UNIVERSITY OF CRETE / FACULTY OF MEDICINE

Μεταπτυχιακό Πρόγραμμα Σπουδών

"Κυτταρική & Γενετική Αιτιολογία, Διαγνωστική & Θεραπευτική των Ασθενειών του Ανθρώπου"

Τ.Θ. 2208, 71003 Ηράκλειο, Κρήτη

Graduate Program

"The Molecular Basis of Human Disease"

P.O.Box 2208, 71003 Heraklion-Crete, Creece

The role of miRNAs in the pathogenesis of pulmonary arterial hypertension

by

KONSTANTINOU K. GEORGIOS, MD

Research Fellow in Dr. Stella Kourembanas' Laboratory

Division of Newborn Medicine / Children's Hospital Boston

Harvard Medical School

Thesis

Submitted for the Degree of Master of Science in the
Department of Medicine at University of Crete

Heraklion, July 2011

Scientific Committee

S. Alex Mitsialis, PhD
Dimitris Kardassis, PhD

Acknowledgements

I would like to offer my gratitude:

To my Professors Dr. Stella Kourembanas and Dr. S. Alex Mitsialis, for their constant supervision, help and support and for giving me the opportunity to be a part of the Children's Hospital of Boston Division of Newborn Medicine.

To my mentor Dr. Vassilis Zannis, for his valuable advice and guidance during the entire period of my undergraduate and postgraduate studies.

To the Graduate Program “The Molecular Basis of Human Disease” and to Dimitrios T. Boumpas, MD, FACP (Director of the Graduate Program, Professor of Medicine) and to Dr. Kardasis, PhD (Associate Director of the Graduate Program, Professor of Biochemistry) for their permission to continue and finish my Master in Boston.

To all members of Dr. Kardasis’ Lab (University of Crete) and Dr. Kourembanas’ Lab (Children’s hospital, Harvard Medical School) for helping me during my first steps as a research scientist. Special thanks to Dr. Eleni Vergadi, Dr. Eleni Maneta and Polina Nikolaou for their time, patience, and understanding.

Table of Contents

Περίληψη

Η πνευμονική αρτηριακή υπέρταση είναι νόσος η οποία χαρακτηρίζεται από αυξημένη πνευμονική αρτηριακή πίεση, αγγειοσυστολή, αναδιαμόρφωση και πάχυνση του αγγειακού τοιχώματος με τελικό αποτέλεσμα την υπερτροφία και ανεπάρκεια της δεξιάς κοιλίας.

Η φλεγμονή του πνευμονικού παρεγχύματος εμπλέκεται στην ανάπτυξη και διατήρηση της αναδιαμόρφωσης των πνευμονικών αγγείων και ένας μεγάλος αριθμός μελετών υποστηρίζει ότι αποτελεί πιθανή αιτία ανάπτυξης πνευμονικής υπέρτασης. Προ-φλεγμονώδεις κυτοκίνες και κυτταροκίνες, όπως είναι ο CCL2 και ο CCL12 (παλαιότερα γνωστοί και ως χημειοτακτικοί παράγοντες των μονοκυττάρων; MCP-1, MCP-5), εμπλέκονται άμεσα στην ανάπτυξη πνευμονικής υπέρτασης. Τα επίπεδα αυτών των κυτοκινών βρέθηκαν να σχετίζονται με την επιβίωση ασθενών. Η μελέτη αυτής της νόσου γίνεται συχνά με μοντέλα ζώων τα οποία εκτίθενται σε υποξία. Έκθεση των ποντικιών σε χρόνιες συνθήκες υποξίας (8.5% O_2 για 21 ημέρες) έχει ως αποτέλεσμα την αγγειοσυστολή και πάχυνση του αγγειακού τοιχώματος (αύξηση έκφρασης ακτίνης λείου μυικού ιστού στα κύτταρα) και αναπαριστά την αύξηση της συστολικής πίεσης της δεξιά κοιλίας και την παθολογία της ανθρώπινης νόσου.

