of MCT treatment (Fig. 14A-B) while control samples without MCT treatment were negative for nestin immunoreactivity (Fig. 14D). Double staining of MCT treated rat lung sections with nestin and α-smooth muscle actin confirmed that nestin is expressed by a sub-population of vascular smooth muscle cells (Fig. 14C).

Nestin expression in *A. pulmonalis* (pulmonary artery) and aorta of MCT treated rats was compared using Western blot analysis. In *A. pulmonalis* of MCT treated samples there is significant increase in nestin expression as compared to respective control, while there is no difference in nestin expression in aorta from control and MCT treated rats (Fig. 14E). This Western blot analysis suggests that MCT induced increase in nestin expression and hence vascular changes are associated with only pulmonary circulation with no or minimal effect on systemic circulation.

Different to hypoxic mouse model, in large arteries of MCT treated rat samples, in addition to vascular smooth muscle cells, nestin staining was also observed in a specific zone at the border of media and adventitia (Fig 15A-C) which was previously described as “vasculogenic zone” harboring stem/progenitor cells (see Klein et al, 2010). Double staining with nestin and α-smooth muscle actin clearly shows that in addition to nestin in vascular smooth muscle cells, nestin$^+$ cells are also present outside the medial layer (Fig. 15 A-B). This data suggests the presence of nestin$^+$ stem/progenitor cells in a certain area of vascular adventitia. Azan staining was also used to clearly differentiate between different layers of vascular wall and to determine the exact location of nestin$^+$ cells shown by nestin immunohistochemistry (Fig. 15D).

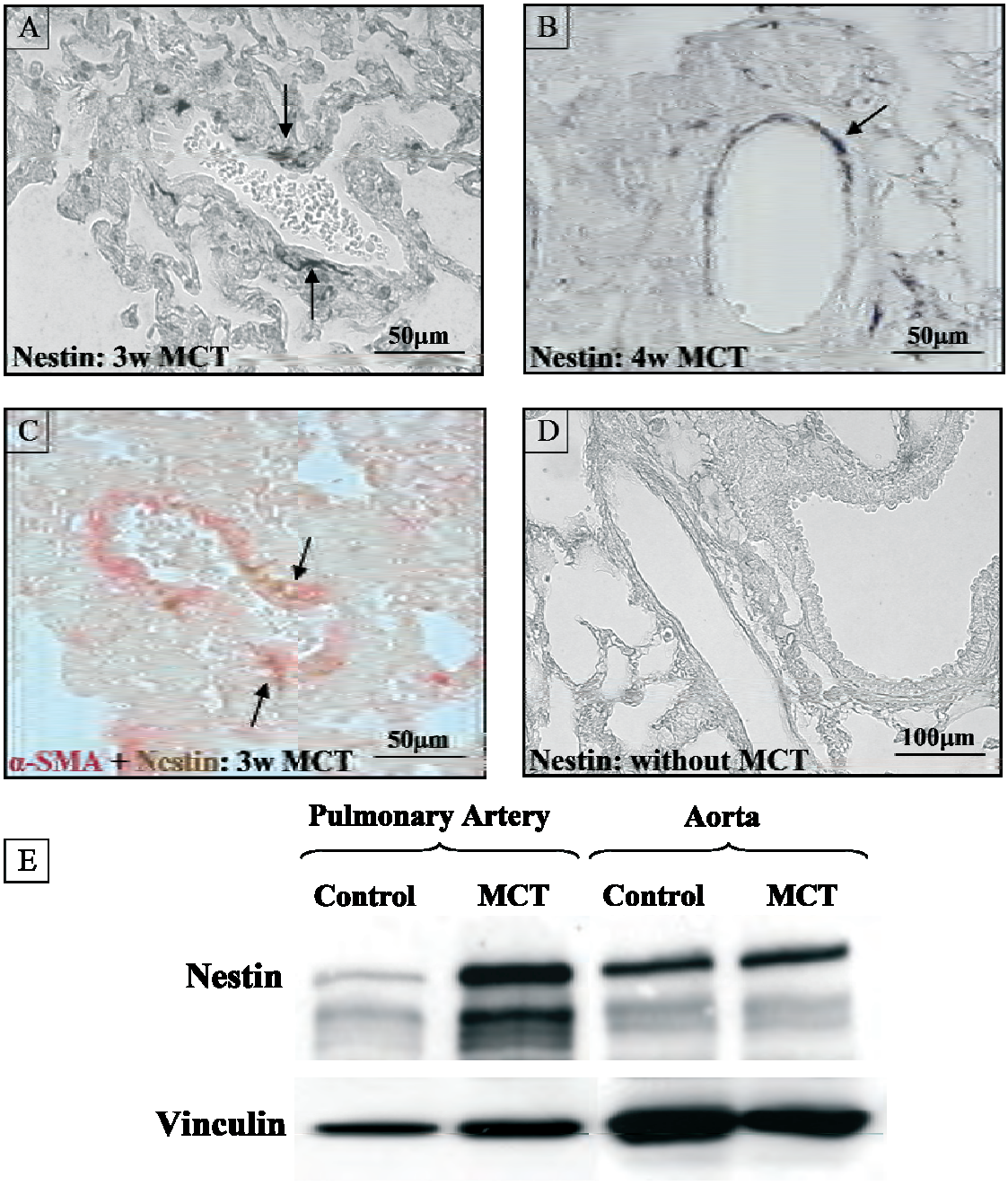


Fig. 14: Nestin expression in monocrotaline treated rat samples. **A-B:** Nestin immunostaining in vascular smooth muscle cells of small vessels (ca. 100 µm diameter) after 3w and 4w of monocrotaline treatment. **C:** Double staining of MCT treated lung sample showing nestin^{+} cells (brown) are smooth muscle cells (red). **D:** Rat lung sample without MCT treatment showing no nestin immunostaining. **E:** Immunoblot showing comparison between nestin expression in MCT treated aorta and *A. pulmonalis*.

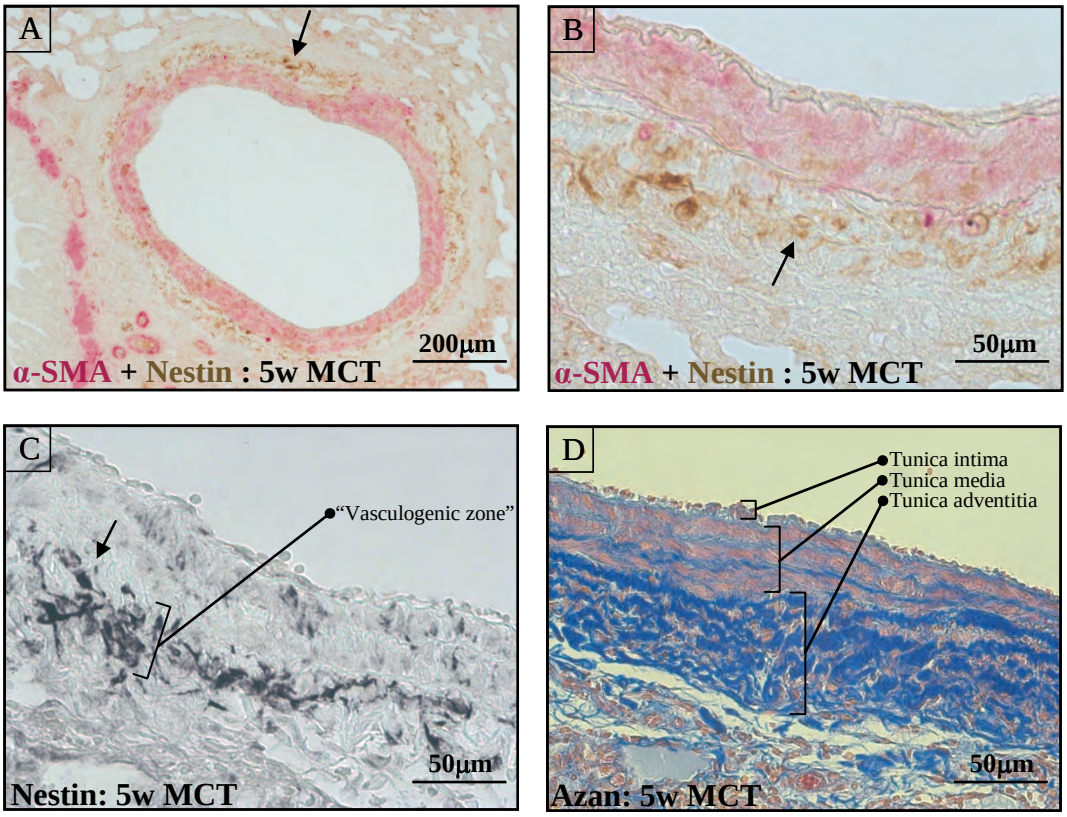


Fig. 15: Nestin staining in large vessels (> 200 µm diameter) of monocrotaline treated rat samples. **A-C:** Nestin immunohistochemistry (C) and double staining (A-B) showing additional nestin expression in adventitial cells (arrows) close to media (previously described as "vasculogenic zone"). **D:** Azan staining of lung samples showing the three vascular layers intima, media and adventitia.

3.4 Nestin in rat samples treated with Sugen 5416 and hypoxia

Nestin expression was also investigated in rats treated with VEGF-2 receptor antagonist Su5416 and hypoxia. In this model, similar to hypoxic mouse and MCT rat models, nestin expression was also found in vascular smooth muscle cells of arteries 5 weeks after treatment with Su5416 and hypoxia (Fig. 16A-C). Double staining and co-localization of nestin and α-smooth muscle actin confirmed the presence of nestin in vascular smooth muscle cells (Fig. 16C). Control samples from animals that were not treated with Su5416 and hypoxia, showed no nestin expression (Fig. 16D). In the Su5416/hypoxia model, however, there are additional characteristic features that do not appear in hypoxic or MCT models, e.g. formation of complex lesions (Abe et al., 2010).

In very small arteries (ca. 30-50 µm diameter), that are supposed to be non-muscular arteries in normal conditions and get muscularized during vascular remodeling, additional nestin immunoreactivity could be observed outside the media (Fig. 17A-D). These nestin$^+$ perivascular cells may contribute to muscularization of previously non-muscular arteries. Double staining with nestin and α-smooth muscle actin confirmed that nestin is also present outside the media (Fig. 17C-D).

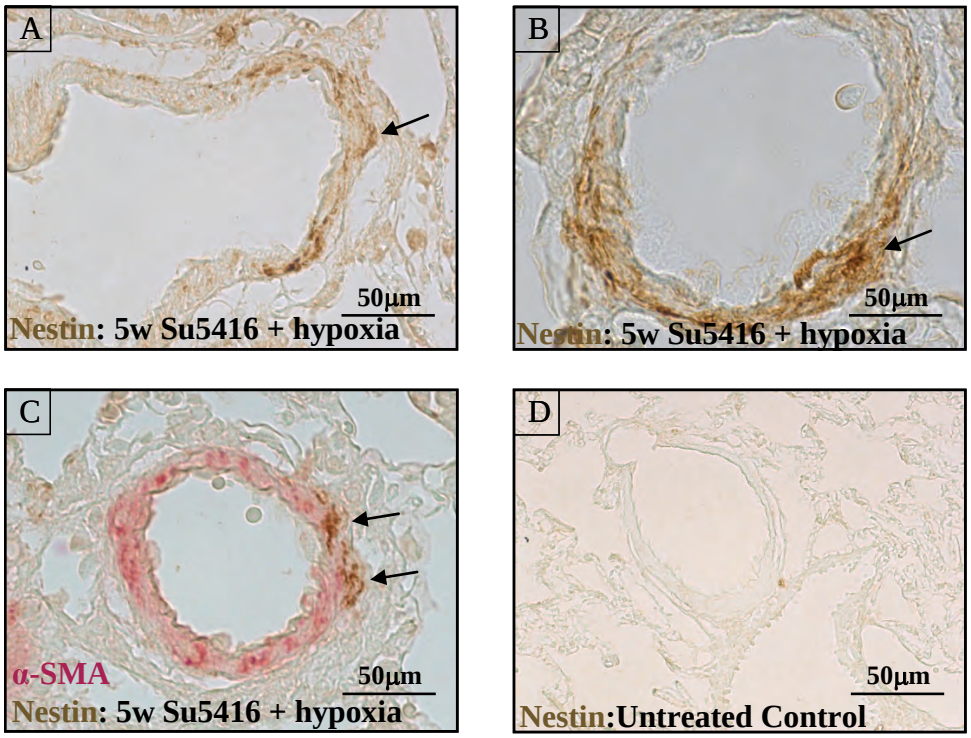


Fig. 16: Nestin expression in rats treated with Sugen5416 and hypoxia. **A-B:** Nestin expression in vascular smooth muscle cells (arrows). **C:** Colocalization of α-smooth muscle actin (red) and nestin (brown) confirming nestin in vascular smooth muscle cells. **D:** Control rat samples without Su5416 and hypoxic treatment showing no nestin in the vasculature.

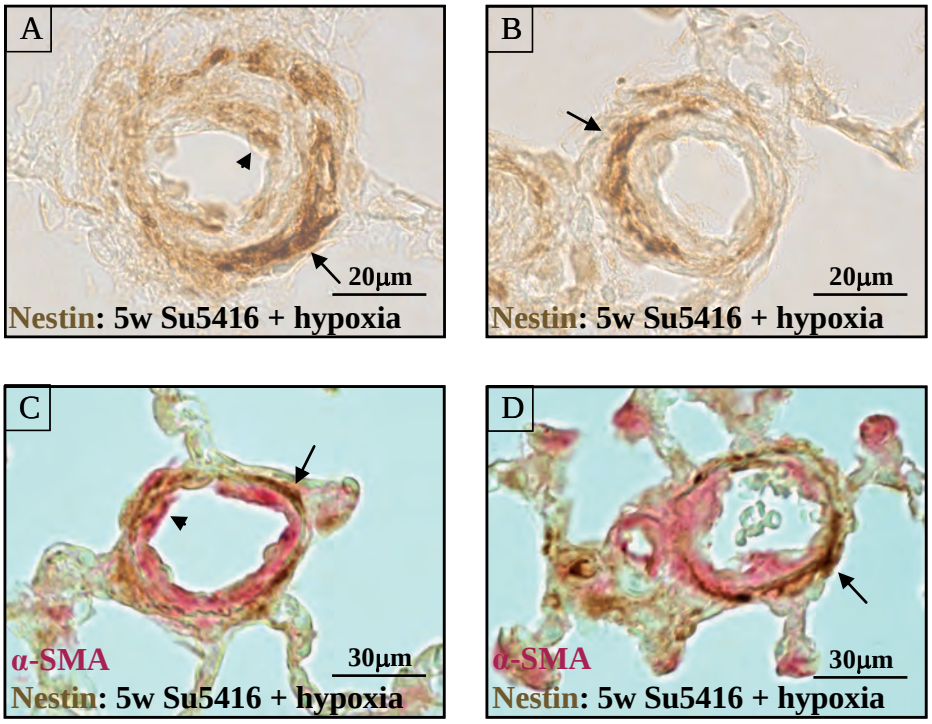


Fig. 17: Nestin expression in very small arteries (ca. 30-50 µm diameter) of rats treated with Sugen5416 and hypoxia. **A-B:** In addition to VSMCs (arrowheads), nestin expression was found outside the media in these very small vessels. **C-D:** Double staining of such vessels with α-smooth muscle actin (red) and nestin (brown) confirming that nestin is present within (arrowhead) and outside the media (arrows).

In small arteries (ca. 70-80µm diameter), different phenotypes of thickened intima could be observed and interestingly, in all such arteries nestin was expressed in vascular smooth muscle cells (Fig. 18A-H). While analyzing different phenotypes of arteries with thickened layer of intima it can be assumed that remodeling of vessels is initiated by accumulation of extracellular matrix in the vessel wall resulting in two layers of vascular smooth muscle cells, an inner thin layer and an outer media layer (Fig. 18A, C). The accumulation of extracellular material seems to be followed by infiltration or migration of nestin$^+$ vascular smooth muscle cells into the extracellular matrix since single nestin$^+$ (also α-SMA$^+$) vascular smooth muscle cells were visible in the extracellular matrix (Fig. 18E-H). In addition, reduction of lumen was also visible. Both processes might contribute to formation of neointima, a characteristic feature of this animal model.

In large vessels (>200 µm diameter), similar to the MCT treated rat samples, nestin immunostaining was found in adventitial cells close to media (Fig. 19A-D). Double staining with nestin and α-smooth muscle actin again confirmed the presence of nestin$^+$ cells outside the media at the border of media and adventitia (Fig. 19C-D).

