Pulmonary arterial hypertension -
smooth muscle cell proliferation and perlecan

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Pulmonary arterial hypertension – smooth muscle cell proliferation and perlecan

Abstract

Pulmonary hypertension (PH) is defined as a mean pulmonary arterial pressure (PAP) above 25 mmHg at rest or 30 mmHg with exercise\(^1\). PH is caused by several different factors, most commonly left heart disease and lung disease, for which the treatment is to optimize the underlying condition\(^2\). Category 1 PH, also known as pulmonary arterial hypertension (PAH) is, in comparison to other forms of PH, a small group but also the group attracting increased attention due to recent discoveries regarding the pathobiology\(^3,4\).

PAH can be either and isolated disease, drug or toxin induced, associated with systemic diseases, or with congenital heart disease (CHD)\(^2\). Regardless of etiology, PAH is a disease of the distal pulmonary arteries characterized by vasoconstriction, vascular remodeling, and coagulation abnormalities\(^3\). Current treatment is focused on the vasoconstriction and coagulation component. However, prognosis is still grim and recent meta-analyses show none or little change in mortality\(^5-7\).

Fortunately, thanks to knowledge of PAH pathobiology acquired in recent years, new therapeutic approaches are being investigated and developed. These potential therapies target mainly the vascular remodeling and smooth muscle cell (SMC) proliferation\(^3\).

Perlecan is the major heparan sulfate proteoglycan (HSPG), binding the majority of heparan sulfate (HS), in the vascular wall. In transgenic Hspg\(^{2\Delta/3\Delta}\) mice, which are deficient in perlecan HS, it has been shown that perlecan HS exert an inhibitory role on SMC proliferation in the peripheral circulation\(^8\). If perlecan HS has the same effect in the pulmonary arteries, novel drugs which increase perlecan levels might prove to be successful in treating pulmonary hypertension, by addressing the vascular remodeling component of the disease.

Here, isolation and culture of pulmonary artery SMCs (paSMCs) from transgenic Hspg\(^{2\Delta/3\Delta}\) mice and wild-type controls was carried out to determine paSMC proliferation in vitro.

In addition, a hypoxic model for PAH was used with Hspg\(^{2\Delta/3\Delta}\) mice and wild-type controls to determine paSMC proliferation, muscularization of pulmonary vessels, and PAH development in vivo.

Results obtained were none conclusive. Regarding the study of paSMC proliferation in vitro, no results were statistically significant. As for the hypoxia study in vivo, results showed an increased proportion of muscularized vessels in the hypoxic group compared to the normoxic group, indicating that the model works. Additionally, a trend was observed where Hspg\(^{2\Delta/3\Delta}\) mice developed more severe muscularization of vessels and PAH compared to wild-type controls. However, this trend was not statistically significant.

In summary, we now have functioning models for mouse paSMC proliferation both in vitro and in vivo. We stand by our initial hypothesis that perlecan HS exert a regulatory role on paSMCs preventing excessive proliferation. New experiments, both in vitro and in vivo, are needed to find out if the observed trends of mouse paSMC proliferation in vivo, muscularization of pulmonary arteries, and development of PAH are true. If so, perlecan may hold promise for future PAH patients.
Background

PH is present when the mean PAP exceed 25 mmHg at rest or 30 mmHg with exercise, although patients usually present with much higher levels of PAP, with only vague and insidious symptoms of increasing fatigue and dyspnea. PH may be caused by several different factors which are categorized according to the revised WHO classification of PH. Category 1 PH also known as PAH is the focus of this thesis. With a prevalence of 15 per million, PAH is a relatively small group compared to PH associated with left heart disease as well as with lung disease and hypoxia. However, PAH is also the group which is attracting increased attention because of recent discoveries regarding its pathobiology. This generates novel therapeutic strategies to be used for PAH and possibly other forms of PH.

PAH can be either an isolated disease (e.g. Idiopathic PAH (IPAH) and Hereditable PAH), drug- and toxin-induced (e.g. fenfluramine and metamphetamine), as well as associated with connective disease (e.g. systemic sclerosis), HIV, portal hypertension, chronic hemolytic anemia, schistosomiasis, and last but not least CHD. CHD occurs in 0.8-1% of live births worldwide and PAH associated with CHD (PAH-CHD) is a major complication in children with left-to-right shunts.