Οι οξυγενάσες της αίμης (heme oxygenases, HO) είναι ένζυμα τα οποία καταλύουν τη μετατροπή της αίμης σε χολερυθρίνη, μονοξείδιο του άνθρακα (CO) και σίδηρο και εμπλέκονται συχνά στη ρύθμιση της φλεγμονώδους απάντησης του οργανισμού. Πρόσφατα αποτελέσματα από το εργαστήριο μας έχουν δείξει πως η συνεχής τοπική έκφραση της οξυγενάσης της αίμης – 1 (HO-1) στον πνεύμονα μπορεί να καταστείλει τόσο την πνευμονική φλεγμονή όσο και τη μετέπειτα εμφάνιση της πνευμονικής υπέρτασης. Επιπλέον η επαγόμενη τοπική έκφραση στον πνεύμονα της HO-1 έχει βρεθεί να προκαλεί μετατροπή στο φαινότυπο των κυψελιδικών μακροφάγων των υποξικών ποντικών, σε προ-φλεγμονώδη τύπο, ο οποίος προστατεύει από την μετέπειτα εμφάνιση πνευμονικής υπέρτασης. Θεραπεία HO -/- ποντικών με ένα από τα προϊόντα της HO-1 το μονοξειδίου του άνθρακα (CO), κατέστειλε την αναδιαμόρφωση και πάχυνση του αγγειακού τοιχώματος αυτών των ποντικών.

Τα μικρομοριακά RNA (microRNAs) ανήκουν σε μια κατηγορία μετά-μεταγραφικών ρυθμιστικών παραγόντων. Ανακαλύφθηκαν για πρώτη φορά το 1993

από τους Victor Ambros, Rosalind Lee και Rhonda Feinbaum κατά τη διάρκεια μιας μελέτης για την ανάπτυξη στο νηματώδη C.elegans. Πρόκειται για σύντομες ~ 22 νουκλεοτιδικές αλληλουχίες RNA που συνδέονται με συμπληρωματικές ακολουθίες στο UTR 3' των πολλαπλών mRNAs στόχων τους. Με τη σύνδεση τους στα mRNA των πρωτεϊνών-στόχων επάγουν την αποδόμηση τους με αποτέλεσμα την καταστολή της σύνθεσης της συγκεκριμένης πρωτεΐνης. Τα microRNAs εμπλέκονται στην ανάπτυξη και πολλαπλασιασμό των κυττάρων, στην διαφοροποίηση, στην απόπτωση, στη ρύθμιση του ανοσοποιητικού συστήματος ακόμη και στον καρκίνο. Μέχρι σήμερα έχουν χαρακτηριστεί περισσότερα από 90 επαγόμενα από την υποξία microRNAs.

Στην παρούσα μελέτη, χρησιμοποιήσαμε ένα διαγονιδιακό μοντέλο ποντικού το οποίο δημιουργήθηκε από τη διασταύρωση ποντικών, που εκφράζουν τον ενεργοποιητή της μεταγραφής της τετρακυκλίνης υπό τον έλεγχο του υποκινητή των επιθηλιακών κυττάρων Clara cells του πνεύμονα με ποντικούς που φέρουν το ανθρώπινο διαγονίδιο της HO-1 υπό την έκφραση του υποκινητή του βακτηριακού οπερονίου της τετρακυκλίνης (TRE), και έτσι υπερεκφράζει HO-1 με επαγόμενο ιστοειδικό τρόπο (tetON σύστημα). Ελέγχοντας έτσι την δράση της HO-1 και κατ' επέκταση της πνευμονικής φλεγμονής, στόχος μας ήταν να διασαφηνίσουμε την φύση της φλεγμονώδους ανοσοαπάντησης στην υποξία και τον ρόλο της στην ανάπτυξη της πνευμονικής υπέρτασης. Διαγονιδιακά ποντίκια με συνεχή έκφραση της HO-1 (SHO1) έχουν επίσης χρησιμοποιηθεί.

Η συστολική πίεση στη δεξιά κοιλία και ο δείκτης Fulton είχαν αυξηθεί σημαντικά στα διαγονιακά μας ποντίκια μετά από 21 ημέρες έκθεση σε υποξία. Η χορήγηση τετρακυκλίνης καθ' όλη την διάρκεια της έκθεσης σε υποξία απέτρεψε την αύξηση της συστολική πίεσης στη δεξιά κοιλία και του δείκτη Fulton στα ποντίκια αυτά σε σύγκριση με τα ποντίκια στα οποία χορηγήθηκε μόνο νερό.