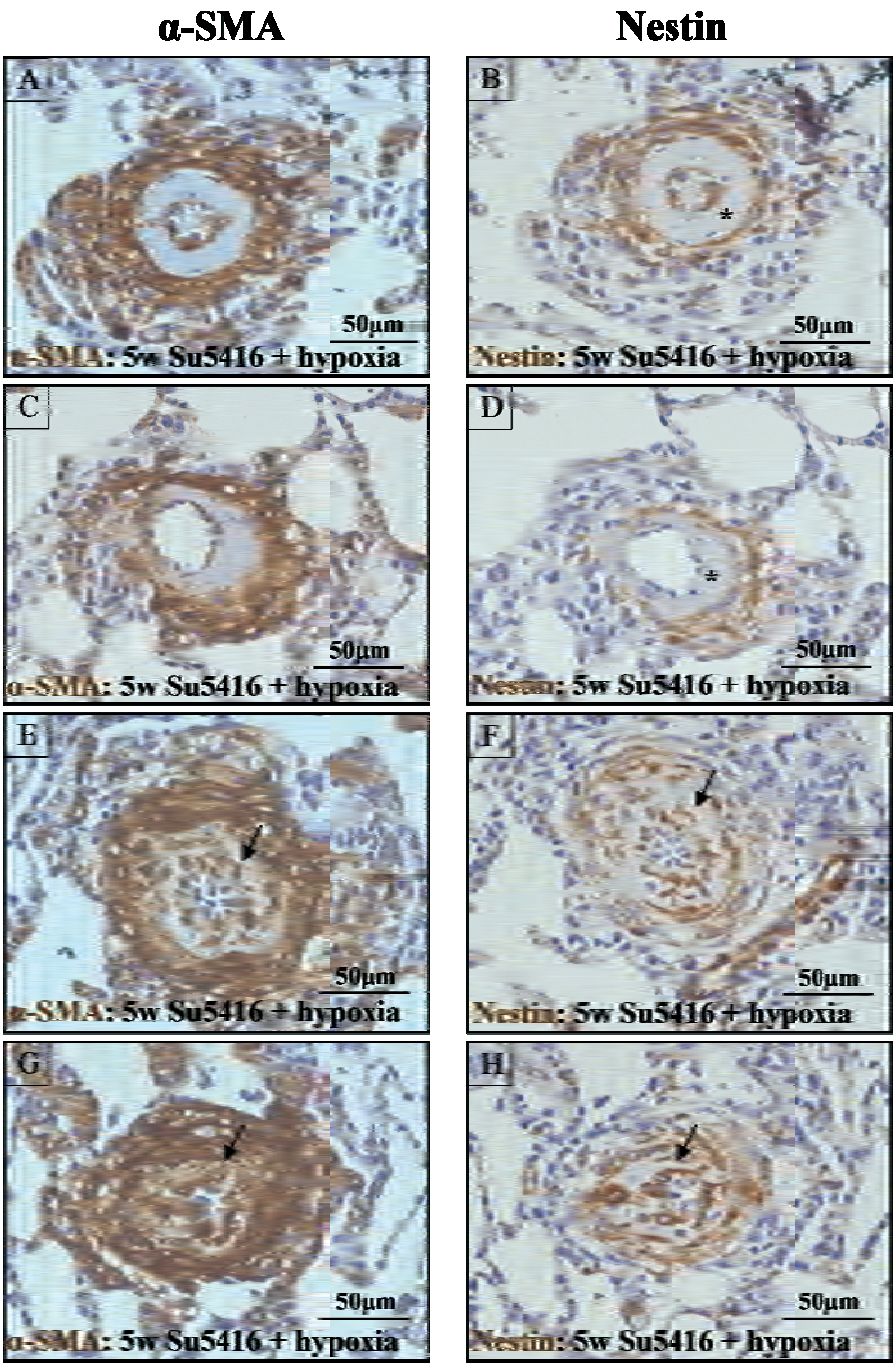


Fig. 18: Different patterns of nestin expression in VSMCs of small arteries (ca. 70-80 µm diameter) suggesting involvement of nestin$^+$ VSMCs in the development of neointima in rats treated with Sugen5416 and hypoxia. **A-H:** α-SMA (A, C, E, G) and nestin (B, D, F, H) expression in serial sections of small arteries indicating colocalization of nestin and α-SMA. **A-D:** Accumulation of extracellular material (asterisks) in media resulting in two layers of VSMCs. **E-H:** Vessels suggesting inward infiltration of nestin$^+$ VSMCs (arrows), resulting in the reduction of lumen.

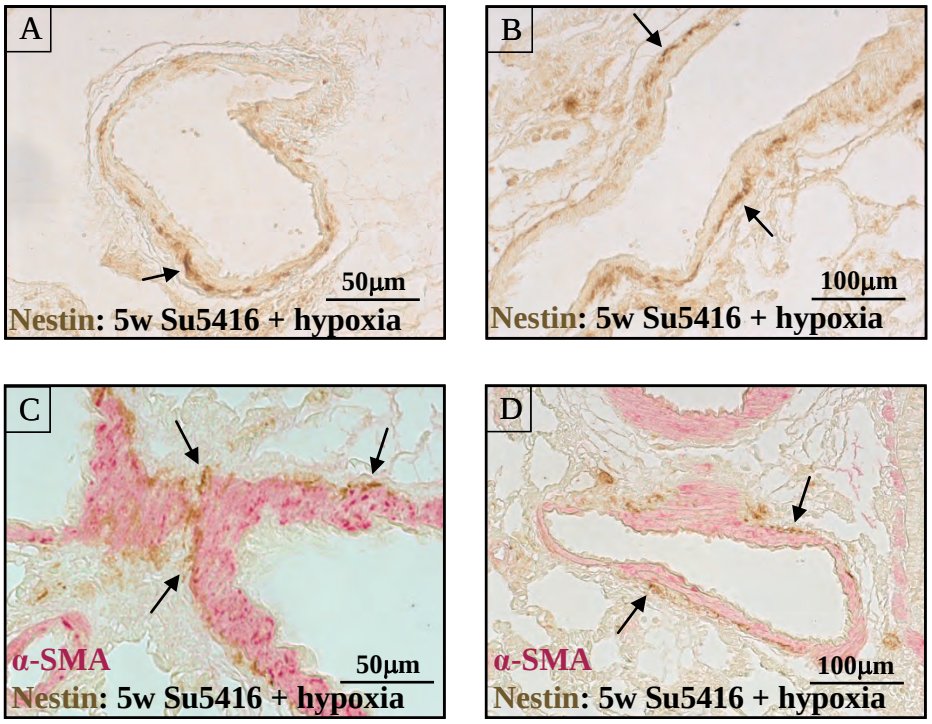


Fig. 19: Nestin expression in large arteries (>200 µm diameter) of rats treated with Sugen5416 and hypoxia. **A, B:** In larger vessels, nestin immunoreactivity in adventitial cells at the junction of media and adventitia (arrows). **C-D:** Double staining showing nestin (brown, arrows) outside the media.

As compared to large arteries of hypoxia/Su5416 rat model which show nestin expression in the small, so-called “vasculogenic zone” between media and adventitia (Fig. 19A-D) which is barely visible in lower magnification (Fig. 20A-B), the large veins show nestin expression in the whole media which is clearly visible even at lower magnification (Fig. 20A-D). Interestingly, these nestin^{+} cells in veins represent cardiomyocytes that can be easily identified in azan stained histological sections (Fig. 20E-H). The presence of cardiomyocytes in large pulmonary veins of rodents has been described before (Josef et. al. 2008). In hypoxia/Su5416 model, as shown before during development, in hypoxic mouse model (Fig. 11) and in MCT rat model (Fig. 14), smooth muscle layer around bronchi and bronchioli do not show any staining for nestin.

Different to hypoxic mouse model, the data from rat models suggests that nestin marks the progenitor cells at different locations within the vessel.

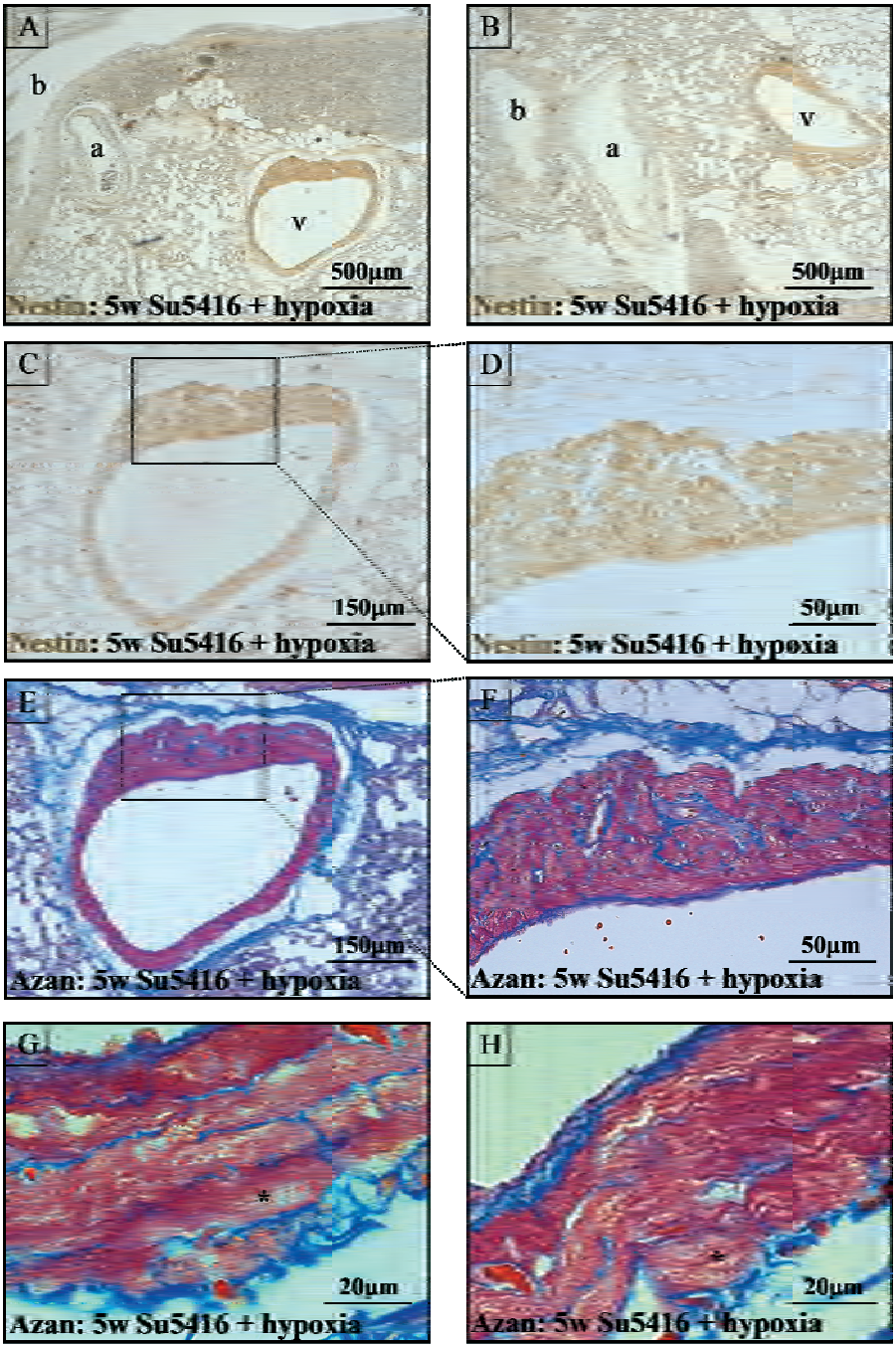


Fig. 20: Nestin expression in large veins (>500 µm diameter) of rats treated with Sugen5416 and hypoxia. **A-D:** Nestin immunostaining in cardiomyocytes present in these large pulmonary veins (a, artery; b, bronchiole; v, vein). **E-H:** Azan staining showing cardiomyocytes in wall of pulmonary veins. **G-H:** Higher magnification showing striated cardiomyocytes (asterisks) in vein.

3.5 Nestin in human samples from patients suffering from pulmonary hypertension

Using immunohistochemistry, nestin expression was investigated in lung samples from patients suffering from pulmonary hypertension. As compared to healthy control lungs, high nestin expression was observed in pulmonary vessels (Fig. 21-22). Nestin expression was observed in vascular smooth muscle cells of very small (ca. 10-20 µm diameter) and small (ca. 50-100 µm diameter) vessels (Fig. 21A-E). Double staining with nestin and α-smooth muscle actin confirmed the vascular smooth muscle cell localization of nestin in small vessels (Fig. 21E). In large arteries (>200 µm diameter), similar to the rat models, in addition to vascular smooth muscle cells there was also nestin expression in adventitial cells in so-called "vasculogenic zone" at the border of media and adventitia (Fig. 21G-H).

Pulmonary hypertension in humans is characterized by specific vascular lesions e.g. concentric lesions and plexiform lesions. Nestin was also found in both concentric (Fig. 22A) and plexiform (Fig. 22B) types of vascular lesions as well as in vessels undergoing recanalization at the site of thrombosis (Fig. 22C) suggesting potential role of nestin$^+$ progenitor cells in development of these lesions.

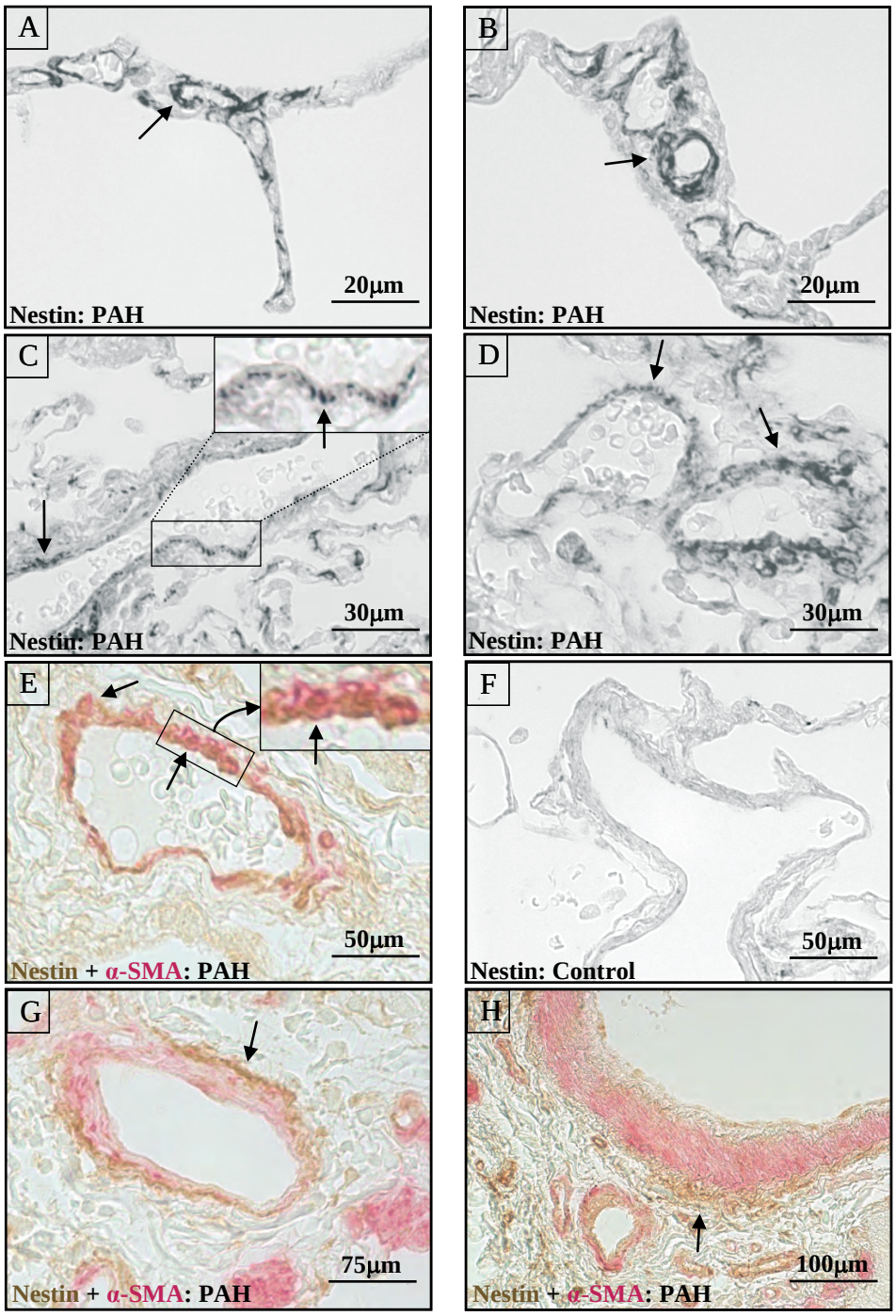


Fig. 21: Nestin immunohistochemistry in very small (ca. 10-20 µm diameter, A-B), small (ca. 50-100 µm diameter, C-F) and large (>200 µm diameter, G-H) arteries of human lung samples from patients suffering from pulmonary hypertension (A-E, G-H) and from healthy control lungs (F). **A-B:** Nestin expression in VSMCs (arrows) of very small lung vessels. **C-E:** Nestin expression in VSMCs (arrows) of small vessels. Double staining and colocalization of nestin (brown) and α-SMA (red) showing nestin in VSMCs (E, arrows). **F:** Section from healthy lung samples showing no nestin immunoreactivity. **G-H:** Double staining with nestin and α-SMA showing nestin in adventitial cells of large vessels outside the media.