Since the etiology differs within the PAH group, so does the underlying pathobiology. However, great similarities also exist, especially regarding histopathology. PAH is a disease affecting the distal pulmonary arteries. Pulmonary vasoconstriction, vascular remodeling (i.e. endothelial cell (EC) and SMC proliferation), and coagulation abnormalities (i.e. thrombosis in situ) are the three major contributors to the narrowing of the pulmonary vessels of both patients and experimental animals with PH. Narrowing of the pulmonary vessels raises the pulmonary vascular resistance (PVR) and PAP, leading to increased right ventricle work load, hypertrophy, and ultimately right sided heart failure and death.

PAH is a serious disease. Untreated, median survival after diagnosis, best characterized for IPAH, is less than 2.8 years. Similar or worse survival is reported for PAH associated with systemic sclerosis and HIV. PAH-CHD is the only category which has notably better prognosis. Progression of PAH-CHD is slower and patients may survive for decades after diagnosis, most likely because the right ventricle has time to compensate for elevated PVR.

In the case of CHD and left-to-right shunting, PAH is caused by increased pulmonary blood flow, which induces increases in shear stress and circumferential stretch leading to endothelial dysfunction. The loss of endothelial barrier may be associated with degradation of extracellular matrix (ECM) and release of growth factors which in turn induce SMC proliferation and vascular remodeling. As mentioned above, vascular remodeling leads to increased PVR and therefore PAH. Once the PVR equals systemic resistance the left-to-right shunt becomes bi-directional, and when the PVR exceeds the systemic resistance, shunt reversal and development of Eisenmenger’s syndrome occurs. It is the most advanced form of PAH-CHD and when developed, it is considered inoperable and the pulmonary lesions irreversible. When it comes to shunt repair of CHD it is the associated PAH, its severity, and its response to therapy, which limit the surgical possibilities. The timing of surgery is critical, since shunt repair prevents blood flow from the right ventricle in any other direction than through the pulmonary vasculature, and may cause right ventricle failure. Surgery must be performed prior the onset of high pulmonary vascular resistance.
It is generally accepted that the vasoconstriction is the most important component early in the disease, but later on, as vascular remodeling develops the arteries become unresponsive to vasodilators. Despite this knowledge, current PAH therapy consists of agents that are primarily vasodilator with modest effects on cell proliferation.

The principal PAH therapies, aside from warfarin, are anti-vasoconstrictive agents such as L-type calcium channel blockers (e.g. nifedipine), prostacyclin (e.g. epoprostenol), endothelin receptor antagonists (e.g. bosentan), inhaled nitric oxide (NO) and phosphodiesterase-5 inhibitors (PDE5-inhibitors) (e.g. sildenafil). Side effects and pharmacokinetics limit their use. Calcium-channel blockers do not work for all patients, responders are identified by a vasodilator challenge where 10-15% are positive and only half of these will receive sustained clinical and hemodynamic benefits. Prostacyclins have a short half life and earlier required constant intravenous infusion. Modern alternatives like Iloprost can instead be inhaled 6-8 times a day, but the long term effects have not yet been established. NO has to be given as constant inhalation although its polyclinical alternative PDE5-inhibitors have shown promising results. Endothelin receptor antagonists have in previous studies shown effect on 6 minute walk distance, New York Heart Association (NYHA) class and mean PAP, but are associated with side effects such as liver function abnormality. Disappointingly, recent meta-analyses of controlled clinical trials in PAH, each of which studied at least one of either prostacyclin, endothelin receptor antagonists and/or PDE5-inhibitors, demonstrated that these agents induced only modest increases in functional capacity and slight hemodynamic improvement with none or little change in mortality.

Fortunately, because of knowledge about the pathobiology of PAH acquired in recent years, several new therapeutic approaches are being considered. These drugs are aimed at reversing sustained vasoconstriction and/or stopping or reversing abnormal cell growth and abnormal ECM protein deposition.