Στη μελέτη μας έχουμε βρει ότι οι μεταγραφικοί παράγοντες fork-head box S1 (Foxs1) και SRY box 17 (Sox17), οι οποίοι σχετίζονται με τη διαφοροποίηση των ενδοθηλιακών κυττάρων και την αναδιαμόρφωση του αγγειακού τοιχώματος, αυξάνονται κατά την έκθεση των ποντικών σε υποξία. Ακόμη, σημαντική ήταν και η αύξηση ενός νέου γονιδίου στόχος του Hypoxia-inducible factor 1 (HIF-1), Ankyrin repeat domain 37 (Ankrd37). Επιπλέον, ο Hypoxia-induced mitogenic factor (HIMF/FIZZ1), τυπικός δείκτης της εναλλακτικής οδού ενεργοποίησης των

μακροφάγων (M2) είχε μειωθεί από την HO-1 τόσο στον πνεύμονα όσο και στα κυψελιδικά μακροφάγα των υποξικών ποντικών. Τέλος οι παράγοντες CCL2 και ο CCL12, μη κανονικοί επαγωγείς του M2 φαινοτύπου βρέθηκαν να επηρεάζονται από την χορήγηση μονοξειδίου του άνθρακα (CO).

Ο πιθανόν ρόλος του miR-155 στη μετατροπή του φαινοτύπου στα μακροφάγα έχει επίσης διερευνηθεί. Συγκεκριμένα η χορήγηση CO αύξησε την έκφραση του miR-155 στον πνεύμονα, το οποίο έχει ήδη αποδειχθεί πως σχετίζεται με τα επίπεδα του υποδοχέα της ιντερλευκίνης-13 (IL-13) και ως εκ τούτου με τον M2 φαινότυπο στα μακροφάγα. Περαιτέρω, για να κατανοήσουμε το μοτίβο έκφρασης των microRNAs τα οποία επάγονται από την HO-1 κατά την διάρκεια των πρώτων σταδίων ανάπτυξης πνευμονικής υπέρτασης, έχουμε κάνει ένα miRNA array.

Η κατανόηση των κυτταρικών και μοριακών μηχανισμών οι οποίοι ευθύνονται για την ανάπτυξη της πνευμονικής υπέρτασης θα βοηθήσει στην ανάπτυξη στοχευμένων φαρμάκων τα οποία θα συμβάλουν στην θεραπεία της νόσου.

Summary

Pulmonary hypertension (PH) is a disease characterized by elevated pulmonary artery pressure, vasoconstriction, cell proliferation and vascular wall remodeling with resultant right ventricular hypertrophy and failure.

Lung inflammation has an important role in the initiation and maintenance of vascular remodeling and an increasing number of studies support the idea that this remodeling is a potential cause for the development of pulmonary hypertension. Pro-inflammatory cytokines and chemokines are involved in the pathogenesis of PH, such as CCL2 and CCL12. The levels of these cytokines are thought to be related to patients' survival. PH is most commonly studied using the hypoxic animal model. Long-term exposure of mice to hypoxia leads to muscularization of small pulmonary arteries - increased expression of smooth muscle actin in cells - and recapitulates the elevation of right ventricular systolic pressure and the pathology of human disease.

Heme oxygenases (HO) are the rate-limiting enzymes that catalyze the conversion of heme into biliverdin, carbon monoxide (CO), and free iron. An increasing number of studies implicate HO-1 in the regulation of inflammation. Results from our lab indicate that lung-specific constitutive expression of HO-1 can suppress both the lung inflammation and the later development of pulmonary hypertension. Furthermore, overexpression in a lung-specific way of HO-1 was found to induce a switch in macrophage polarity toward an anti-inflammatory phenotype, and this effect was associated with protection from PH. Interestingly treatment of HO-$1^{-/-}$ mice with inhaled CO also protected them from pulmonary vascular remodeling.

MicroRNAs (miRNAs) are small noncoding transcripts of 16 to 29 nucleotide RNAs that regulate gene expression posttranscriptionally by targeting mRNAs. MicroRNAs have been shown to play important biological roles in various contexts; during development, cell differentiation, and immune regulation and also in pathologies such as cancer. To date more than 90 hypoxia-regulated miRNAs (HRMs) have been discovered.

In the present study, we utilized a bitransgenic mouse model, that was generated by crossing of mice that express the reverse tetracycline transactivator (rtTA) under the control of Clara cell secretory protein promoter (CC10) with mice

that harbor the human HO-1 transgene under the control of the bacterial tetracycline response element (TRE) and expressed HO-1 in a lung-specific, inducible way (tetOn system). By turning on and off HO-1 activity and modulating lung inflammatory response, our goal was to shed light on the nature of this hypoxia-induced inflammatory response and its role in the later development of the disease. Transgenic (SH01) mice for the HO-1 gene were also used.