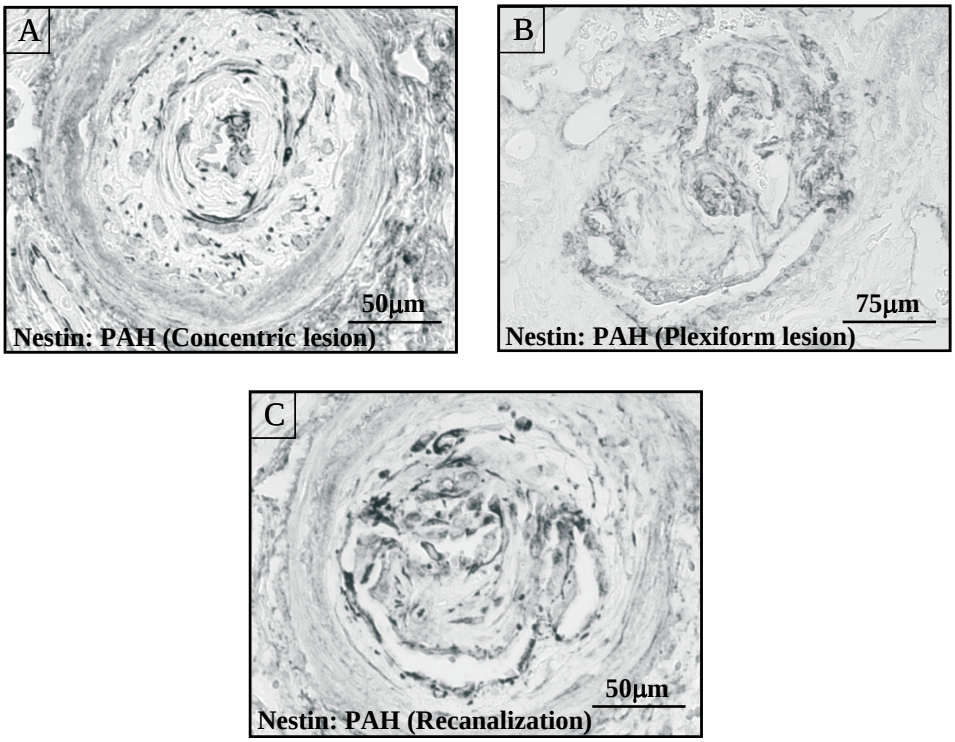


Fig. 22: Nestin immunohistochemistry in complex lesions of arteries in case of progressive remodeling of human lungs from patients suffering from pulmonary hypertension. **A**: Nestin expression in a typical concentric lesion found in pulmonary hypertension. **B**: Nestin expression observed in a plexiform lesion of pulmonary hypertension. **C**: Nestin expression found at the site of recanalization of an artery after thrombosis.

3.6 Nestin in human samples from patients suffering from pulmonary fibrosis

As pulmonary fibrosis is also characterized by vascular changes (Parra et al, 2005; Farkas et al, 2011), nestin immunohistochemistry was also performed in lung samples from patients suffering from pulmonary fibrosis. Enhanced expression of nestin was found in vascular smooth muscle cells of large (>200 µm diameter) (Fig. 23A) and small (<150 µm diameter) vessels (Fig. 23C-H) as well as in some capillaries (Fig. 23D marked by arrowheads). In control samples from healthy persons, there was no nestin expression (Fig 23I-J).

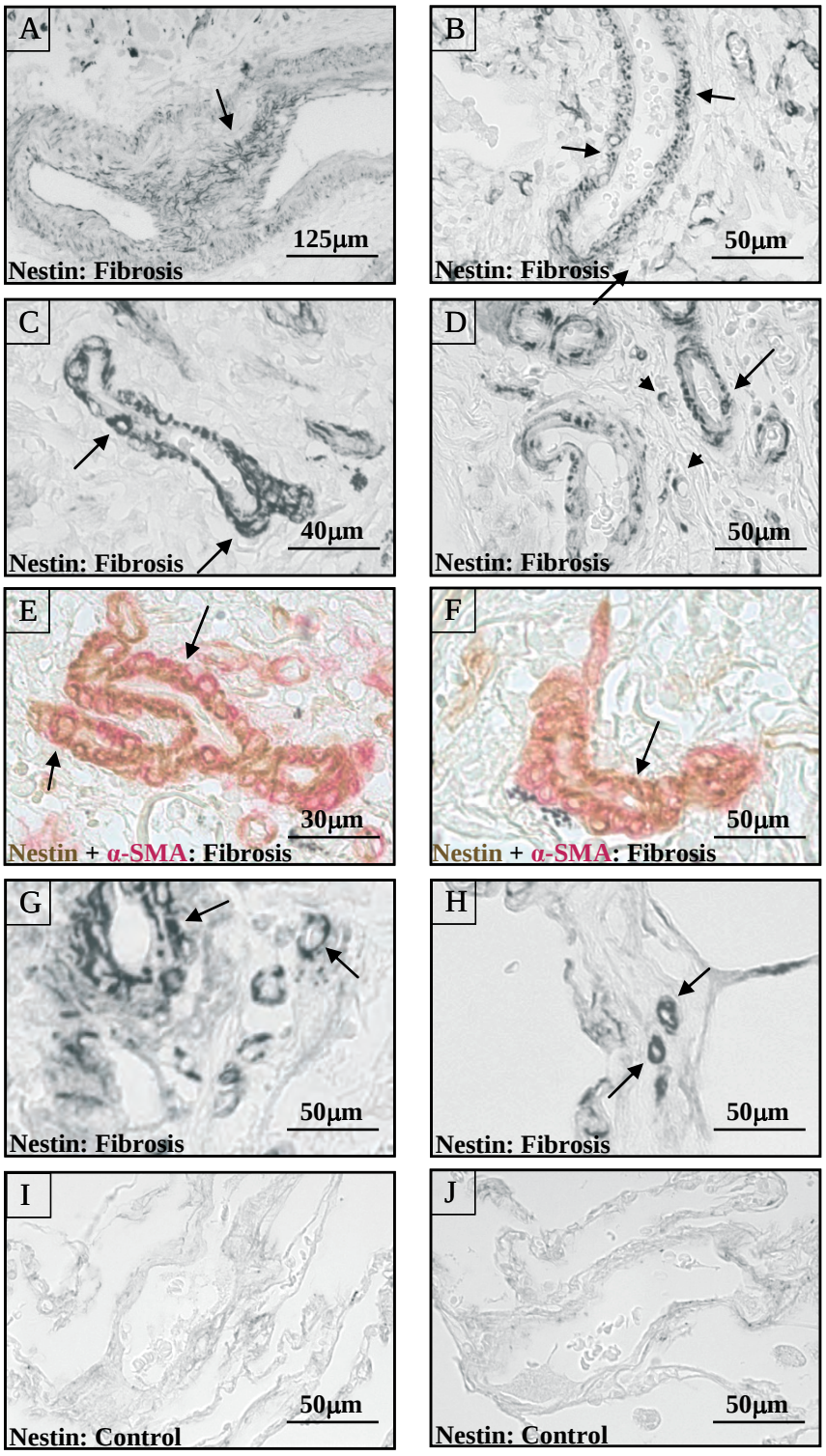


Fig. 23: Nestin immunohistochemistry in human lung samples from patients suffering from pulmonary fibrosis (A-H) and from healthy control lungs (I-J). **A**: Nestin expression in VSMCs (arrows) of large arteries (>200 µm diameter). **B-H**: Nestin^{+} VSMCs (arrows) in small arteries (<150 µm diameter). **E-F**: Colocalization of nestin (brown) and α-SMA (red) confirming nestin in VSMCs (arrows). **I-J**: Sections from healthy lung samples showing no nestin immunoreactivity.

4 Discussion

In this study it is shown that nestin expression and vascular smooth muscle cell proliferation increased in early postnatal development. Nestin was found in vasculature of lungs from different animal models (hypoxic mouse model, MCT and Su5416/hypoxia rat models) and human samples. In hypoxic mouse model, nestin was exclusively found in vascular smooth muscle cells. These nestin$^+$ vascular smooth muscle cells were found to proliferate from 3 days to 1 week of hypoxic exposure and nestin expression and vascular smooth muscle cell proliferation reduced to normoxic level after 3 weeks under hypoxia. In MCT and Su5416/hypoxia treated rats and human samples from patients suffering from pulmonary hypertension, there was enhanced nestin expression in vascular smooth muscle cells as compared to controls. In rat models and human samples, there was additional nestin expression in certain cells of adventitia i.e. in the so called vasculogenic zone which is also known to harbor stem/progenitor cells. Nestin$^+$ progenitor cells may play significant role in vascular changes both during postnatal development and progression of diseases like pulmonary hypertension.

We were able to demonstrate nestin expression and localization in vasculature of lungs during development as well as in vascular remodeling in adults using different animal models and human samples. Nestin, that was initially identified as a marker of neural stem and progenitor cells (Lendahl et al., 1990), is expressed in various cell types in embryonic and adult tissues. Nestin expression is associated with stem/progenitor cell populations with capability to proliferate, migrate and differentiate into different cell types (see Wiese et al., 2004). There are only few studies showing nestin expression in lung. (Amoh et al., 2005; Amoh et al., 2006).

For the first time, we were able to show nestin localization predominantly in vascular smooth muscle cells, both during development and vascular remodeling. In this context, there are only few studies that describe nestin expression in vascular smooth muscle cells *in vivo* or *in vitro* but no data showing nestin localization in vascular smooth muscle cells of lung. Nestin$^+$ stem/progenitor cells were suggested to play a role in development of atherosclerosis as nestin expression was found in smooth muscle cells

of coronary atherosclerotic lesions (Suguta et al., 2007). Progenitor cells of testosterone producing Leydig cells were revealed by nestin expression in vascular smooth muscle cells and pericytes of testicular blood vessels (Davidoff et al., 2004). Furthermore, a recent study described nestin expression in medial vascular smooth muscle cells of rat aorta from late embryogenesis to adulthood (Oikawa et al., 2010) showing transient nestin expression in vascular smooth muscle cells which decreases after birth in region dependent manner. The *in vitro* studies showed that nestin expression in cultured vascular smooth muscle cells is induced by serum and is regulated by extracellular signal-regulated kinase (ERK) pathway mediated through PDGF and EGF receptors (Huang et al., 2008; Huang et al., 2009; Oikawa et al., 2010).

4.1 Postnatal development

Birth itself represents a drastic change in the environment of lung with lung liquid being replaced by air. Postnatal development of lung is also characterized by dramatic changes in lung structure.

In this context, we found significant differences in nestin expression and vascular smooth muscle cell proliferation between postnatal day 1 and adult. At birth, human lungs are immature with conductive airways and peripheral respiratory airspaces. The air spaces at this stage are of classical saccular type. The walls of these air spaces are made of thick septa with a central layer of connective tissue sandwiched between two layers of capillary networks. At this stage the capillaries face the airspace from only one side. On the other hand, the adult setup is entirely different. The inter-alveolar wall is accompanied by only a single capillary network exposed to airspaces on both sides of alveolar wall as it meanders through the axial tissue framework (Zeltner and Burri, 1987). The pre-acinar arteries are already present in the lungs of 20 week old fetus (Hislop and Reid, 1972) while intra-acinar arteries appear during later embryonic stages and postnatal period, side by side with the formation of alveolar duct and alveoli (Haworth, 1995). After birth, the size and number of these arteries increases rapidly during early post natal development at the same rate as number of alveoli. Simultaneously, the thickness of pulmonary arteries especially the media decreases by means of reduction in overlap of vascular smooth muscle cells without any decrease in

number of vascular smooth muscle cells hence reducing the mean diameter of media (Hislop and Reid, 1973; Allen and Haworth, 1988).

While studying nestin expression and VSMC proliferation in a series of samples from postnatal development, we found highest numbers of nestin expressing vascular smooth muscle cells, which were able to proliferate, between day 3 and day 10 after birth suggesting strong correlation between nestin expression and proliferation. This data is in agreement with previous findings (Oikawa et al., 2010) where during postnatal development in rats, nestin expression was also found in vascular smooth muscle cells residing in media of aortic arch and abdominal aorta 6 weeks after birth. Interestingly, these nestin$^+$ smooth muscle cells were found to be proliferating as marked by BrdU. Moreover, the vasculature of mouse trachea, which is frequently used as model to study airway vasculature (McDonald, 1994; Baluk et al., 2005), undergoes rapid reorganization and changes pattern after birth. Tracheal blood vessels regress from postnatal day 1 to day 3 and new vessels appear between day 3 and day 7. Our observation of increase in nestin expression and proliferation of vascular smooth muscle cells between postnatal day 3 and day 10 is consistent with increased number of vascular sprouts and pericytes during remodeling of tracheal vasculature (Ni et al., 2010). Mice are born with immature lungs at saccular stage. The alveolar stage starts at around postnatal day 4 and alveolarization completes at around postnatal day 14 (Amy et al., 1977). The increased proliferation of nestin expressing VSMCs can be related to remodeling of existing vasculature, formation of alveoli and accompanying capillary network during alveolar stage after birth.

Here it can be speculated that nestin$^+$ vascular smooth muscle cells my also contribute to development of bronchial tree and formation of alveoli as a potential cellular source. This speculation is supported by previous findings of differentiation of nestin$^+$ vascular smooth muscle cells and pericytes into Leydig cells (Davidoff et al., 2004) and differentiation of pericytes into skeletal muscles (Dellavalle et al., 2007).

Our preliminary data shows an increase in polysialated NCAM in vascular smooth muscle cells of lungs from postnatal day 7 to day 15 that appeared after the up-regulation of nestin indicating growth of vascular smooth muscle cells. In agreement, during development and maturation of neurons, PSA-NCAM is transiently expressed

after nestin (Seki and Arai, 1993; von Bohlen, 2007). Polysialated NCAM may contribute to the molecular mechanisms inducing cell proliferation and migration not only during development but also in the adult (Bonfanti and Theodosis, 1994; Lledo et al., 2006).

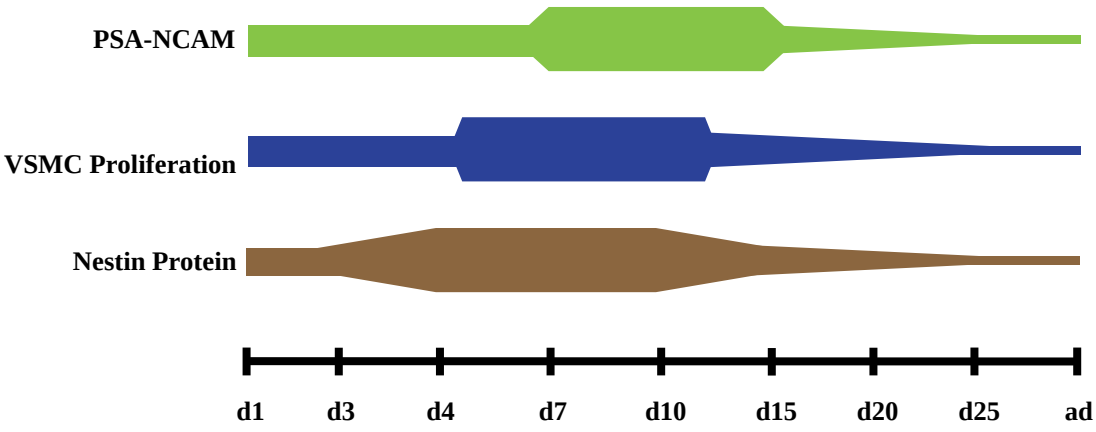


Fig. 24: Timeline of lung postnatal development showing overview of changes in nestin expression (brown), vascular smooth muscle cell proliferation (blue) and PSA-NCAM (Green).

4.2 Pulmonary hypertension

Pulmonary hypertension is characterized by structural changes in pulmonary arteries (Durmowicz and Stenmark, 1999; Mandegar et al., 2004). We used three frequently used animal models (Hypoxic mouse model, MCT and Su5416/hypoxia rat models) and human samples from patients suffering from pulmonary hypertension and pulmonary fibrosis to investigate nestin expression in vascular remodeling during progression of pulmonary hypertension. In all animal models as well as in human samples, we were able to demonstrate an increase in nestin expression in vasculature of lung.

4.2.1 Hypoxic mouse model

In the hypoxic mouse model, we found up-regulation of nestin mRNA expression after 2 days of hypoxic exposure while peak of nestin protein was found one day later after 3 days of hypoxic exposure which remained up-regulated till 1 week and came to normal after 3 weeks of hypoxic exposure. In mice, hypoxia induced pulmonary hypertension

develops in 3 weeks which can be clearly identified histologically and clinically i.e. peak of nestin expression precedes pulmonary hypertension.

Analyzing the hypoxic samples for VSMC proliferation, we found increased number of proliferating vascular smooth muscle cells during the same time period where we found increased nestin levels i.e. at 3 days and 4 days after hypoxia that decreased towards 1 week and was absent in 3 week hypoxic samples. In previous studies, while analyzing 2 days, 4 days, 6 days, 10 days, 16 days and 3 weeks hypoxic lungs, Paddenberg and colleagues described hypoxia induced proliferation of VSMCs within first week of hypoxic exposure (Paddenberg et al., 2007). In another study, where only 2 days, 4 days, 6 days and 3 weeks hypoxic samples were analyzed, enhanced VSMC proliferation was observed in 4 days and 6 days hypoxic samples with no proliferation at 3 weeks after hypoxic treatment (Quinlan et al., 2000).

It is of interest that all the vascular smooth muscle cells were not $Nestin^{+}$. It was only a subpopulation of vascular smooth muscle cells which was $Nestin^{+}$ and able to proliferate as marked by a labeling of Nestin and proliferation marker Ki67. This data is consistent with data of nestin in testicular blood vessels where only a sub-population of vascular smooth muscle cells was $nestin^{+}$ and proliferating (Davidoff et al., 2004).