Rho-kinase signaling plays a key role in the PAH pathogenesis of various animal models, and the rho-kinase inhibitor fasudil has caused dramatic reduction in PAP in PAH animal models where traditional vasodilators had little or no effect.

Regarding cell proliferation, novel therapies attracting attention inhibit platelet derived growth factor (PDGF) e.g. Imatinib and PPARγ agonists. PPARγ is a nuclear receptor recently indicated to be central in both the pathobiology and possible treatment of PAH. Imatinib is approved for treatment of chronic myeloid leukemia, which is associated with a high PAH prevalence, and is shown to act on a wide variety of tyrosine kinases, e.g. the PDGF receptor β. It is shown to be capable of reversing PH in an animal model and there are case reports demonstrating that Imatinib is beneficial in reducing PAP and improving symptoms in human patients with PH. What may limit its use is that Imatinib is potentially cardio toxic. However, since more selective therapies targeting intracellular kinases are widely being considered to be the future of cancer treatment, this might hold promise for the future of PH patients as well.
Perlecan is a large HSPG, produced by both vascular ECs and SMCs, and a major component of the vascular ECM. Proteglycans are macromolecules consisting of a protein core with covalently bound glycosaminoglycan (GAG) side chains. The majority of proteglycan bound GAGs in the vascular wall are either of chondroitin-, dermatan-, or heparan sulfate (HS) subclass. Perlecan usually has three large HS side chains attached to the N-terminal of the molecule. HSPGs are structurally and functionally similar to heparin which is a potent inhibitor of SMC proliferation in both peripheral and pulmonary arteries. Perlecan inhibits proliferation by sequestering and limiting the levels of bioavailable growth factors such as PDGF, but perlecan may also stabilize growth factor-receptor complexes and thereby promote proliferation.

Null mutations of the perlecan Hspg2 gene are embryonically lethal. However, a targeted deletion of exon-3 in domain-I, thereby removing the major binding sites for HS results in live mice with perlecan molecules deficient in HS, i.e. Hspg2^{Δ3/Δ3}, previously described by Rossi et al. In peripheral vascular SMCs isolated from Hspg2^{Δ3/Δ3} mice, there is an increase of SMC proliferation in vitro in response to stimulation with various growth factors. Additionally, in a model of vascular injury where the carotid artery is ligated, Hspg2^{Δ3/Δ3} mice show increased arterial SMC proliferation in vivo compared to wild-type controls.

Hence, perlecan HS have an important regulatory role in the control of SMC proliferation in peripheral vascular disease. Previous studies have shown that heparin can inhibit paSMC proliferation in vitro. However, the role of endogenous perlecan HS in the development of pulmonary vascular remodeling and subsequent development of PAH is currently unknown.

Our hypothesis is that perlecan bound HS exert a regulatory role on paSMCs and prevent excessive proliferation. We aim to show that Hspg2^{Δ3/Δ3} mice, deficient of perlecan bound HS, have an accelerated development of PAH compared to normal wild-type controls in vivo and that isolated paSMCs from Hspg2^{Δ3/Δ3} mice show accelerated proliferation in vitro.
Materials and methods

Materials
Ham’s F-12 medium, Penicillin-Streptomycin (PEST), fetal calf serum (FCS), Trypsin, Versene, phosphate buffered saline (PBS) were purchased from GIBCO Invitrogen; bovine serum albumin (BSA), DAPI from SIGMA-Aldrich; L-ascorbic acid (AA) from Merck, Collagenase type II from Worthington and cell culture ware from Corning incorporated. Paraffin, Zinc formaldehyde solution 4%, and Pertex were purchased from Histolab; Tissue clear, DIVA Decloaker, Peroxidased, Background sniper, Rodent block, buffer Da Vinci Green, Negative control mouse, triss buffered saline (TBS), MACH 2 Double stain kit 1, Cardassian diaminobenzidine (DAB), Vulcan fast red from Biocare Medical; Monoclonal Mouse Anti-Human Smooth Muscle actin, polyclonal rabbit anti-Human von Willebrand factor (vWf), Fluorescent mounting medium from DAKO; Hematoxylin eosin (HE), normal horse serum (NHS), and DyLight 594 Horse Anti-Mouse IgG Antibody from Vector Laboratories.