Right ventricular systolic pressure, the Fulton Index, and the medial wall thickness index, were significantly elevated after 21 days of hypoxia in our bitransgenic mice. Doxycycline administration for the entire course of hypoxia prevented the increase in right ventricular systolic pressure, the Fulton Index, and the medial wall thickness index in a comparison with the water treaded control mice.

It has been shown in our study that hypoxia leads to induction of the fork-head box S1 (Foxs1) and the SRY box 17 (Sox17) transcription factors, which are found to be involved in the regulation of endothelial cell differentiation and vessel remodeling. Furthermore a striking increase of the Ankyrin repeat domain 37 (Ankrd37) a novel Hypoxia-inducible factor 1 (HIF-1) target gene was also observed. Hypoxia-induced mitogenic factor (HIMF/FIZZ1), a well-defined marker of alternative activated macrophages (M2) was altered by HO-1 expression both in the total lung and in the alveolar macrophages. Finally, the chemokine (C-C motif) ligand 2 (CCL2) and the chemokine (C-C motif) ligand 12 (CCL12) noncanonical inducers of M2 polarization were found to be suppressed by CO administration.

A potential role of miR-155 in the polarization of macrophages was demonstrated. Specifically CO induced the expression of miR-155 in total lung, which has been reported to target and decrease IL-13 receptor levels and subsequently the expression of the M2 phenotype. Moreover in order to identify the expression pattern of miRNAs induced by HO-1 during the first stages of pulmonary hypertension a miRNA array was performed.

Further understanding of the cellular and molecular pathways that contribute to the progression of pulmonary hypertension is needed, in order to lead to the development of targeted therapies, which may raise hope for the cure.

A. Introduction

a. Pulmonary arterial hypertension

Pulmonary hypertension (PH) is a disease characterized by elevated pulmonary artery pressure, which often results in right ventricular hypertrophy and failure. Pulmonary hypertension in the literature is defined as a mean pulmonary artery pressure greater than 25 mmHg at rest or greater than 30 mmHg during exercise (measured by right heart catheterization).[1] It may be idiopathic, familial, or associated with multiple other diseases.[2] PH occurs in men and women of any race or age.

PH was previously considered as either primary or secondary; however, at the present time, this classification is thought to be insufficient. The revised World Health Organization (WHO) classification system is now used to characterize and classify patients with PH.[3-4] WHO classification system categorizes patients with PH in five groups.(Table 1) Patients in the first group are considered to have pulmonary arterial hypertension (PAH), whereas patients in the remaining four groups are considered to have pulmonary hypertension (PH). The term PH is generally used when all five groups are discussed collectively.

The prognosis of PH is variable and depends on the severity of both the PH and the underlying disease. Early identification and treatment of pulmonary hypertension (PH) is commonly recommended as advanced disease may be less responsive to therapy.[5-7] Patients with idiopathic pulmonary arterial hypertension (IPAH) who do not receive treatment have an average survival of approximately three years. Symptomatic patients with PAH that is associated with another disease (eg, liver disease, systemic sclerosis) usually have worse prognosis than patients with IPAH.[8] Patients with severe PAH or right heart failure die sooner, usually within one year when left without treatment.

Factors that may indicate a poor prognosis include age at presentation greater than 45 years, pericardial effusion, large right atrial size, elevated right atrial pressure, septal shift during diastole, decreased pulmonary arterial capacitance, increased N-terminal brain natriuretic peptide level, and perhaps hypocapnia.[9-10]

Table 1 **Updated Clinical Classification of Pulmonary Hypertension (Dana Point, 2008)**

1. Pulmonary arterial hypertension (PAH)
1.1. Idiopathic PAH
1.2. **Heritable**
1.2.1. **BMPR2**
1.2.2. **ALK1, endoglin (with or without hereditary hemorrhagic telangiectasia)**
1.2.3. **Unknown**
1.3. Drug- and toxin-induced
1.4. Associated with
1.4.1. Connective tissue diseases
1.4.2. HIV infection
1.4.3. Portal hypertension
1.4.4. Congenital heart diseases
1.4.5. **Schistosomiasis**
1.4.6. **Chronic hemolytic anemia**
1.5 Persistent pulmonary hypertension of the newborn

1′. **Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)**

2. Pulmonary hypertension owing to left heart disease
2.1. **Systolic dysfunction**
2.2. **Diastolic dysfunction**
2.3. Valvular disease