In this context it is noteworthy that the tunica media of pulmonary arteries is composed of different populations of vascular smooth muscle cells (Frid et al., 1994; Yoshida and Owens, 2005). Depending upon the morphology and expression of molecular markers, these different populations of vascular smooth muscle cells can be divided into “contractile” and “synthetic” types. Contractile vascular smooth muscle cells can be characterized by typical spindle shaped nuclei and elongated cell phenotype. They express molecular markers like smooth muscle myosin heavy chain (SM-MHC), calponin and caldesmon (Owens, 1995; Sobue et al., 1999). Synthetic type vascular smooth muscle cells are cuboidal shaped cells which lack contractile markers and express non-muscular myosin heavy chain A and B (Kuro et al., 1991; Stenmark and Frid, 1998). We were able to differentiate between two distinct subpopulations of smooth muscle cells in vasculature using calponin as marker of contractile cells. One contractile phenotype with strong calponin immunoreactivity and the other phenotype with down-regulated calponin expression suggesting change of phenotype in a

subpopulation of vascular smooth muscle cells during enhanced proliferative activity. One of the most interesting features of smooth muscle cells is that they are not terminally differentiated in mature vascular tissue, allowing modulation of their phenotype under certain conditions. SMC dedifferentiation and change of phenotype is suggested to be an important aspect of vascular remodeling (Owens et al., 2004; Muto et al., 2007). Only few factors are identified that are implicated in smooth muscle cells' phenotype switching and PDGF-BB (see below) is one of them (Kawai and Owens, 2006; Muto et al., 2007).

Interestingly, the smooth muscle cell layer of the airways, which did not express nestin, comprised only calponin^{+} smooth muscle cell population. The presence of nestin only in vascular smooth muscle cells but not in airway smooth muscle cells also support the hypothesis of occurrence of two different phenotypes of vascular smooth muscle cells. In agreement, nestin was only expressed in vascular smooth muscle cells with no nestin in peritubular contractile cells in testis (Davidoff et al., 2004).

By demonstrating nestin expression in a subpopulation of vascular smooth muscle cells, we propose nestin as marker of synthetic type of vascular smooth muscle cells that are thought to proliferate and play important role in progression of vascular diseases like pulmonary hypertension (Stenmark and Frid, 1998).

It is worth mentioning that in our hypoxic mouse model, nestin expression and cell proliferation could only be observed in vascular smooth muscle cells while endothelial cells were negative for nestin and did not show any proliferation. In agreement to our findings, it is described in a recent study that there is no increase in endothelial cell proliferation in hypoxia induced rat or mouse models of pulmonary hypertension (Yu and Hales, 2010). In addition, human pulmonary artery endothelial cells do not show any significant cell proliferation and cell cycle progression under hypoxia in vitro (Yu and Hales, 2010). Moreover, nestin expression is described in proliferating endothelial cells during tumor angiogenesis (Sugawara et al., 2002; Mokry et al., 2004) including lungs (Amoh et al., 2005; Amoh et al., 2006) but according to the magnification shown in above mentioned publications it is only speculative that nestin derived green fluorescent protein is really present in capillary endothelial cells. It may also be present in the pericytes as the pericytes and endothelial cells are covered by the same basement

membrane (Diaz-Flores et al., 2009) and it is extremely difficult to differentiate between them using normal microscopy unless you use electron microscopy or specific markers. Instead, nestin has been found preferentially in pericytes and vascular smooth muscle cells of tumor vessels (Friedrich et al., 2012).

Several growth factors and signaling pathways are known to be involved in progression of pulmonary hypertension including VEGF, FGF, TGF-β, PDGF (Schermuly et al., 2011). We were able to demonstrate a slight increase in expression of PDGFR-β during progression of pulmonary hypertension while the expression of phosphorylated (activated) PDGFR-β showed a peak between 4 days and one week after hypoxia which precede up-regulation of nestin expression and the onset of vascular smooth muscle cell proliferation under hypoxia, hence, confirming involvement of PDGF-BB/ PDGFR-β signaling in progression of hypoxia induced pulmonary hypertension. In support of our observation, under hypoxic conditions *in vitro*, arterial smooth muscle cells showed higher PDGFR-β expression and proliferation (Chanakira et al., 2012). PDGF promotes the proliferation and migration of vascular smooth muscle cells and PDGF-BB/PDGFR-β signaling is known to be involved in progression of pulmonary hypertension (Schermuly et al., 2005; Perros et al., 2008; Li et al., 2010; Bhola et al., 2011). PDGF-BB is reported to stimulate the proliferation of vascular smooth muscle cells during vascular remodeling through a HIF-1 dependent mechanism (Abedi and Zachary, 1995; Muto et al., 2007) PDGF inhibitors result in reversal of pulmonary vascular remodeling/ pulmonary hypertension (Schermuly et al., 2005).

PDGFR-β has also been described in vascular progenitor cells of tissues other than pulmonary vasculature. Newly generated Leydig cells as well as their progenitors (vascular smooth muscle cells and pericytes) showed PDGFR-β expression (Davidoff et al., 2004). PDGFR-β is also used as meanwhile established marker of pericytes (Bergers and Song, 2005) and circulating smooth muscle progenitor cells (Sugiyama et al., 2006). The thickening of arteries and muscularization of previously non-muscular arteries were suggested to be due to differentiation of precursor pericytes and “intermediate cells” (Meyrick and Ried, 1980) Intermediate cells were defined as intermediates between pericytes and smooth muscle cells.

There is increasing evidence that pericytes represent or are at least closely related to mesenchymal stem cells (Crisan et al., 2008). These cells were shown to be capable of renewing a variety of cell types (Crisan et al., 2008; Kovacic et al, 2009) such as Leydig cells (Davidoff et al., 2004), skeletal muscle cells (Dellavalle et al., 2007) osteoblasts, chondrocytes and adipocytes (see Crisan et al., 2008). Most recently, even the ischemic limbs were shown to be healed by cells exhibiting the features of vascular pericytes, when using multipotent progenitor cells derived from embryonic stem cells and induced pluripotent stem cell-derived regenerative units (Dar et al., 2012; Peault, 2012). Recently, we found that pericytes, in pulmonary vasculature, also express nestin (Berndt, 2011).

Apart from a role in vascular smooth muscle cell proliferation and migration PDGF receptors are also involved in early anti-apoptotic responses. PDGF-B/ PDGFR-β signaling in isolated neurons, is involved in cell survival and protection from pro-apoptotic stimuli for up to 48h under hypoxia (Zhang et al., 2003). In brainstem of adult rats, hypoxia induced phosphorylation of PDGFR-β was associated with activation of anti-apoptotic mechanisms through Akt/BAD signaling pathway (Simakajornboon et al., 2010). Our unpublished results also show results in the same direction as we observed increase in PDGFR-β phosphorylation as early as 6 hours – 15 hours under hypoxia.

VEGF is up-regulated in rats with hypoxic treatment but down-regulated in MCT treated rats (Partovian et al., 1998). Hypoxia, in combination with VEGF receptor inhibition using VEGF receptor antagonist Su5416, results in severe pulmonary hypertension (Taraseviciene-Stewart et al., 2001), a model for experimental pulmonary hypertension that we also used in this study (see 1.2.3).

Thioredoxin (Trx) is a ubiquitously expressed protein that is important for maintaining the oxidative balance within the cell. There are two isoforms of theoredoxin, Trx-1 and Trx-2 (Collet, 2010). Trx-1 levels in hypoxic lung samples also showed characteristic pattern similar to the expression patterns of nestin expression and vascular smooth muscle cells proliferation (Data not shown) suggesting that redox signaling may also be involved in hypoxia induced vascular remodeling. Trx-1 protein expression was recently described to be necessary for hypoxia induced proliferation of pulmonary

artery smooth muscle cells (Nelin, 2011). Trx-1 is known to enhance neovascularization in chronic myocardial infarction (Adluri et al., 2011). Increased thioredoxin levels at the time of increased nestin expression and vascular smooth muscle cell proliferation could be associated with vascular remodeling. It is thinkable that under conditions of reduced oxygen concentration, Trx-1 and nestin might be up-regulated via the same hypoxia induced transcriptional control.

Summarizing the data from hypoxic mouse model, the timeline of events taking place in this model are as follows. Nestin mRNA shows peak after 2 days under hypoxia. Nestin protein up-regulation occurs at 3 days after hypoxic exposure followed by increase in vascular smooth muscle cell proliferation and in the end phosphorylation of PDGFR-β after 4 days – 1 week of hypoxic treatment. Expression of all the proteins and proliferation of vascular smooth muscle cells comes to normoxic level before week 3 of hypoxic exposure when the complete histological characteristic and the distinct clinical symptoms are visible.

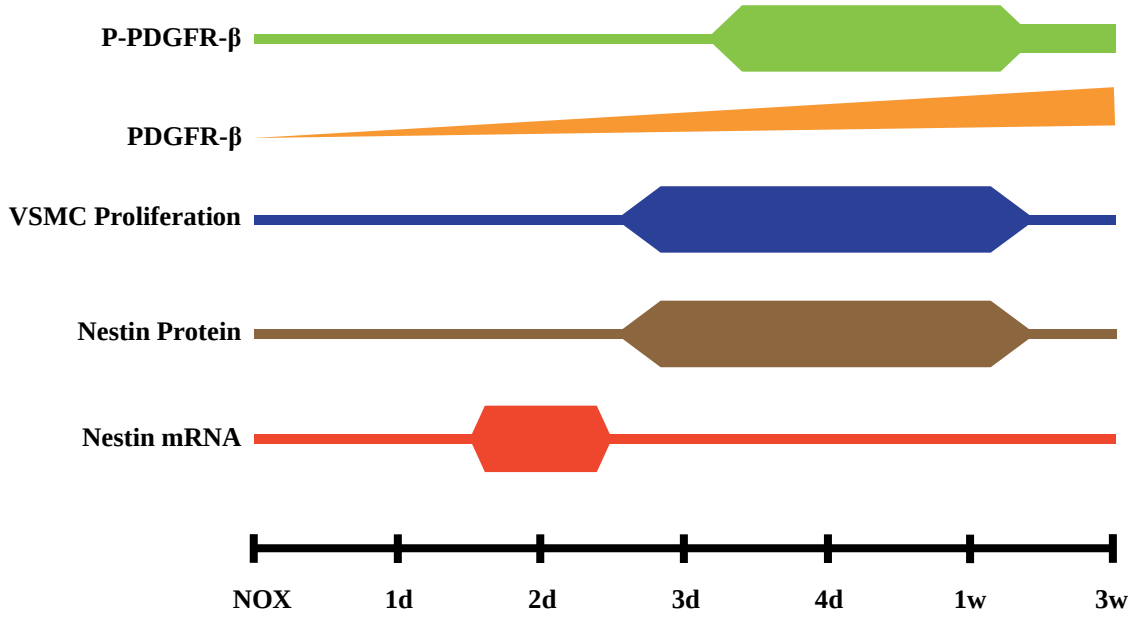


Fig. 25: Timeline of experimental pulmonary hypertension (mice under hypoxia) showing overview of changes in nestin mRNA (red) nestin protein (brown), PDGFR-β (orange) and p-PDGFR-β (green) expression and vascular smooth muscle cell proliferation (Blue).

4.2.2 MCT rat model

Monocrotaline treated rats are widely used as model for experimental pulmonary hypertension (Stenmark et al., 2009; Gomez-Arroyo et al., 2012). Increased nestin expression was also observed in vascular smooth muscle cells of monocrotaline treated rat lung samples. In addition to vascular smooth muscle cells, nestin^{+} cells were also observed in the adventitia of pulmonary vessels. Vascular adventitia has been designated as a niche of potential progenitor cells that contribute to pathogenesis of pulmonary hypertension by proliferating and migrating to intima or media (Hinz et al., 2007). In recent years, it has been shown that resident stem and progenitor cells of large and medium sized arteries are predominantly localized in a specific zone at the border of media and adventitia. This zone is named as "vasculogenic zone" (Ergun et al., 2011). Our observation of nestin in vascular adventitia may represent nestin in stem/progenitor cells in this so-called vasculogenic zone.

In MCT rat model, we found nestin in vascular tree in a time dependent manner i.e. in smaller vessels after 3 weeks and 4 weeks of MCT treatment and in large vessels after 5 weeks of MCT treatment. It is known that vascular remodeling during pathogenesis of pulmonary hypertension starts in smaller vessels and spreads through the vascular tree into large vessels. Muscularization of small previously non-muscular arteries starts as early as 2 days under hypoxia (Quinlan et al., 2000)

While comparing nestin expression in *A. pulmonalis* and aorta of MCT treated and control rats, we found that, nestin up-regulation was found only in *A. pulmonalis* while there was no difference in nestin expression in aorta of MCT treated and untreated rats that suggest that in MCT induced pulmonary hypertension, the increase of nestin in vascular smooth muscle cells during proliferation may be specific for the pulmonary circulation with no effect in systemic vasculature. This particular effect may be due to the reason that MCT is activated in the liver and pulmonary vasculature represents the first major vascular bed distal to liver (Marsboom and Janssens, 2004) and the activated metabolic product of MCT, "MCT pyrrole" is quickly degraded in aqueous solutions like plasma (Wilson et al., 1992).

It is known that along with vascular remodeling, the application of MCT also activates other processes e.g. peri-vascular inflammation and platelet activation (Wilson et al., 1992). These additional MCT effects might coincide with an increase of degradation of certain cytoplasmic proteins such as GSK-3 (Müller, personal communication) and nestin. It is thinkable that in this model, the variety of nestin immunoreactive signals (nestin degradation products) in Western blot is also responsible for immunostaining of further cell types other than vascular smooth muscle cells e.g. cells of adventitia.

4.2.3 Su5416/Hypoxia model

Hypoxia in combination with VEGF receptor antagonist, Sugen 5416, which results in formation of characteristic lesions of pulmonary hypertension, is also used as animal model to study vascular remodeling (Taraseviciene-Stewart et al., 2001; Sakao et al., 2005; Abe et al., 2010). In this model, after 5 weeks of Su5416 injection and hypoxic treatment we were able to demonstrate nestin expression in both arteries and veins. Similar to the monocrotaline rat model, nestin was observed in vascular smooth muscle cells of the media of arteries. This animal model resembles the traditional monocrotaline model of pulmonary hypertension with prominent perivascular inflammatory infiltrates (Stenmark et al., 2009). In the Su5416/hypoxia model, however, there are additional characteristics in nestin localization depending on the size of vessels and the degree of remodeling.

One of the characteristic features of hypoxia induced pulmonary hypertension is muscularization of previously nonmuscular arteries (Jones et al., 2008; Rabinovitch, 2008; Stenmark et al., 2009). In very small arteries (ca. 30-50 µm diameter) of the Su5416/hypoxia model, which could derive from such non muscular arteries, nestin was observed in a perivascular pattern outside the media. These nestin^{+} cells might represent special perivascular cells that are suggested to increase during development of pulmonary hypertension and contribute to neo-muscularization of small arteries (Sluiter et al., 2012).

In certain small arteries (ca. 70-80µm diameter), where nestin was found in vascular smooth muscle cells, it can be suggested that extracellular matrix accumulates in vessel wall resulting in two layers of vascular smooth muscle cells, one inner thin layer close

to lumen and another outer layer of media smooth muscle cells. This observation is consistent with previous findings in fibrosis of lamina propria in testes, regularly consisting of multiple layers, where accumulation of extracellular material also resulted in two layers of contractile cells (Volkmann et al., 2011).

The accumulation of extracellular material seems to be the first step in remodeling of these vessels followed by infiltration or migration of nestin$^+$ vascular smooth muscle cells into the extracellular matrix that might contribute to formation of neointima. In agreement, it is reported that smooth muscle cells present in neointimal lesions are locally derived from the vessel wall and not from bone marrow cells (Bentzon et al., 2007; Hagensen et al., 2010). De-differentiated smooth muscle cells that are found to be highly proliferative, migrate toward the lumen of the vessel (from the tunica media into the tunica intima), thus resulting in the development of a neointimal lesion (Owens et al., 2004; Muto et al., 2007). The proposed hypothesis of extracellular material accumulation followed by inward migration of vascular smooth muscle cells is only based upon observation of different phenotypes of arteries. Detailed analysis, however, of formation of such lesions at different time points of Su5416/hypoxia treatment is still to be performed.

Another interesting finding in this model was the nestin expression in large pulmonary veins. The media of these large veins was mainly comprised of nestin$^+$ cardiomyocytes. Cardiomyocytes were described in pulmonary veins of mice, rats and humans (Mueller-Hoecker et al., 2008). Nestin has also been reported in cardiomyocytes (Scobioala et al., 2008; Mokry et al., 2008). Nestin$^+$ cardiomyocyte-like cells with striated pattern were also shown to reside in adult heart that contributes in healing the infarcted heart (El-Helou et al., 2005). In Su5416/hypoxia model, we were able to analyze nestin expression only 5 weeks after Su5416 injection and hypoxic treatment and the detailed expression pattern of nestin after different time periods of Su5416 and hypoxic treatment still remains to be investigated.

Nestin expression in rats is found in multiple cell types as compared to mice that showed nestin predominantly in vascular smooth muscle cells. To support our observation, it is described that under hypoxia, mice develop less pulmonary arterial thickening than rats and the vascular remodeling is less pronounced in mice as

compared to rats (Hoshikawa et al., 2001; Hoshikawa et al., 2002). In addition, it has been demonstrated that there is heterogeneous remodeling of pulmonary arteries in hypoxic and MCT rat models in different segments of pulmonary arterial tree (van Suylen et al., 1998). Higher degree of vascular remodeling in rats and heterogeneous remodeling in different segments of pulmonary arterial tree might explain the presence of additional nestin^{+} progenitor cells in so-called "vasculogenic zone". In large vessels, vessel wall resident progenitor cells were described in a distinct zone at the border between media and adventitia (Zengin et al., 2006; Klein et al., 2010; Ergun et al., 2011) and Zengin and colleagues termed this zone as "vasculogenic zone". Further explaining the presence of progenitor cells in vasculogenic zone at the border between media and adventitia, one can think of the German term of "letzte wiese". "Letzte wiese" is the area in heart or brain which is deprived of oxygen because it is present at the border zone of blood supply from different arteries. The oxygen deprived areas develop infarcts known as borderzone infarct or watershed infarct (Mangla et al., 2011). In large vessels of rats and humans, where we found nestin^{+} cells in "vasculogenic zone", one can assume that this zone being present at the border between media and the adventitia might also be deprived of oxygen. Stronger nestin expression in progenitor cells might be induced due to hypoxic conditions in this region.