Cells were cultured using routinely F-12 with 50 μg/ml L-ascorbic acid, 50 IU/ml penicillin, 50μg/ml streptomycin and 20 % FCS (F-12/20 % FCS). Cell cultures were split by washing with Versene followed by trypsinization. Passages 4 through 6 were used for experiments. Regarding all other procedures using F-12, the same concentrations of AA and PEST were consistently used.

Transgenic mice
We used transgenic Hspg2Δ3/Δ3 mice lacking exon 3 of the Perlecain Hspg2 gene, thus lacking attachment site for 3 HS side chains, previously described by Rossi et al.25. Mice in our experiments have been backbred for over 12 generations to the C57BL/6 background. Littermates were used as control. For paSMC isolation both male and female mice were used and for hypoxia experiments only female mice. Animals were fed standard rodent chow and water ad libitum. All experiments were approved by the local animal ethics committee.

Pulmonary artery SMCs - primary isolation and cell culture
Initially, primary isolation and culturing of distal paSMCs was tried by following the same protocol as below, except we did not isolate the pulmonary artery (PA) but the entire lungs. The lungs were digested as noted below in collagenase and cultured under optimized conditions for mouse SMCs with F-12/20% FCS. A homologous cell culture was obtained with characteristics of SMCs. However, we found no possibility of distinguishing vascular SMCs from bronchial SMCs and therefore did no further experiments with this cell line.

Subsequently, primary isolation of proximal paSMCs was achieved from Hspg2Δ3/Δ3 and wild-type C57BL/6 mice. Animals were euthanized by CO₂ asphyxiation. A small horizontal incision was made in the abdominal skin, followed by stripping of the entire skin making the exposed abdominal and thorax wall sterile. The abdominal wall was cut open, intestines put aside and the diaphragm cut open. Because of positive thoracic pressure, the lungs were deflated, and further thoracic operation enabled. The thoracic wall was cut along the axillary line and flipped up. With the heart exposed, a 1 ml syringe was used to draw blood from the right ventricle, minimizing subsequent bleeding.
Under Leica EZ4D stereomicroscope, the thymus was carefully removed, exposing the aorta, the left and right vena cava superior, and the PA trunk. The PA originates from the right ventricle descending in between the aorta and left vena cava superior. To access the PA, the aorta and both vena cava were removed and bleeding stopped using a compress. The PA and its branches to each lung were then dissected free from surrounding tissue, isolated and placed in a tube with F-12, AA and PEST. The groups of mice in each setting were 3 $Hspg2^{-/-/-}$ and 3 wild-type mice and the PAs in each group were pooled together.

In a laminar flow hood, further micro dissection of the isolated PAs was performed to separate the adventitia from the media layer. Sterile PBS was used to flush the vessel in a sterile Petri dish, making the adventitia swell and easier to remove. The PAs were then cut into small pieces and placed in 0.1% Collagenase type II in F-12 and incubated at 37°C for 4-6 hours with a shaker, Janke&Kunkel VX8. Collagenase was removed by centrifugation and the cell pellet seeded in culture medium F-12/20 % FCS.

**Immunocytochemistry**

$Hspg2^{-/-/-}$ and wild-type paSMCs from passage 4 were cultured in chamber slides for 24 hours. Fixation slides were treated with zinc formaldehyde 4 % for 10 min. Blocking of unspecific epitopes was made by both Rodent block and PBS/5% NHS during 20 min respectively. For SMC specific alpha-actin staining, the cells were incubated for 60 min with a monoclonal Mouse Anti-Human Smooth Muscle actin antibody (1:100 in PBS/5 %NHS), followed by 45 min incubation with DyLight secondary conjugated antibody (1:200 in PBS/5 %NHS). Staining for cell nuclei was made by incubation with DAPI fluorescence (1:20 000 in distilled water) for 15 min, which binds to A-T rich regions of DNA. Slides were subsequently mounted with DAKO fluorescent mounting medium. PBS was used as wash buffer between each step. Negative controls were performed. For microscopic evaluation of the staining Leica DMRB fluorescence microscope was used and pictures taken by Leica TCS SP5 confocal microscope.