4.2.4 Human samples

Significantly increased nestin expression was observed in different cell types in human samples from patients suffering from pulmonary hypertension. Pulmonary hypertension in humans is characterized by specific lesions (Wagenvoort and Wagenvoort, 1970, Pietra et al., 2004) and most prominent are plexiform and concentric lesions. While comparing human pulmonary hypertension with animal models, these characteristic lesions are not developed in most of the animal models which is quite a draw back in investigating the pathogenesis of these structures in human pulmonary hypertension. It is of interest that in addition to nestin in vascular smooth muscle cells and in cells of "vasculogenic zone" between media and adventitia of large vessels, nestin was found in all types of lesions in currently investigated human samples. We assume nestin is expressed in smooth muscle cells and other proliferating progenitor cells present in these lesions. Concentric lesion is composed of proliferating myofibroblasts and medial smooth muscle cells resulting in narrowing of lumen (Wagenvoort and Wagenvoort,

1970). Regarding the cell components of plexiform lesion, there were different views in the past. The cell types in plexiform lesions were described as smooth muscle cells, endothelial cells or myofibroblasts (Naeye and Vennart, 1960; Wagenvoort and Wagenvoort, 1977; Smith and Heath, 1979) but later on, studies using molecular markers have addressed this concern and endothelial cells were found to be the predominant cell type along with smooth muscle cells and inflammatory cells (Tuder et al., 1994; Cool et al., 1999). The presence of nestin in plexiform lesion may represent proliferating endothelial progenitor cells involved in pathogenesis of these lesions that comes either from circulating source or the resident pool in "vasculogenic zone" as described previously (Zengin et al., 2006). Moreover, a recent study demonstrated that there was no evidence of enhanced endothelial cell proliferation in pulmonary vasculature of rat and mice under hypoxia (Yu and Hales, 2011).

Pulmonary fibrosis is known to be associated with vascular changes as patients suffering from pulmonary fibrosis also develop severe pulmonary hypertension (Pitsiou et al., 2011). In this context we found very high nestin expression in vascular smooth muscle cells of arteries in human samples from patients suffering from pulmonary fibrosis. The vascular changes start with destruction of pulmonary capillaries by fibrotic tissues (Renzoni et al., 2003) followed by neovascularization in fibrotic tissue and increase in vascularization in nonfibrotic or least fibrotic tissue (Renzoni et al., 2003; Ebina et al., 2004). Our observation of nestin in fibrotic samples may support the hypothesis that increased vascular density in least fibrotic tissues has a role in regeneration of alveolar septa that is damaged by fibrotic process (Ebina et al., 2004). On the other hand, nestin$^+$ cells in the vessels of fibrotic samples may contribute to fibrotic process serving as the source of myofibroblasts. In agreement to this assumption, vascular smooth muscle cells and pericytes were suggested to be involved in vessel repair and are suggested to contribute to fibrosis in scleroderma (Rajkumar et al., 2005; Hinz et al., 2007).

In case of human samples, we only got samples from patients that were at the end stage of disease and were considered for lung transplant. It is not possible to get human samples from different stages of disease, and due to this discrepancy, it is not possible to investigate in detail, the fate of nestin$^+$ cells during the progression of disease and we have to rely on animal models.

4.3 Nestin as marker of proliferating stem/progenitor cells

After investigating different animal models and human samples and describing nestin expression in lung vasculature, we postulate that certain resident progenitor cells could be identified in vasculature using nestin as a marker of proliferating resident progenitor cells. Nestin expression has been described in a variety of cell types in different organs during development as well as in adults (Wiese et al., 2004; Krupkova et al., 2010). Davidoff et al. demonstrated nestin as marker of proliferating vascular smooth muscle cells and pericytes that represented progenitors of Leydig cell (Davidoff et al., 2004). Hair follicle sheath cells marked by nestin (Li et al., 2003) were described as source of dermal blood vessels (Amoh et al., 2004) which play important role in survival and wound healing after skin transplant (Aki et al., 2010). Nestin expression was widely observed in newly formed blood vessels at the site of tumor angiogenesis (Chen et al., 2010; Krupkova et al., 2010; Ishiwata et al., 2011; Friedrich et al., 2012) and is suggested to be a useful marker to identify neovasculature in the cancerous tissue (Teranishi et al., 2007). Nestin$^+$ vascular wall cells were also suggested to contribute to tumorigenesis (Friedrich et al., 2012). Nestin expression in combination with proliferation markers Ki67 or PCNA in different tumors has been designated as marker for angiogenesis (Gravdal et al., 2009; Yamahatsu et al., 2012).

4.4 Nestin as prognostic or diagnostic marker

Another important finding in our hypoxic mouse model was up-regulation of nestin quite early (after 3 days of hypoxic exposure) compared to histological and clinical features that appear after 3 weeks of hypoxic treatment. This observation suggests that nestin expression might be used as a prognostic or diagnostic marker to deal with certain diseases like pulmonary hypertension. In support of this idea, nestin expression has been suggested as diagnostic and prognostic marker of malignancy in certain tumors (Ehrmann et al., 2005; Svachova et al., 2011; Arai et al., 2012). Nestin expression was described as prognostic indicator of poor survival probability for patients suffering from resected non-small cell lung cancer that might be used to select patients who should receive adjuvant chemotherapy (Ryuge et al., 2011).

Furthermore, due to its early appearance in the progression of disease, nestin could be a potential novel molecular target to inhibit vascular remodeling in vascular diseases like pulmonary hypertension. In agreement, nestin is suggested to be a potential therapeutic target to inhibit tumor angiogenesis (Yamahatsu et al., 2012).

4.5 Nestin$^+$ cells in potential therapeutic and healing strategies

After establishing the fact that nestin$^+$ cells are involved in tissue repair in adults, in recent years, many studies have evaluated the plasticity of nestin$^+$ cells isolated form different organs and these nestin$^+$ cells, when grown in appropriate conditions, give rise to neurons, astrocytes, oligodendrocytes, blood vessels and cardiomyocytes (see Calderone, 2012). It has been suggested that nestin$^+$ cells could be isolated to be used in cell based therapies (Birbrair et al., 2011). The possibility of nestin$^+$ cells in healing the infarcted heart has been reviewed in a recent publication (Calderone, 2012).

4.6 Function of nestin

Nestin expression has been described in a variety of cell types in different organs during development as well as in adults (Wiese et al., 2004; Krupkova et al., 2010) but its exact function and mechanisms involved in up-regulation of nestin in proliferation of progenitor cells is still unclear. One of the properties of nestin is that it cannot polymerize itself and co-polymerize with other intermediate filament proteins like vimentin, desmin and α-internexin (Sjöberg et al., 1994; Marvin et al., 1998; Eliasson et al., 1999). Phosphorylation and dephosphorylation of nestin may modulate assembly and disassembly of further intermediate filaments (Steinert et al., 1999) and these processes may play a significant role in increased cytoplasmic trafficking in stem/progenitor cells undergoing proliferation, migration and differentiation (Lendahl et al., 1990; Kachinsky et al., 1995; Vaittenen et al., 2001). In this context, we observed that nestin is associated with proliferating progenitor cells in vascular wall; therefore, it is thinkable that nestin in progenitor cells may participate in or promote the proliferation of progenitor cells. In agreement, our preliminary data using nestin siRNA transfected pulmonary arterial VSMCs provide evidence of inhibition of VSMC proliferation in the absence of nestin in vitro. Furthermore, nestin has been proposed to promote mesangial cell proliferation in vitro suggesting a supporting role of nestin in reparative action

against injury (Daniel et al., 2008). It has also been demonstrated that nestin is important for survival and self-renewal of neural stem cells (Park et al., 2010) Nestin might also play an anti-apoptotic function via Cdk5-induced phosphorylation of BCL-2 that leads to inhibition of caspase-9 hence preventing the downstream caspase-3 activation in rat vascular smooth muscle cells (Huang et al., 2009). In podocytes, nestin was suggested to protect from apoptosis by another mechanism i.e. by maintaining the regular arrangement of actin cytoskeleton in a rat puromycin aminonucleoside (PAN) induced model of nephrosis (Wen et al., 2011). Along with determining the fate of cells as stem/progenitor cells, nestin was suggested to play an important functional role in differentiation of pancreatic stem cells into insulin secreting cells (Kim et al., 2010). This process of differentiation did not take place in the absence of nestin.

4.7 Mechanisms inducing nestin

Similar to the functional role of nestin, mechanisms inducing or stimulating nestin expression are also a matter of debate. External stimuli may trigger nestin expression in certain cell types. In this context, hypoxia can be such one of such stimuli as used by us in one of the animal models that resulted in significant nestin up-regulation after 3 days of hypoxic exposure. In line with our observation, nestin is suggested to be induced by hypoxia in cochlear stem/progenitor cells in the inner ear (Chen et al., 2011) and Müller glial cells in adult rat retina (Xue et al., 2011). Hypoxia inducible factor-1 and HIF like factor (HLF) are known to act on their consensus enhancer element 5'-CACGTG-3', it was found that at least one such candidate site was present on the first intron of nestin. It is still to be examined whether a HIF/HLF-dependent expression of nestin exists and whether this candidate site could be involved (Sugawara et al., 2002).

Similarly, extra-cellular soluble factors secreted by neighboring cells might also trigger gene expression; pulmonary artery fibroblasts were suggested to sense hypoxia and release soluble factors that stimulated pulmonary artery smooth muscle cell proliferation (Rose et al., 2002). Furthermore, proliferation of arterial smooth muscle cells could only occur when incubated with hypoxic endothelial cell conditioned medium suggesting the stimulation of vascular smooth muscle cell proliferation as a result of paracrine activity (Chanakira et al., 2012).

In conclusion, nestin expression was found in vasculature of lungs and nestin^{+} cells showed proliferation during development and vascular remodeling. Certain vascular wall cells capable of proliferation could be identified by nestin expression in lungs and may be used as prognostic and diagnostic markers and new target for therapeutic interventions of diseases like pulmonary hypertension.

5 Summary

Vascular wall cells distinguished by expression of the neuronal stem cell marker nestin may represent stem cell-like progenitor cells for tissues in various organs. In one of our previous studies, we found that nestin expressing vascular smooth muscle cells and pericytes in testicular blood vessels are the progenitors of testosterone producing Leydig cells. The aim of this study was to investigate nestin expression and nestin^{+} cells in postnatal and adult lungs.

To analyze the expression pattern of nestin and its role as marker for proliferating progenitor cells in the lung, nestin expression and localization was investigated during postnatal development and vascular remodeling in a hypoxia induced pulmonary hypertension model in mice. Nestin data was compared with expression of proliferation markers (PCNA, Ki67) and PDGF receptors. Nestin expression and localization was also investigated in two rat models of pulmonary hypertension, the monocrotaline model and the Su5416/hypoxia model (VEGF receptor-2 blockage by its antagonist Sugen 5416 in combination with hypoxia) as well as in human samples from patients suffering from pulmonary hypertension and pulmonary fibrosis.

Nestin was found in a subpopulation of vascular smooth muscle cells in lung vasculature. As compared to adults, significantly higher nestin expression was observed in pulmonary vasculature of postnatal lungs. While investigating the time course of postnatal development, peaks of nestin expression and vascular smooth muscle cell proliferation were found from postnatal day 3 to day 10 that reduced gradually with no nestin and vascular smooth muscle cell proliferation in adults suggesting a strong correlation between nestin expression in and proliferation of vascular smooth muscle cells. Similarly, as compared to normoxic controls, in adult lungs nestin upregulation was found between 3 days and 1 week of hypoxic exposure but not at later time points when pulmonary hypertension became clinically and histologically evident. In hypoxic lungs peak of phosphorylated (activated) PDGF receptor-ß was observed between 4 days and 1 week of hypoxic treatment. Nestin up-regulation correlated well with an increase of cell proliferation and PDGFR-ß phosphorylation/activation.

Higher nestin expression was also found in vasculature of lungs from monocrotaline and Su5416/hypoxia treated rat samples. In small arteries, nestin was found in vascular smooth muscle cells. In large arteries of lungs from both rat models, in addition to vascular smooth muscle cells, nestin was also found in cells present in so-called "vasculogenic zone" at the border of media and adventitia. In the Su5416/hypoxia model, nestin-positive vascular smooth muscle cells were also detected in more complex lesions with neointima, a characteristic feature of this model. Along with arteries, large veins of this model also showed strong nestin immunoreactivity and interestingly, nestin^{+} cells in these veins were found to be cardiomyocytes.

In human samples from patients of pulmonary hypertension, nestin was found in vascular smooth muscle cells in small vessels, in "vasculogenic zone" in large vessels as well as in vascular lesions e.g. concentric lesions, plexiform lesions and recanalization of thrombosed arteries. In lung samples from patients of pulmonary fibrosis, nestin expression was predominantly found in vascular smooth muscle cells.

Certain vascular wall cells capable of proliferation could be identified by nestin expression in lungs and may be used as prognostic and diagnostic markers and new target for therapeutic interventions of diseases like pulmonary hypertension.

6 Zusammenfassung

Es wird vermutet, dass bestimmte Blutgefäßzellen, die sich durch die Expression des neuronalen Stammzellmarkers Nestin auszeichnen, als Vorläuferzellen für den Ersatz von geschädigtem Gewebe in verschiedenen Organen dienen können. So haben frühere Studien gezeigt, dass Nestin-exprimierende glatte Muskelzellen und Perizyten von Blutgefäßen des Hodens zur Neubildung von Leydigzellen in der Lage sind. Im Rahmen dieser Doktorarbeit sollte untersucht werden, welche Rolle Nestin-exprimierende Gefäßzellen in der Lunge während der postnatalen Entwicklung und unter pathologischen Bedingungen, z. B. den bei pulmonaler Hypertonie stattfindenden Umbauvorgängen der Gefäßwand (vaskuläres Remodeling), spielen.

Immunhistochemische und Immunoblot-Analysen von Mauslungen sowie Untersuchungen an GFP-Nestin-Mäusen zeigten übereinstimmend eine dramatisch höhere Nestin-Expression während der frühen postnatalen Entwicklung als in der adulten Lunge. Interessanterweise konnte ein Maximum der Nestin-Expression zwischen Tag 3 und Tag 10 postnatal festgestellt werden. Die Nestin-exprimierenden Zellen wurden als eine Subpopulation der glatten Muskelzellen charakterisiert, die zudem durch die Expression von Proliferationsmarkern (PCNA, Ki67) gekennzeichnet sind. Die Studien deuten somit auf eine bisher nicht beschriebene Rolle von Nestin-exprimierenden glatten Muskelzellen bei Proliferationsvorgängen während der postnatalen Lungenentwicklung hin.

Für die Untersuchungen der Nestinexpression unter Bedingungen von pulmonaler Hypertonie wurden verschiedene Tiermodelle eingesetzt, bei denen das Krankheitsbild durch chronische Hypoxie (Maus) oder Monocrotalin (Ratte) induziert wird. Daneben konnten Lungen von Ratten genutzt werden, bei denen pulmonale Hypertonie durch eine Kombination von chronischer Hypoxie und Behandlung mit VEGF-Rezeptor2-Antagonisten (Su5416) erzeugt wurde.

Hypoxie induzierte eine starke Zunahme der Nestin-Proteinexpression in Mauslungen 3 Tage nach Beginn der Behandlung, die nach weiteren 4 Tagen wieder zurückging. Die Zunahme der Nestin-Proteinexpression korrelierte mit einer Zunahme an Zellproliferation (PCNA/Ki67-positiven Zellen) und PDGF-Rezeptor β-Aktivität

(Phosphorylierung). Diese Studien weisen auf eine wesentliche und zeitlich definierbare Rolle von Nestin-exprimierenden glatten Muskelzellen beim Prozess des vaskulären Remodeling während der Entstehung von pulmonaler Hypertonie (volle Ausprägung nach 3 Wochen Hypoxie) hin.

Vergleichbare Änderungen der Nestinexpression wurden auch in den beiden Rattenmodellen der pulmonalen Hypertonie gefunden. In kleinen Arterien war Nestin ausschließlich in glatten Gefäßmuskelzellen detektierbar. In großen Arterien konnte Nestin zusätzlich auch in Zellen der so genannten "vaskulogenen Zone" an der Grenze zwischen Media und Adventitia gefunden. Interessanterweise ließ sich im Su5416/Hypoxie-Modell Nestin auch in glatten Muskelzellen komplexer Läsionen mit Neointima und in großen Venen nachweisen. Deren nestinpositiven Zellen zeigten erstaunlicherweise die klassischen Merkmale von Kardiomyozyten.

In menschlichen Proben von Patienten mit Lungenhypertonie wurde Nestin in glatten Gefäßmuskelzellen von kleinen Gefäßen, in vaskulogenen Zonen der großen Gefäße sowie an Stellen von Gefäßverletzungen (z. B. konzentrische Läsionen, plexiforme Läsionen) gefunden. In Lungenproben von Patienten mit Lungenfibrose war die Nestinexpression weitgehend auf glatte Gefäßmuskelzellen beschränkt.

Die in dieser Arbeit erhaltenen Befunde zur Rolle von Nestin-exprimierenden Zellen bei Proliferationsvorgängen in der Lunge unter pathophysiologischen Belastungen könnten von diagnostischem Nutzen sein und die Basis für neue therapeutische Optionen darstellen.