**Cell Proliferation**

$Hspg2^{-/-/-}$ and wild-type paSMCs were plated 5000 cells/well in a 24-well plate. The area of each well was 1,5 cm². Cell counting was made using a Coulter counter VDA 140 from Analys Instrument AB and/or Kova Glastic slide 10 with grids from Hycor. Cells were cultured for 24 hours in F-12/20 % FCS before synchronization in F-12/0,5 % FCS for 24 hours. Day 0 cells were activated with F-12/5 % FCS or F-12/20 % FCS. Wells were trypsinized and cells counted day 0, day 3, and day 5.

**DNA synthesis**

Numerous attempts were made to determine $Hspg2^{-/-/-}$ and wild-type paSMC DNA synthesis activity in vitro using Bromodeoxyuridine (BrdU) Colorimetric kit from Roche. BrdU is incorporated into the DNA of proliferating cells in the S-phase and can then be detected by using an enzyme conjugated anti-BrdU antibody, followed by colorimetric detection in an ELISA reader. PaSMCs were plated 1000-2000 cells/well in 96-well plates, cultured, and synchronized as in the proliferation study. Cells were subsequently activated for 15 hours using F-12/5-20 % FCS as well as F-12/0-0,5% FCS + PDGF 10-40 ng/mL. BrdU was added and cells additionally incubated for 2-6 hours. However, this study yielded no results because of too low ELISA readings.
Hypoxia
A well established in vivo hypoxia model for PAH was used where Hspg2−/− and wild-type C57BL/6 mice were kept in a specially designed hypoxic chamber where oxygen content was lowered to 10% by substitution with nitrogen (Picture 1)\(^{16}\). This is equivalent to the oxygen level at 5000-6000 m above sea level and leads to increased paSMC proliferation, pulmonary hypertension and right sided heart hypertrophy\(^{27}\). As negative controls Hspg2−/− and wild-type mice were kept in normoxic but otherwise similar conditions. After 6 weeks animals were euthanized by CO\(_2\) asphyxiation. The thoracic wall was opened as noted above for paSMC isolation. With the heart exposed, a 1 ml syringe was used to inject the right ventricle with PBS, rinsing the pulmonary vasculature from blood. Lungs were perfused zinc formaldehyde via trachea to fixate the lung tissue. Subsequently, lungs were harvested and embedded in paraffin.

Immunohistochemistry and determination of muscularized vessels
Paraffin embedded mouse lung was sectioned in 5μm thick sections using a microtome and plated on glassslides. To rid the sections of paraffin, Tissue clear and alcohol 99-96-70% were used. The slides were then heated with DIVA Decloaker in a Histolab 2100-RETRIEVER during 20 minutes for antigen retrieval. Endogenous peroxidaze activity was quenched by incubation in Peroxidazed for 5 min. Blocking of unspecific epitopes was performed using Background sniper for 20 min. Primary antibodies, monoclonal mouse anti-human SMC alpha-actin (1:100 in buffer Da Vinci green) and polyclonal rabbit anti-Human vWf (1:1000 in buffer Da Vinci Green), were added and incubated 60 min together. Subsequently, double stain kit 1 from Biocare Medical was used. As chromogens, DAB and Vulcan fast red were used and staining evaluated by a Zeiss Axiostar light microscope. The sections were then counterstained with HE and mounted with Pertex. TBS was used as wash buffer between each step. Negative controls were performed.

For each animal, 3 sections of lung were stained, 1 for a negative control and 2 for analysis. With a Nikon Optiphot-2 microscope, histological evaluation was made at 10x magnification, and 16 photos/lung were taken for further analysis. ECs and therefore all vessels are vWF positive. SMCs stain specifically for SMC alpha-actin. Muscularized and non-muscularized vessels were counted and the percentage of muscularized vessels determined. Both photographing and analysis were performed by a blinded observer.

Statistical analysis
Differences between the experimental groups analyzed in vitro and in vivo were tested by unpaired Student’s t test. Data are expressed as mean with standard error of the mean (SE). P-value < 0.05 was considered significant.
Results

Characterization of paSMCs

$Hspg2^{+/+}$ and wild-type cell isolations from passage 4 were characterized as paSMCs. From the primary cell isolation, the yield from $Hspg2^{+/+}$ was smaller than that from the wild-type mice. Hence, fewer cells and lower cell concentrations were obtained in the $Hspg2^{+/+}$ cell line. This difference was aggravated by each passage and is illustrated by picture 2 A–B.