7 References

Abe K, Toba M, Alzoubi A, Ito M, Fagan KA, Cool CD, Voelkel NF, McMurtry IF, Oka M (2010). Formation of plexiform lesions in experimental severe pulmonary arterial hypertension. *Circulation* 121:2747-2754.

Abedi H, Zachary I (1995). Signalling mechanisms in the regulation of vascular cell migration. *Cardiovasc Res* 30:544-556.

About I, Laurent-Maquin D, Lendahl U, Mitsiadis TA (2000). Nestin expression in embryonic and adult human teeth under normal and pathological conditions. *Am J Pathol* 157:287-295.

Adluri RS, Thirunavukkarasu M, Zhan L, Akita Y, Samuel SM, Otani H, Ho YS, Maulik G, Maulik N (2011). Thioredoxin 1 enhances neovascularization and reduces ventricular remodeling during chronic myocardial infarction: a study using thioredoxin 1 transgenic mice. *J Mol Cell Cardiol* 50:239-247.

Aihara M, Sugawara K, Torii S, Hosaka M, Kurihara H, Saito N, Takeuchi T (2004). Angiogenic endothelium-specific nestin expression is enhanced by the first intron of the nestin gene. *Lab Invest* 84:1581-1592.

Aki R, Amoh Y, Li L, Katsuoka K, Hoffman RM (2010). Nestin-expressing interfollicular blood vessel network contributes to skin transplant survival and wound healing. *J Cell Biochem* 110:80-86.

Allen K, Haworth SG (1988). Human postnatal pulmonary arterial remodeling. Ultrastructural studies of smooth muscle cell and connective tissue maturation. *Lab Invest* 59:702-709.

Alva JA, Iruela-Arispe ML (2004). Notch signaling in vascular morphogenesis. *Curr Opin Hematol* 11:278-283.

Amoh Y, Bouvet M, Li L, Tsuji K, Moossa AR, Katsuoka K, Hoffman RM (2006). Visualization of nascent tumor angiogenesis in lung and liver metastasis by differential dual-color fluorescence imaging in nestin-linked-GFP mice. *Clin Exp Metastasis* 23:315-322.

Amoh Y, Li L, Yang M, Moossa AR, Katsuoka K, Penman S, Hoffman RM (2004). Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells. *Proc Natl Acad Sci U S A* 101:13291-13295.

Amoh Y, Yang M, Li L, Reynoso J, Bouvet M, Moossa AR, Katsuoka K, Hoffman RM (2005). Nestin-linked green fluorescent protein transgenic nude mouse for imaging human tumor angiogenesis. *Cancer Res* 65:5352-5357.

Amy RW, Bowes D, Burri PH, Haines J, Thurlbeck WM (1977). Postnatal growth of the mouse lung. *J Anat* 124:131-151.

Andrae J, Gallini R, Betsholtz C (2008). Role of platelet-derived growth factors in physiology and medicine. *Genes Dev* 22:1276-1312.

Arai H, Ikota H, Sugawara KI, Nobusawa S, Hirato J, Nakazato Y (2012). Nestin expression in brain tumors: its utility for pathological diagnosis and correlation with the prognosis of high-grade gliomas. *Brain Tumor Pathol*.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275:964-967.

Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG, Hicklin DJ, Jeltsch M, Petrova TV, Pytowski B, Stacker SA, Yla-Herttuala S, Jackson DG, Alitalo K, McDonald DM (2005). Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J Clin Invest* 115:247-257.

Beers MF, Morrisey EE (2011). The three R's of lung health and disease: repair, remodeling, and regeneration. *J Clin Invest* 121:2065-2073.

Bentzon JF, Sondergaard CS, Kassem M, Falk E (2007). Smooth muscle cells healing atherosclerotic plaque disruptions are of local, not blood, origin in apolipoprotein E knockout mice. *Circulation* 116:2053-2061.

Bergers G, Song S (2005). The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol* 7:452-464.

Berndt, Claudia. (2011). “Expression und Lokalisation des neuronalen Stammzellmarkers Nestin in den Gefäßwänden der Lunge von neugeborenen und adulten Mäusen”. Jusltus Liebig Universität, Gießen, Germany. Dissertation.
http://geb.uni-giessen.de/geb/volltexte/2012/8540/

Birbrair A, Wang ZM, Messi ML, Enikolopov GN, Delbono O (2011). Nestin-GFP transgene reveals neural precursor cells in adult skeletal muscle. *PLoS One* 6:e16816.

Bonfanti L, Theodosis DT (1994). Expression of polysialylated neural cell adhesion molecule by proliferating cells in the subependymal layer of the adult rat, in its rostral extension and in the olfactory bulb. *Neuroscience* 62:291-305.

Burg ED, Remillard CV, Yuan JX (2008). Potassium channels in the regulation of pulmonary artery smooth muscle cell proliferation and apoptosis: pharmacotherapeutic implications. *Br J Pharmacol* 153 Suppl 1:S99-S111.

Burri PH (2006). Structural aspects of postnatal lung development - alveolar formation and growth. *Biol Neonate* 89:313-322.

Calderone A (2012). Nestin^{+} cells and healing the infarcted heart. *Am J Physiol Heart Circ Physiol* 302:H1-9.

Campos AH, Wang W, Pollman MJ, Gibbons GH (2002). Determinants of Notch-3 receptor expression and signaling in vascular smooth muscle cells: implications in cell-cycle regulation. *Circ Res* 91:999-1006.

Caplan AI (2008). All MSCs are pericytes? *Cell Stem Cell* 3:229-230.

Carmeliet P (2003). Angiogenesis in health and disease. *Nat Med* 9:653-660.

Cattaneo E, McKay R (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature* 347:762-765.

Chan SY, Loscalzo J (2008). Pathogenic mechanisms of pulmonary arterial hypertension. *J Mol Cell Cardiol* 44:14-30.

Chanakira A, Dutta R, Charboneau R, Barke R, Santilli SM, Roy S (2012). Hypoxia differentially regulates arterial and venous smooth muscle cell proliferation via PDGFR-beta and VEGFR-2 expression. *Am J Physiol Heart Circ Physiol* 302:H1173-1184.

Chang L, Goldman RD (2004). Intermediate filaments mediate cytoskeletal crosstalk. *Nat Rev Mol Cell Biol* 5:601-613.

Chen HC, Sytwu HK, Chang JL, Wang HW, Chen HK, Kang BH, Liu DW, Chen CH, Chao TT, Wang CH (2011). Hypoxia enhances the stemness markers of cochlear stem/progenitor cells and expands sphere formation through activation of hypoxia-inducible factor-1 alpha. *Hear Res* 275:43-52.

Chen J, Boyle S, Zhao M, Su W, Takahashi K, Davis L, Decaestecker M, Takahashi T, Breyer MD, Hao CM (2006). Differential expression of the intermediate filament protein nestin during renal development and its localization in adult podocytes. *J Am Soc Nephrol* 17:1283-1291.

Chen Z, Wang T, Luo H, Lai Y, Yang X, Li F, Lei Y, Su C, Zhang X, Lahn BT, Xiang AP (2010). Expression of nestin in lymph node metastasis and lymphangiogenesis in non-small cell lung cancer patients. *Hum Pathol* 41:737-744.

Collet JF, Messens J (2010). Structure, function, and mechanism of thioredoxin proteins. *Antioxid Redox Signal* 13:1205-1216.

Cool CD, Stewart JS, Werahera P, Miller GJ, Williams RL, Voelkel NF, Tuder RM (1999). Three-dimensional reconstruction of pulmonary arteries in plexiform pulmonary hypertension using cell-specific markers. Evidence for a dynamic and heterogeneous process of pulmonary endothelial cell growth. *Am J Pathol* 155:411-419.

Crisan M, Corselli M, Chen CW, Peault B (2011). Multilineage stem cells in the adult: a perivascular legacy? *Organogenesis* 7:101-104.

Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng PN, Traas J, Schugar R, Deasy BM, Badylak S, Buhring HJ, Giacobino JP, Lazzari L, Huard J, Peault B (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 3:301-313.

da Silva Meirelles L, Chagastelles PC, Nardi NB (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 119:2204-2213.

Dahal BK, Heuchel R, Pullamsetti SS, Wilhelm J, Ghofrani HA, Weissmann N, Seeger W, Grimminger F, Schermuly RT (2011). Hypoxic pulmonary hypertension in mice with constitutively active platelet-derived growth factor receptor-beta. *Pulm Circ* 1:259-268.

Dahlstrand J, Zimmerman LB, McKay RD, Lendahl U (1992). Characterization of the human nestin gene reveals a close evolutionary relationship to neurofilaments. *J Cell Sci* 103 (Pt 2):589-597.

Daniel C, Albrecht H, Ludke A, Hugo C (2008). Nestin expression in repopulating mesangial cells promotes their proliferation. *Lab Invest* 88:387-397.

Daniel JM, Sedding DG (2011). Circulating smooth muscle progenitor cells in arterial remodeling. *J Mol Cell Cardiol* 50:273-279.

Dar A, Domev H, Ben-Yosef O, Tzukerman M, Zeevi-Levin N, Novak A, Germanguz I, Amit M, Itskovitz-Eldor J (2012). Multipotent vasculogenic pericytes from human pluripotent stem cells promote recovery of murine ischemic limb. *Circulation* 125:87-99.

Davidoff MS, Middendorff R, Enikolopov G, Riethmacher D, Holstein AF, Muller D (2004). Progenitor cells of the testosterone-producing Leydig cells revealed. *J Cell Biol* 167:935-944.

Davidoff MS, Middendorff R, Mayer B, Holstein AF (1995). Nitric oxide synthase (NOS-I) in Leydig cells of the human testis. *Arch Histol Cytol* 58:17-30.

Davie N, Haleen SJ, Upton PD, Polak JM, Yacoub MH, Morrell NW, Wharton J (2002). ET(A) and ET(B) receptors modulate the proliferation of human pulmonary artery smooth muscle cells. *Am J Respir Crit Care Med* 165:398-405.

Dellavalle A, Sampaolesi M, Tonlorenzi R, Tagliafico E, Sacchetti B, Perani L, Innocenzi A, Galvez BG, Messina G, Morosetti R, Li S, Belicchi M, Peretti G, Chamberlain JS, Wright WE, Torrente Y, Ferrari S, Bianco P, Cossu G (2007). Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nat Cell Biol* 9:255-267.

deMello DE, Reid LM (2000). Embryonic and early fetal development of human lung vasculature and its functional implications. *Pediatr Dev Pathol* 3:439-449.

deMello DE, Sawyer D, Galvin N, Reid LM (1997). Early fetal development of lung vasculature. *Am J Respir Cell Mol Biol* 16:568-581.

Diaz-Flores L, Gutierrez R, Madrid JF, Varela H, Valladares F, Acosta E, Martin-Vasallo P, Diaz-Flores L, Jr. (2009). Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. *Histol Histopathol* 24:909-969.

Dore-Duffy P, Katychev A, Wang X, Van Buren E (2006). CNS microvascular pericytes exhibit multipotential stem cell activity. *J Cereb Blood Flow Metab* 26:613-624.

Durmowicz AG, Stenmark KR (1999). Mechanisms of structural remodeling in chronic pulmonary hypertension. *Pediatr Rev* 20:e91-e102.

Ebina M, Shimizukawa M, Shibata N, Kimura Y, Suzuki T, Endo M, Sasano H, Kondo T, Nukiwa T (2004). Heterogeneous increase in CD34-positive alveolar capillaries in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 169:1203-1208.

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 (2006). Cross talk between endothelial and smooth muscle cells in pulmonary hypertension: critical role for serotonin-induced smooth muscle hyperplasia. *Circulation* 113:1857-1864.

Edelman GM (1986). Cell adhesion molecules in neural histogenesis. *Annu Rev Physiol* 48:417-430.

Ehrmann J, Kolar Z, Mokry J (2005). Nestin as a diagnostic and prognostic marker: immunohistochemical analysis of its expression in different tumours. *J Clin Pathol* 58:222-223.

El-Helou V, Dupuis J, Proulx C, Drapeau J, Clement R, Gosselin H, Villeneuve L, Manganas L, Calderone A (2005). Resident nestin^{+} neural-like cells and fibers are detected in normal and damaged rat myocardium. *Hypertension* 46:1219-1225.

Eliasson C, Sahlgren C, Berthold CH, Stakeberg J, Celis JE, Betsholtz C, Eriksson JE, Pekny M (1999). Intermediate filament protein partnership in astrocytes. *J Biol Chem* 274:23996-24006.

Ergun S, Tilki D, Klein D (2011). Vascular wall as a reservoir for different types of stem and progenitor cells. *Antioxid Redox Signal* 15:981-995.

Farkas L, Gauldie J, Voelkel NF, Kolb M (2011). Pulmonary hypertension and idiopathic pulmonary fibrosis: a tale of angiogenesis, apoptosis, and growth factors. *Am J Respir Cell Mol Biol* 45:1-15.

Fischer LG, Honemann CW, Patrie JT, Durieux ME, Rich GF (2000). Ropivacaine attenuates pulmonary vasoconstriction induced by thromboxane A2 analogue in the isolated perfused rat lung. *Reg Anesth Pain Med* 25:187-194.

Fishman AP (2001). Clinical classification of pulmonary hypertension. *Clin Chest Med* 22:385-391, vii.

Frid MG, Kale VA, Stenmark KR (2002). Mature vascular endothelium can give rise to smooth muscle cells via endothelial-mesenchymal transdifferentiation: in vitro analysis. *Circ Res* 90:1189-1196.

Frid MG, Moiseeva EP, Stenmark KR (1994). Multiple phenotypically distinct smooth muscle cell populations exist in the adult and developing bovine pulmonary arterial media in vivo. *Circ Res* 75:669-681.

Friedrich RE, Holstein AF, Middendorff R, Davidoff MS (2012). Vascular wall cells contribute to tumourigenesis in cutaneous neurofibromas of patients with neurofibromatosis type 1. A comparative histological, ultrastructural and immunohistochemical study. *Anticancer Res* 32:2139-2158.

Frojdman K, Pelliniemi LJ, Lendahl U, Virtanen I, Eriksson JE (1997). The intermediate filament protein nestin occurs transiently in differentiating testis of rat and mouse. *Differentiation* 61:243-249.

Fuchs E, Weber K (1994). Intermediate filaments: structure, dynamics, function, and disease. *Annu Rev Biochem* 63:345-382.

Gaine SP, Rubin LJ (1998). Primary pulmonary hypertension. *Lancet* 352:719-725.

Gambaryan N, Cohen-Kaminsky S, Montani D, Girerd B, Huertas A, Seferian A, Humbert M, Perros F (2012). Circulating fibrocytes and pulmonary arterial hypertension. *Eur Respir J* 39:210-212.

Gao Y, Raj JU (2010). Regulation of the pulmonary circulation in the fetus and newborn. *Physiol Rev* 90:1291-1335.

Geraci MW, Gao B, Shepherd DC, Moore MD, Westcott JY, Fagan KA, Alger LA, Tuder RM, Voelkel NF (1999). Pulmonary prostacyclin synthase overexpression in transgenic mice protects against development of hypoxic pulmonary hypertension. *J Clin Invest* 103:1509-1515.

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N (1998). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 273:30336-30343.

Gomez-Arroyo JG, Farkas L, Alhussaini AA, Farkas D, Kraskauskas D, Voelkel NF, Bogaard HJ (2012). The monocrotaline model of pulmonary hypertension in perspective. *Am J Physiol Lung Cell Mol Physiol* 302:L363-369.

Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA (2009). Proliferation of immature tumor vessels is a novel marker of clinical progression in prostate cancer. *Cancer Res* 69:4708-4715.

Grieb G, Steffens G, Pallua N, Bernhagen J, Bucala R (2011). Circulating fibrocytes--biology and mechanisms in wound healing and scar formation. *Int Rev Cell Mol Biol* 291:1-19.

Guignabert C, Raffestin B, Benferhat R, Raoul W, Zadigue P, Rideau D, Hamon M, Adnot S, Eddahibi S (2005). Serotonin transporter inhibition prevents and reverses monocrotaline-induced pulmonary hypertension in rats. *Circulation* 111:2812-2819.

Hagensen MK, Shim J, Thim T, Falk E, Bentzon JF (2010). Circulating endothelial progenitor cells do not contribute to plaque endothelium in murine atherosclerosis. *Circulation* 121:898-905.

Hall SM, Hislop AA, Haworth SG (2002). Origin, differentiation, and maturation of human pulmonary veins. *Am J Respir Cell Mol Biol* 26:333-340.

Hall SM, Hislop AA, Pierce CM, Haworth SG (2000). Prenatal origins of human intrapulmonary arteries: formation and smooth muscle maturation. *Am J Respir Cell Mol Biol* 23:194-203.

Hatano S, Strasser R (1975). Primary pulmonary hypertension. Geneva: World Health Organization.

Haworth SG (1995). Development of the normal and hypertensive pulmonary vasculature. *Exp Physiol* 80:843-853.

Herrmann H, Aebi U (2000). Intermediate filaments and their associates: multi-talented structural elements specifying cytoarchitecture and cytodynamics. *Curr Opin Cell Biol* 12:79-90.

Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G (2007). The myofibroblast: one function, multiple origins. *Am J Pathol* 170:1807-1816.

Hislop A, Reid L (1972). Intra-pulmonary arterial development during fetal life-branching pattern and structure. *J Anat* 113:35-48.

Hislop A, Reid L (1973). Pulmonary arterial development during childhood: branching pattern and structure. *Thorax* 28:129-135.

Hislop AA (2002). Airway and blood vessel interaction during lung development. *J Anat* 201:325-334.

Hockfield S, McKay RD (1985). Identification of major cell classes in the developing mammalian nervous system. *J Neurosci* 5:3310-3328.

Hoendermis ES (2011). Pulmonary arterial hypertension: an update. *Neth Heart J* 19:514-522.

Hoshikawa Y, Nana-Sinkam P, Moore MD, Sotto-Santiago S, Phang T, Keith RL, Morris KG, Kondo T, Tuder RM, Voelkel NF, Geraci MW (2003). Hypoxia induces different genes in the lungs of rats compared with mice. *Physiol Genomics* 12:209-219.

Hoshikawa Y, Voelkel NF, Gesell TL, Moore MD, Morris KG, Alger LA, Narumiya S, Geraci MW (2001). Prostacyclin receptor-dependent modulation of pulmonary vascular remodeling. *Am J Respir Crit Care Med* 164:314-318.

Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q (2004). Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. *J Clin Invest* 113:1258-1265.

Huang YL, Shi GY, Jiang MJ, Lee H, Chou YW, Wu HL, Yang HY (2008). Epidermal growth factor up-regulates the expression of nestin through the Ras-Raf-ERK signaling axis in rat vascular smooth muscle cells. *Biochem Biophys Res Commun* 377:361-366.

Huang YL, Wu CM, Shi GY, Wu GC, Lee H, Jiang MJ, Wu HL, Yang HY (2009). Nestin serves as a prosurvival determinant that is linked to the cytoprotective effect of epidermal growth factor in rat vascular smooth muscle cells. *J Biochem* 146:307-315.

Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkel NF, Rabinovitch M (2004). Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol* 43:13S-24S.

Hyde DM, Blozis SA, Avdalovic MV, Putney LF, Dettorre R, Quesenberry NJ, Singh P, Tyler NK (2007). Alveoli increase in number but not size from birth to adulthood in rhesus monkeys. *Am J Physiol Lung Cell Mol Physiol* 293:L570-579.

Ishiwata T, Matsuda Y, Naito Z (2011). Nestin in gastrointestinal and other cancers: effects on cells and tumor angiogenesis. *World J Gastroenterol* 17:409-418.

Izikki M, Guignabert C, Fadel E, Humbert M, Tu L, Zadigue P, Dartevelle P, Simonneau G, Adnot S, Maitre B, Raffestin B, Eddahibi S (2009). Endothelial-derived FGF2 contributes to the progression of pulmonary hypertension in humans and rodents. *J Clin Invest* 119:512-523.

Jeffery TK, Wanstall JC (2001). Comparison of pulmonary vascular function and structure in early and established hypoxic pulmonary hypertension in rats. *Can J Physiol Pharmacol* 79:227-237.

Jin S, Hansson EM, Tikka S, Lanner F, Sahlgren C, Farnebo F, Baumann M, Kalimo H, Lendahl U (2008). Notch signaling regulates platelet-derived growth factor receptor-beta expression in vascular smooth muscle cells. *Circ Res* 102:1483-1491.

Jones R, Jacobson M, Steudel W (1999). alpha-smooth-muscle actin and microvascular precursor smooth-muscle cells in pulmonary hypertension. *Am J Respir Cell Mol Biol* 20:582-594.

Jones RC, Capen DE, Cohen KS, Munn LL, Jain RK, Duda DG (2008). A protocol for phenotypic detection and characterization of vascular cells of different origins in a lung neovascularization model in rodents. *Nat Protoc* 3:388-397.

Kachinsky AM, Dominov JA, Miller JB (1995). Intermediate filaments in cardiac myogenesis: nestin in the developing mouse heart. *J Histochem Cytochem* 43:843-847.

Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, Voelkel NF (2000). Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 106:1311-1319.

Kato M, Staub NC (1966). Response of small pulmonary arteries to unilobar hypoxia and hypercapnia. *Circ Res* 19:426-440.

Kawai-Kowase K, Owens GK (2007). Multiple repressor pathways contribute to phenotypic switching of vascular smooth muscle cells. *Am J Physiol Cell Physiol* 292:C59-69.

Kierszenbaum, A. L. 2007. Histology and Cell Biology: An Introduction to Pathology. 2nd Edition. St. Louis: Mosby.

Kim SY, Lee S, Hong SW, Min BH, Lee KU, Bendayan M, Park IS (2010). Nestin action during insulin-secreting cell differentiation. *J Histochem Cytochem* 58:567-576.

Klein D, Hohn HP, Kleff V, Tilki D, Ergun S (2010). Vascular wall-resident stem cells. *Histol Histopathol* 25:681-689.

Kovacic JC, Boehm M (2009). Resident vascular progenitor cells: an emerging role for non-terminally differentiated vessel-resident cells in vascular biology. *Stem Cell Res* 2:2-15.

Krupkova O, Jr., Loja T, Zambo I, Veselska R (2010). Nestin expression in human tumors and tumor cell lines. *Neoplasma* 57:291-298.

Kuro-o M, Nagai R, Nakahara K, Katoh H, Tsai RC, Tsuchimochi H, Yazaki Y, Ohkubo A, Takaku F (1991). cDNA cloning of a myosin heavy chain isoform in embryonic smooth muscle and its expression during vascular development and in arteriosclerosis. *J Biol Chem* 266:3768-3773.

Le Cras TD, Hardie WD, Fagan K, Whitsett JA, Korfhagen TR (2003). Disrupted pulmonary vascular development and pulmonary hypertension in transgenic mice overexpressing transforming growth factor-alpha. *Am J Physiol Lung Cell Mol Physiol* 285:L1046-1054.

Lendahl U, Zimmerman LB, McKay RD (1990). CNS stem cells express a new class of intermediate filament protein. *Cell* 60:585-595.

Li JC, Pan JQ, Huang GQ, Tan X, Sun WD, Liu YJ, Wang XL (2010). Expression of PDGF-beta receptor in broilers with pulmonary hypertension induced by cold temperature and its association with pulmonary vascular remodeling. *Res Vet Sci* 88:116-121.

Li L, Mignone J, Yang M, Matic M, Penman S, Enikolopov G, Hoffman RM (2003). Nestin expression in hair follicle sheath progenitor cells. *Proc Natl Acad Sci U S A* 100:9958-9961.

Li P, Oparil S, Sun JZ, Thompson JA, Chen YF (2003). Fibroblast growth factor mediates hypoxia-induced endothelin-- a receptor expression in lung artery smooth muscle cells. *J Appl Physiol* 95:643-651; discussion 863.

Liem RK (1993). Molecular biology of neuronal intermediate filaments. *Curr Opin Cell Biol* 5:12-16.

Lledo PM, Alonso M, Grubb MS (2006). Adult neurogenesis and functional plasticity in neuronal circuits. *Nat Rev Neurosci* 7:179-193.

Machado RD, Eickelberg O, Elliott CG, Geraci MW, Hanaoka M, Loyd JE, Newman JH, Phillips JA, 3rd, Soubrier F, Trembath RC, Chung WK (2009). Genetics and genomics of pulmonary arterial hypertension. *J Am Coll Cardiol* 54:S32-42.

MacLean MR, Herve P, Eddahibi S, Adnot S (2000). 5-hydroxytryptamine and the pulmonary circulation: receptors, transporters and relevance to pulmonary arterial hypertension. *Br J Pharmacol* 131:161-168.

Majesky MW, Dong XR, Regan JN, Hoglund VJ (2011). Vascular smooth muscle progenitor cells: building and repairing blood vessels. *Circ Res* 108:365-377.

Mandegar M, Fung YC, Huang W, Remillard CV, Rubin LJ, Yuan JX (2004). Cellular and molecular mechanisms of pulmonary vascular remodeling: role in the development of pulmonary hypertension. *Microvasc Res* 68:75-103.

Mangla R, Kolar B, Almast J, Ekholm SE (2011). Border zone infarcts: pathophysiologic and imaging characteristics. *Radiographics* 31:1201-1214.

Marsboom GR, and Janssens SP (2004). Models for pulmonary hypertension. Drug Discovery Today: Disease Models 1(3), 289-296

Marvin MJ, Dahlstrand J, Lendahl U, McKay RD (1998). A rod end deletion in the intermediate filament protein nestin alters its subcellular localization in neuroepithelial cells of transgenic mice. *J Cell Sci* 111 (Pt 14):1951-1961.

Mayer EJ, Hughes EH, Carter DA, Dick AD (2003). Nestin positive cells in adult human retina and in epiretinal membranes. *Br J Ophthalmol* 87:1154-1158.

McDonald DM (1994). Endothelial gaps and permeability of venules in rat tracheas exposed to inflammatory stimuli. *Am J Physiol* 266:L61-83.

McGrath JC, Deighan C, Briones AM, Shafaroudi MM, McBride M, Adler J, Arribas SM, Vila E, Daly CJ (2005). New aspects of vascular remodelling: the involvement of all vascular cell types. *Exp Physiol* 90:469-475.

Messam CA, Hou J, Berman JW, Major EO (2002). Analysis of the temporal expression of nestin in human fetal brain derived neuronal and glial progenitor cells. *Brain Res Dev Brain Res* 134:87-92.

Metzger RJ, Klein OD, Martin GR, Krasnow MA (2008). The branching programme of mouse lung development. *Nature* 453:745-750.

Meyrick B, Reid L (1980). Hypoxia-induced structural changes in the media and adventitia of the rat hilar pulmonary artery and their regression. *Am J Pathol* 100:151-178.

Meyrick BO, Perkett EA (1989). The sequence of cellular and hemodynamic changes of chronic pulmonary hypertension induced by hypoxia and other stimuli. *Am Rev Respir Dis* 140:1486-1489.

Mietens A, Tasch S, Feuerstacke C, Eichner G, Volkmann J, Schermuly RT, Grimminger F, Muller D, Middendorff R (2012). Phosphodiesterase 5 (PDE5) inhibition, ANP and NO rapidly reduce epididymal duct contractions, but long-term PDE5 inhibition in vivo does not. *Mol Cell Endocrinol* 349:145-153.

Mokry J, Cizkova D, Filip S, Ehrmann J, Osterreicher J, Kolar Z, English D (2004). Nestin expression by newly formed human blood vessels. *Stem Cells Dev* 13:658-664.

Mokry J, Pudil R, Ehrmann J, Cizkova D, Osterreicher J, Filip S, Kolar Z (2008). Re-expression of nestin in the myocardium of postinfarcted patients. *Virchows Arch* 453:33-41.

Morrisey EE, Hogan BL (2010). Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev Cell* 18:8-23.

Moudgil R, Michelakis ED, Archer SL (2006). The role of k^+ channels in determining pulmonary vascular tone, oxygen sensing, cell proliferation, and apoptosis: implications in hypoxic pulmonary vasoconstriction and pulmonary arterial hypertension. *Microcirculation* 13:615-632.

Mueller-Hoecker J, Beitinger F, Fernandez B, Bahlmann O, Assmann G, Troidl C, Dimomeletis I, Kaab S, Deindl E (2008). Of rodents and humans: a light microscopic and ultrastructural study on cardiomyocytes in pulmonary veins. *Int J Med Sci* 5:152-158.

Muller D, Hida B, Guidone G, Speth RC, Michurina TV, Enikolopov G, Middendorff R (2009). Expression of guanylyl cyclase (GC)-A and GC-B during brain development: evidence for a role of GC-B in perinatal neurogenesis. *Endocrinology* 150:5520-5529.

Muller D, Mukhopadhyay AK, Speth RC, Guidone G, Potthast R, Potter LR, Middendorff R (2004). Spatiotemporal regulation of the two atrial natriuretic peptide receptors in testis. *Endocrinology* 145:1392-1401.

Mund SI, Stampanoni M, Schittny JC (2008). Developmental alveolarization of the mouse lung. *Dev Dyn* 237:2108-2116.

Muto A, Fitzgerald TN, Pimiento JM, Maloney SP, Teso D, Paszkowiak JJ, Westvik TS, Kudo FA, Nishibe T, Dardik A (2007). Smooth muscle cell signal transduction: implications of vascular biology for vascular surgeons. *J Vasc Surg* 45 Suppl A:A15-24.

Naeye RL, Vennart GP (1960). The structure and significance of pulmonary plexiform structures. *Am J Pathol* 36:593-605.

Nelin, Viktoria. (2011). "The Proliferation Of Vascular Smooth Muscle Cells Depends On Thioredoxin 1 Protein In A Model Of Pulmonary Hypertension" Honors College Capstone Experience/Thesis Projects. Paper 329. http://digitalcommons.wku.edu/stu_hon_theses/329

Ni A, Lashnits E, Yao LC, Baluk P, McDonald DM (2010). Rapid remodeling of airway vascular architecture at birth. *Dev Dyn* 239:2354-2366.

Oikawa H, Hayashi K, Maesawa C, Masuda T, Sobue K (2010). Expression profiles of nestin in vascular smooth muscle cells in vivo and in vitro. *Exp Cell Res* 316:940-950.

Owens GK (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev* 75:487-517.

Owens GK, Kumar MS, Wamhoff BR (2004). Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* 84:767-801.

Paddenberg R, Stieger P, von Lilien AL, Faulhammer P, Goldenberg A, Tillmanns HH, Kummer W, Braun-Dullaeus RC (2007). Rapamycin attenuates hypoxia-induced pulmonary vascular remodeling and right ventricular hypertrophy in mice. *Respir Res* 8:15.

Pak O, Aldashev A, Welsh D, Peacock A (2007). The effects of hypoxia on the cells of the pulmonary vasculature. *Eur Respir J* 30:364-372.

Park D, Xiang AP, Mao FF, Zhang L, Di CG, Liu XM, Shao Y, Ma BF, Lee JH, Ha KS, Walton N, Lahn BT (2010). Nestin is required for the proper self-renewal of neural stem cells. *Stem Cells* 28:2162-2171.

Parra ER, David YR, da Costa LR, Ab'Saber A, Sousa R, Kairalla RA, de Carvalho CR, Filho MT, Capelozzi VL (2005). Heterogeneous remodeling of lung vessels in idiopathic pulmonary fibrosis. *Lung* 183:291-300.

Partovian C, Adnot S, Eddahibi S, Teiger E, Levame M, Dreyfus P, Raffestin B, Frelin C (1998). Heart and lung VEGF mRNA expression in rats with monocrotaline- or hypoxia-induced pulmonary hypertension. *Am J Physiol* 275:H1948-1956.

Partovian C, Ladoux A, Eddahibi S, Teiger E, Raffestin B, Frelin C, Adnot S (1998). Cardiac and lung VEGF mRNA expression in chronically hypoxic and monocrotaline-treated rats. *Chest* 114:45S-46S.

Passman JN, Dong XR, Wu SP, Maguire CT, Hogan KA, Bautch VL, Majesky MW (2008). A sonic hedgehog signaling domain in the arterial adventitia supports resident $Sca1^+$ smooth muscle progenitor cells. *Proc Natl Acad Sci U S A* 105:9349-9354.

Pearson JD (2010). Endothelial progenitor cells--an evolving story. *Microvasc Res* 79:162-168.

Peault B (2012). Are mural cells guardians of stemness?: From pluri- to multipotency via vascular pericytes. *Circulation* 125:12-13.

Perros F, Montani D, Dorfmuller P, Durand-Gasselin I, Tcherakian C, Le Pavec J, Mazmanian M, Fadel E, Mussot S, Mercier O, Herve P, Emilie D, Eddahibi S, Simonneau G, Souza R, Humbert M (2008). Platelet-derived growth factor expression and function in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 178:81-88.

Pietra GG, Capron F, Stewart S, Leone O, Humbert M, Robbins IM, Reid LM, Tuder RM (2004). Pathologic assessment of vasculopathies in pulmonary hypertension. *J Am Coll Cardiol* 43:25S-32S.

Pitsiou G, Papakosta D, Bouros D (2011). Pulmonary hypertension in idiopathic pulmonary fibrosis: a review. *Respiration* 82:294-304.

Psaltis PJ, Harbuzariu A, Delacroix S, Holroyd EW, Simari RD (2011). Resident vascular progenitor cells--diverse origins, phenotype, and function. *J Cardiovasc Transl Res* 4:161-176.

Quinlan TR, Li D, Laubach VE, Shesely EG, Zhou N, Johns RA (2000). eNOS-deficient mice show reduced pulmonary vascular proliferation and remodeling to chronic hypoxia. *Am J Physiol Lung Cell Mol Physiol* 279:L641-650.

Rabinovitch M (2008). Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Invest* 118:2372-2379.

Rajkumar VS, Howell K, Csiszar K, Denton CP, Black CM, Abraham DJ (2005). Shared expression of phenotypic markers in systemic sclerosis indicates a convergence of pericytes and fibroblasts to a myofibroblast lineage in fibrosis. Arthritis Res Ther 7:R1113-1123.

Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2008

Renzoni EA, Walsh DA, Salmon M, Wells AU, Sestini P, Nicholson AG, Veeraraghavan S, Bishop AE, Romanska HM, Pantelidis P, Black CM, Du Bois RM (2003). Interstitial vascularity in fibrosing alveolitis. *Am J Respir Crit Care Med* 167:438-443.

Rose F, Grimminger F, Appel J, Heller M, Pies V, Weissmann N, Fink L, Schmidt S, Krick S, Camenisch G, Gassmann M, Seeger W, Hanze J (2002). Hypoxic pulmonary artery fibroblasts trigger proliferation of vascular smooth muscle cells: role of hypoxia-inducible transcription factors. *FASEB J* 16:1660-1661.