However, both $Hspg2^{+/+}$ and wild-type cell lines had characteristics of mouse SMCs. Additionally, cells from passage 4 were stained by fluorescent immunocytochemistry with DAPI and SMC specific alpha-actin. >99% of all visible cells, both $Hspg2^{+/+}$ and wild-type, were positive for SMC alpha-actin (picture 3).

Picture 3. Immunocytochemistry of mouse paSMCs. DAPI (blue) stain cell nuclei and SMC alpha-actin (red) stain SMCs specifically. >99% of all DAPI positive cells were SMC alpha-actin positive.
PaSMC proliferation in vitro

Mutant Hspg2Δ3/Δ3 and wild-type paSMCs from passage 6 were plated 5000 cells/well. Each group and time point consisted of 3-4 wells. Cells were cultured, synchronized and subsequently activated by F-12/5% FCS (Figure 1 A) or F-12/20% FCS (Figure 1 B). Cells were counted day 0, day 3, and day 5. Trends where mutant Hspg2Δ3/Δ3 paSMCs proliferate in less extent than wild-type paSMCs are discernible but not statistically significant.

Figure 1 A. PaSMCs were plated 5000 cells/well, synchronized by F-12/0.5% FCS and activated by F-12/5% FCS. Cell number is shown as mean ± standard error of the mean.

Figure 1 B. PaSMCs were plated 5000 cells/well, synchronized by F-12/0.5% FCS and activated by F-12/5% FCS. Cell number is shown as mean ± standard error of the mean.
PaSMC proliferation in vivo – Hypoxia

Mutant Hspg2\(^{Δ3/Δ3}\) and wild-type mice were kept in either hypoxic or normoxic conditions during 6 weeks. Subsequently, the lungs were harvested and stained for SMC alpha-actin, vWF and HE (picture 4). The percentage of SMC alpha-actin positive, i.e. muscularized vessels, was determined. The experiment was initially made with 3 mice in each group (figure 2 A) and repeated with 6 mice in each hypoxic group and 3 mice in each normoxic group (figure 2 B). Figure 2 A show statistical significant difference between the hypoxic and normoxic groups (P<0.05). Between mutant Hspg2\(^{Δ3/Δ3}\) and wild-type mice in the hypoxic group, a trend is discernible where Hspg2\(^{Δ3/Δ3}\) mice show a higher percentage of muscularized vessels, compared to wild-type controls. However, this trend is not statistically significant in neither figure 2 A nor 2 B. Due to staining difficulties and unspecific alpha-actin uptake in the second experiment, figure 2 B show a generally higher percentage of muscularized vessels compared to figure 2 A.

![Picture 4. Light microscopy x10 of mouse lung. Vessels indicated by vWF (brown) and muscularized vessels by SMC alpha-actin (red). Arrows indicate vessels positive or negative for alpha-actin.](image)

Figure 2 A-B. The percentage of muscularized vessels is shown as mean ± 2 standard error of the mean. Figure 2 A show statistical significant difference between the hypoxic and normoxic groups (*P<0.05).
Discussion

PAH can be either an isolated disease, drug or toxin induced, associated with systemic diseases, or with CHD\(^2\). PAH-CHD is a major complication in children with left-to-right shunts\(^10\). Regardless of etiology PAH is a disease of the distal pulmonary arteries characterized by vasoconstriction, vascular remodeling and coagulation abnormalities\(^3\). Recent meta-analyses show none or little change in mortality with current treatment, which mainly targets vasoconstriction and coagulation abnormalities\(^5\)\(^\text{-}\)\(^7\). Because of this, and thanks to recent discoveries in PAH pathobiology, new therapeutic possibilities are being researched. These potential therapies target mainly the vascular remodeling and SMC proliferation\(^3\).

Perlecan is the major HSPG in the vascular wall, binding the majority of HS there. It is previously shown in peripheral vascular SMCs from transgenic \(Hspg2^{\text{43/43}}\) mice, deficient in perlecan HS, that there is an increase in SMC proliferation \textit{in vitro} and also that \(Hspg2^{\text{43/43}}\) mice show increased SMC proliferation in peripheral arteries \textit{in vivo} compared to wild-type controls\(^8\). Perlecan is considered to exert a regulatory role in peripheral vascular SMCs, preventing excessive proliferation.

To determine whether perlecan has the same role in pulmonary arteries, an \textit{in vitro} proliferation study was performed with primary isolation of paSMCs from \(Hspg2^{\text{43/43}}\) and wild-type mice. We also performed an \textit{in vivo} study where \(Hspg2^{\text{43/43}}\) and wild-type mice were kept in hypoxic conditions during 6 weeks and subsequently determined the percentage of muscularized vessels in the lungs.

Regarding the \textit{in vitro} study, none of the results were statistically significant. A trend is however discernible where \(Hspg2^{\text{43/43}}\) proliferate less than wild-type paSMCs. This could possibly be explained by how the paSMCs were isolated and cultured. During primary isolation, the yield of paSMCs from \(Hspg2^{\text{43/43}}\) was smaller than that from wild-type mice. When culturing and splitting the cells, compensation and correction of differences in cell numbers were not made. Hence, the \(Hspg2^{\text{43/43}}\) paSMCs had to proliferate more than the wild-type paSMCs, making every passage more strenuous for the \(Hspg2^{\text{43/43}}\) cells. Since the proliferation experiment was made with paSMCs from passage 6, this is likely to have affected outcome. Because of this, and also because the differences were not statistically significant, new primary isolation of paSMCs and new proliferation studies need to be carried out to determine the influence of perlecan HS on mouse paSMC proliferation \textit{in vitro}.

The \textit{in vivo} hypoxia experiment was carried out twice. Statistical significant differences between the hypoxic and normoxic group, in both \(Hspg2^{\text{43/43}}\) and wild-type mice, are present in the first experiment. This shows that the hypoxic model is a functioning model for PAH in mouse and in our hands. In both hypoxia experiments results show a trend where \(Hspg2^{\text{43/43}}\) mice develop more severe PAH than wild-type controls. However, this trend did not reach statistical significance. This could be because of too few animals in the different groups. Also, in the second experiment, due to staining difficulties and unspecific alpha-actin uptake a generally higher percentage of muscularized vessels, i.e. alpha-actin positive vessels, is shown in figure 2 B.
In summary, we now have functioning models for mouse paSMC proliferation both in vitro and in vivo. However, we have no conclusive results at this point. Considering the above results, we stand by our initial hypothesis that perlecan HS exert a regulatory role on paSMCs preventing excessive proliferation. To further investigate this we are planning to repeat the in vitro study where we now have a functioning protocol for mouse paSMC primary isolation. In each split cell counting will be performed and corrections made to adjust cell numbers in Hspg2^+/− and wild-type cell lines. By doing so, we will gain reliable proliferation data on paSMCs proliferation in culture. Additional in vitro DNA synthesis experiments are also planned. Regarding the in vivo hypoxia experiments, we now have a functioning model for PAH in mouse. New experiments are needed including more animals to find out if the observed trends in mouse paSMC proliferation in vivo, muscularization of pulmonary arteries and development of PAH are indeed true. If so, perlecan might hold promise for future PAH patients.

My part

This thesis is a presentation of my 30 Hp Degree project in Medicine at Uppsala University. It has been carried out under individual supervision by Kiet Tran, MD PhD, Department of Surgical Sciences, Uppsala University. Regarding practical work, I have carried out the entire paSMC proliferation in vitro study, from planning and creating protocols for primary isolation to managing cell culture, proliferation studies, and analyzing results. As for the hypoxia in vivo study, what is presented in my thesis is what I have performed together with Ya-Ting Chang, MD PhD-student, Department of Molecular Medicine, Karolinska Institute. I have retrieved results and taken part in discussion and analysis of results. Additionally, I have attended a course in laboratory animal science and am now certified to work independently with the animal experiments described in this thesis, as well as in future studies.

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