Rubin LJ (1997). Primary pulmonary hypertension. *N Engl J Med* 336:111-117.

Rubin LJ, Galie N (2004). Pulmonary arterial hypertension: a look to the future. *J Am Coll Cardiol* 43:89S-90S.

Ryuge S, Sato Y, Wang GQ, Matsumoto T, Jiang SX, Katono K, Inoue H, Satoh Y, Masuda N (2011). Prognostic significance of nestin expression in resected non-small cell lung cancer. *Chest* 139:862-869.

Saga H, Kimura K, Hayashi K, Gotow T, Uchiyama Y, Momiyama T, Tadokoro S, Kawashima N, Jimbou A, Sobue K (1999). Phenotype-dependent expression of alpha-smooth muscle actin in visceral smooth muscle cells. *Exp Cell Res* 247:279-292.

Sakao S, Taraseviciene-Stewart L, Lee JD, Wood K, Cool CD, Voelkel NF (2005). Vascular endothelial growth factor receptor blockade by Su5416 combined with pulsatile shear stress causes apoptosis and subsequent proliferation of apoptosis-resistant endothelial cells. *Chest* 128:610S-611S.

Sakao S, Tatsumi K, Voelkel NF (2009). Endothelial cells and pulmonary arterial hypertension: apoptosis, proliferation, interaction and transdifferentiation. *Respir Res* 10:95.

Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, Lai YJ, Weissmann N, Seeger W, Grimminger F (2005). Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* 115:2811-2821.

Schermuly RT, Ghofrani HA, Wilkins MR, Grimminger F (2011). Mechanisms of disease: pulmonary arterial hypertension. *Nat Rev Cardiol* 8:443-455.

Schittny JC, Mund SI, Stampanoni M (2008). Evidence and structural mechanism for late lung alveolarization. *Am J Physiol Lung Cell Mol Physiol* 294:L246-254.

Scobioala S, Klocke R, Kuhlmann M, Tian W, Hasib L, Milting H, Koenig S, Stelljes M, El-Banayosy A, Tenderich G, Michel G, Breithardt G, Nikol S (2008). Up-regulation of nestin in the infarcted myocardium potentially indicates differentiation of resident cardiac stem cells into various lineages including cardiomyocytes. *FASEB J* 22:1021-1031.

Sejersen T, Lendahl U (1993). Transient expression of the intermediate filament nestin during skeletal muscle development. *J Cell Sci* 106 (Pt 4):1291-1300.

Seki T, Arai Y (1993). Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system. *Neurosci Res* 17:265-290.

Shimizu T, Sugawara K, Tosaka M, Imai H, Hoya K, Takeuchi T, Sasaki T, Saito N (2006). Nestin expression in vascular malformations: a novel marker for proliferative endothelium. *Neurol Med Chir (Tokyo)* 46:111-117.

Simakajornboon N, Kuptanon T, Jirapongsuwan P (2010). The effect of prenatal nicotine exposure on PDGFR-mediated anti-apoptotic mechanism in the caudal brainstem of developing rat. *Neurosci Lett* 478:46-50.

Simonneau G, Robbins IM, Beghetti M, Channick RN, Delcroix M, Denton CP, Elliott CG, Gaine SP, Gladwin MT, Jing ZC, Krowka MJ, Langleben D, Nakanishi N,

Souza R (2009). Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol* 54:S43-54.

Simper D, Stalboerger PG, Panetta CJ, Wang S, Caplice NM (2002). Smooth muscle progenitor cells in human blood. *Circulation* 106:1199-1204.

Sjoberg G, Jiang WQ, Ringertz NR, Lendahl U, Sejersen T (1994). Colocalization of nestin and vimentin/desmin in skeletal muscle cells demonstrated by three-dimensional fluorescence digital imaging microscopy. *Exp Cell Res* 214:447-458.

Sluiter I, van Heijst A, Haasdijk R, Kempen MB, Boerema-de Munck A, Reiss I, Tibboel D, Rottier RJ (2012). Reversal of pulmonary vascular remodeling in pulmonary hypertensive rats. *Exp Mol Pathol* 93:66-73.

Smith P, Heath D (1979). Electron microscopy of the plexiform lesion. *Thorax* 34:177-186.

Sobue K, Hayashi K, Nishida W (1999). Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation. *Mol Cell Biochem* 190:105-118.

Steinert PM, Chou YH, Prahlad V, Parry DA, Marekov LN, Wu KC, Jang SI, Goldman RD (1999). A high molecular weight intermediate filament-associated protein in BHK-21 cells is nestin, a type VI intermediate filament protein. Limited co-assembly in vitro to form heteropolymers with type III vimentin and type IV alpha-internexin. *J Biol Chem* 274:9881-9890.

Stenmark KR, Abman SH (2005). Lung vascular development: implications for the pathogenesis of bronchopulmonary dysplasia. *Annu Rev Physiol* 67:623-661.

Stenmark KR, Davie N, Frid M, Gerasimovskaya E, Das M (2006). Role of the adventitia in pulmonary vascular remodeling. *Physiology (Bethesda)* 21:134-145.

Stenmark KR, Frid MG (1998). Smooth muscle cell heterogeneity: role of specific smooth muscle cell subpopulations in pulmonary vascular disease. *Chest* 114:82S-90S.

Stenmark KR, Meyrick B, Galie N, Mooi WJ, McMurtry IF (2009). Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol* 297:L1013-1032.

Sugawara K, Kurihara H, Negishi M, Saito N, Nakazato Y, Sasaki T, Takeuchi T (2002). Nestin as a marker for proliferative endothelium in gliomas. *Lab Invest* 82:345-351.

Sugiyama S, Kugiyama K, Nakamura S, Kataoka K, Aikawa M, Shimizu K, Koide S, Mitchell RN, Ogawa H, Libby P (2006). Characterization of smooth muscle-like cells in circulating human peripheral blood. *Atherosclerosis* 187:351-362.

Suguta M, Nakano A, Hoshino Y, Endoh M, Hatori T, Hasegawa A, Aihara M, Takeuchi T, Kurabayashi M (2007). Nestin, a maker for multilineage potential of cells from human primary and restenotic coronary artery plaques. *Int J Cardiol* 121:53-56.

Sun XY, An J (2004). Expression of nestin, an intermediate filament protein, in human fetal hepatic stem cells. *Di Yi Jun Yi Da Xue Xue Bao* 24:207-209.

Svachova H, Kovarova L, Stossova J, Potacova A, Pour L, Hajek R (2011). Impact of nestin analysis in multiple myeloma. *Klin Onkol* 24 Suppl:S53-57.

Taraseviciene-Stewart L, Kasahara Y, Alger L, Hirth P, Mc Mahon G, Waltenberger J, Voelkel NF, Tuder RM (2001). Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J* 15:427-438.

Teranishi N, Naito Z, Ishiwata T, Tanaka N, Furukawa K, Seya T, Shinji S, Tajiri T (2007). Identification of neovasculature using nestin in colorectal cancer. *Int J Oncol* 30:593-603.

Tilki D, Hohn HP, Ergun B, Rafii S, Ergun S (2009). Emerging biology of vascular wall progenitor cells in health and disease. *Trends Mol Med* 15:501-509.

Timmermans F, Plum J, Yoder MC, Ingram DA, Vandekerckhove B, Case J (2009). Endothelial progenitor cells: identity defined? *J Cell Mol Med* 13:87-102.

Toma JG, Akhavan M, Fernandes KJ, Barnabe-Heider F, Sadikot A, Kaplan DR, Miller FD (2001). Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 3:778-784.

Torsney E, Xu Q (2011). Resident vascular progenitor cells. *J Mol Cell Cardiol* 50:304-311.

Tuder RM, Groves B, Badesch DB, Voelkel NF (1994). Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol* 144:275-285.

Tuder RM, Yun JH (2008). Vascular endothelial growth factor of the lung: friend or foe. *Curr Opin Pharmacol* 8:255-260.

Vaittinen S, Lukka R, Sahlgren C, Hurme T, Rantanen J, Lendahl U, Eriksson JE, Kalimo H (2001). The expression of intermediate filament protein nestin as related to vimentin and desmin in regenerating skeletal muscle. *J Neuropathol Exp Neurol* 60:588-597.

van Suylen RJ, Smits JF, Daemen MJ (1998). Pulmonary artery remodeling differs in hypoxia- and monocrotaline-induced pulmonary hypertension. *Am J Respir Crit Care Med* 157:1423-1428.

Volkmann J, Muller D, Feuerstacke C, Kliesch S, Bergmann M, Muhlfeld C, Middendorff R (2011). Disturbed spermatogenesis associated with thickened lamina propria of seminiferous tubules is not caused by dedifferentiation of myofibroblasts. *Hum Reprod* 26:1450-1461.

von Bohlen Und Halbach O (2007). Immunohistological markers for staging neurogenesis in adult hippocampus. *Cell Tissue Res* 329:409-420.

Wagenvoort CA, Wagenvoort A, (1977). Pathology of pulmonary hypertension. New York, John Wiley & Sons.

Wagenvoort CA, Wagenvoort N, (1970). Primary pulmonary hypertension: a pathologic study of the lung vessels in 156 clinically diagnosed cases. *Circulation* 42:1163–1184.

Warburton D (2008). Developmental biology: order in the lung. *Nature* 453:733-735.

Warburton D, El-Hashash A, Carraro G, Tiozzo C, Sala F, Rogers O, De Langhe S, Kemp PJ, Riccardi D, Torday J, Bellusci S, Shi W, Lubkin SR, Jesudason E (2010). Lung organogenesis. *Curr Top Dev Biol* 90:73-158.

Warburton D, Gauldie J, Bellusci S, Shi W (2006). Lung development and susceptibility to chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 3:668-672.

Wedgwood S, Devol JM, Grobe A, Benavidez E, Azakie A, Fineman JR, Black SM (2007). Fibroblast growth factor-2 expression is altered in lambs with increased pulmonary blood flow and pulmonary hypertension. *Pediatr Res* 61:32-36.

Weissmann N, Dietrich A, Fuchs B, Kalwa H, Ay M, Dumitrascu R, Olschewski A, Storch U, Mederos y Schnitzler M, Ghofrani HA, Schermuly RT, Pinkenburg O, Seeger W, Grimminger F, Gudermann T (2006). Classical transient receptor potential channel 6 (TRPC6) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. *Proc Natl Acad Sci U S A* 103:19093-19098.

Weissmann N, Manz D, Buchspies D, Keller S, Mehling T, Voswinckel R, Quanz K, Ghofrani HA, Schermuly RT, Fink L, Seeger W, Gassmann M, Grimminger F (2005). Congenital erythropoietin over-expression causes "anti-pulmonary hypertensive" structural and functional changes in mice, both in normoxia and hypoxia. *Thromb Haemost* 94:630-638.

Wen D, You L, Zhang Q, Zhang L, Gu Y, Hao CM, Chen J (2011). Upregulation of nestin protects podocytes from apoptosis induced by puromycin aminonucleoside. *Am J Nephrol* 34:423-434.

Wiese C, Rolletschek A, Kania G, Blyszczuk P, Tarasov KV, Tarasova Y, Wersto RP, Boheler KR, Wobus AM (2004). Nestin expression--a property of multi-lineage progenitor cells? *Cell Mol Life Sci* 61:2510-2522.

Wilson DW, Segall HJ, Pan LC, Lame MW, Estep JE, Morin D (1992). Mechanisms and pathology of monocrotaline pulmonary toxicity. *Crit Rev Toxicol* 22:307-325.

Xue L, Ding P, Xiao L, Hu M, Hu Z (2011). Nestin is induced by hypoxia and is attenuated by hyperoxia in Muller glial cells in the adult rat retina. *Int J Exp Pathol* 92:377-381.

Yamahatsu K, Matsuda Y, Ishiwata T, Uchida E, Naito Z (2012). Nestin as a novel therapeutic target for pancreatic cancer via tumor angiogenesis. *Int J Oncol* 40:1345-1357.

Yang J, Cheng L, Yan Y, Bian W, Tomooka Y, Shiurba R, Jing N (2001). Mouse nestin cDNA cloning and protein expression in the cytoskeleton of transfected cells. *Biochim Biophys Acta* 1520:251-254.

Yeager ME, Frid MG, Stenmark KR (2011). Progenitor cells in pulmonary vascular remodeling. *Pulm Circ* 1:3-16.

Yoshida T, Owens GK (2005). Molecular determinants of vascular smooth muscle cell diversity. *Circ Res* 96:280-291.

Yu L, Hales CA (2011). Hypoxia does neither stimulate pulmonary artery endothelial cell proliferation in mice and rats with pulmonary hypertension and vascular remodeling nor in human pulmonary artery endothelial cells. *J Vasc Res* 48:465-475.

Yu Y, Fantozzi I, Remillard CV, Landsberg JW, Kunichika N, Platoshyn O, Tigno DD, Thistlethwaite PA, Rubin LJ, Yuan JX (2004). Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. *Proc Natl Acad Sci U S A* 101:13861-13866.

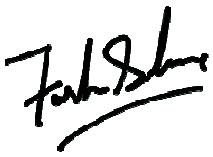
Zeltner TB, Burri PH (1987). The postnatal development and growth of the human lung. II. Morphology. *Respir Physiol* 67:269-282.

Zengin E, Chalajour F, Gehling UM, Ito WD, Treede H, Lauke H, Weil J, Reichenspurner H, Kilic N, Ergun S (2006). Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development* 133:1543-1551.

Zhang SX, Gozal D, Sachleben LR, Jr., Rane M, Klein JB, Gozal E (2003). Hypoxia induces an autocrine-paracrine survival pathway via platelet-derived growth factor (PDGF)-B/PDGF-beta receptor/phosphatidylinositol 3-kinase/Akt signaling in RN46A neuronal cells. *FASEB J* 17:1709-1711.

8 Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.



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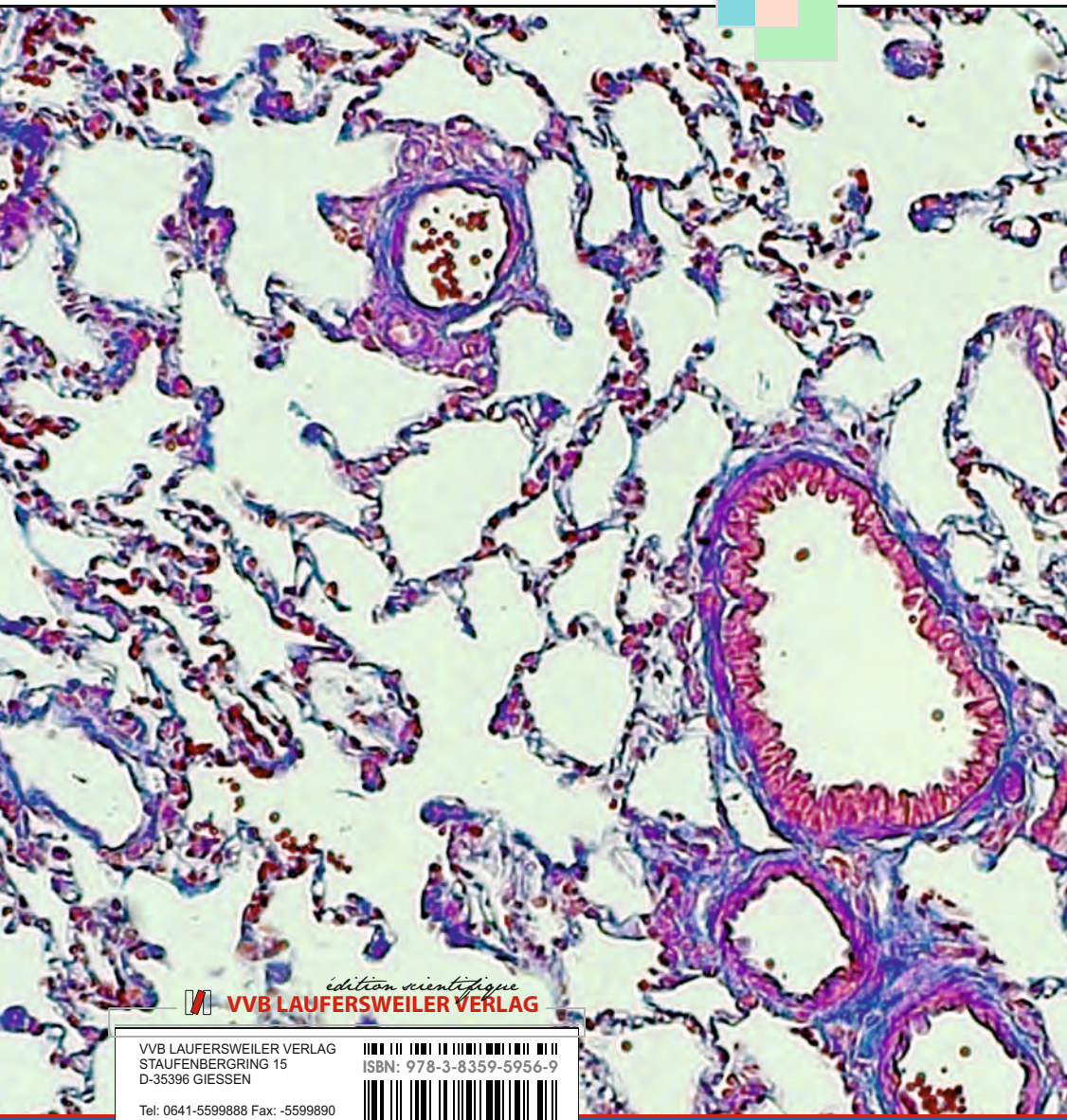

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