3. Pulmonary hypertension owing to lung diseases and/or hypoxia
3.1. Chronic obstructive pulmonary disease
3.2. Interstitial lung disease
3.3. **Other pulmonary diseases with mixed restrictive and obstructive pattern**
3.4. Sleep-disordered breathing
3.5. Alveolar hypoventilation disorders
3.6. Chronic exposure to high altitude
3.7. Developmental abnormalities

4. **Chronic thromboembolic pulmonary hypertension (CTEPH)**

5. **Pulmonary hypertension with unclear multifactorial mechanisms**
5.1. **Hematologic disorders: myeloproliferative disorders, splenectomy**
5.2. **Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis:** lymphangioleiomyomatosis, neurofibromatosis, vasculitis
5.3. **Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders**
5.4. **Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis**

Table 1. Dana Point classification 4th World Symposium on PH, Dana Point, California 2008

Pathogenesis of pulmonary hypertension

The pathogenesis of pulmonary arterial hypertension (PAH) is complex, involving multiple modulating genes and environmental factors. Multifactorial impairment of the physiologic balance can lead to vasoconstriction, cell proliferation, fibrosis, inflammation, remodeling and in-situ thrombosis.[11] The pathological findings include intimal hyperplasia and fibrosis, medial hypertrophy, and in situ thrombi of the small pulmonary arteries and arterioles.[12-13]

PAH is a disorder with a proliferative nature, thus it is considered by some to be analogous to cancer. A multiple hit hypothesis has been formed. In other words, patients with PAH may have an underlying genetic predisposition to pulmonary vascular disease and a superimposed second hit then activates the disease process.[14-15] The process may involve increased endothelin levels (a vasoconstrictor and mitogen), decreased nitric oxide levels (nitric oxide is a vasodilator and is antiproliferative), and/or decreased prostacyclin levels (prostacyclin is a vasodilator, is antiproliferative, and inhibits platelet function).[12] As a result advanced therapies have emerged that inhibit endothelin, promote nitric oxide activity, and replace prostacyclin.

Predisposition to pulmonary vascular disease may be associated with genetic mutations in the bone morphogenetic protein receptor type II (BMPR2), activin-like kinase type 1, and/or 5-hydroxytryptamine (serotonin) transporter (5HTT) genes. BMPR2 is considered a member of the transforming growth factor beta family. Abnormal BMPR2 may play an important role in the pathogenesis of IPAH, with up to 25 percent of patients with IPAH having abnormal BMPR2 structure or function.[16] The BMPR2 pathway induces apoptosis in some types of cells and it has been hypothesized that abnormal pathway activity may permit excess endothelial cell growth and proliferation in response to a variety of injuries.[17-18] This is supported by a number of studies: transgenic mice with smooth muscle specific deletion of BMPR2 have pulmonary hypertension.[19] The activin-like kinase type 1 receptor is another member of the transforming growth factor beta family. Mutations have been identified in some patients with hereditary hemorrhagic telangiectasia and PAH.[20] 5HTT activity is related to pulmonary artery smooth muscle hypertrophy. The L-allelic variant of the 5HTT gene promoter is associated with increased activity of

5HTT and is found in a greater percentage of patients with idiopathic PAH compared to controls.[21]

The second hit that activates PAH may also be another genetic disorder, increased flow secondary to a congenital left-to-right shunt, an infectious organism such as human herpes virus-8 (HHV-8) or human immunodeficiency virus (HIV), altered structure or function of a membrane ion channel, drugs (anorexigens), inflammatory mediators, or cytokines.[13, 22-24]

The role of inflammation in the development of pulmonary hypertension

Inflammation plays an important role in the initiation and maintenance of vascular remodeling in the most common animal models of pulmonary hypertension (PH). An increasing number of studies support the idea of inflammation-mediated remodeling as a potential cause for the development of pulmonary hypertension.[25] Inflammatory cell infiltrates, mainly composed of T and B lymphocytes, macrophages, mast cells and dendritic cells (DCs) have been found in the walls of pulmonary arteries and perivascular areas of the plexiform lesions from patients with PAH. [26-27] Perros et al. [28] demonstrated immature DC population infiltrating vascular lesions in both IPAH patients and in experimental PH. Accumulation of these professional antigen-presenting cells may involve presentation of antibodies to endothelial cells, fibroblasts and nuclear antigens that are found in the serum of patients with IPAH and collagen vascular disease-associated PAH. [29]

Pro-inflammatory cytokines and chemokines are involved in the pathogenesis. High levels of proinflammatory cytokines such as interleukin (IL)-1 and IL- 6, are detected in pulmonary arteries, of patients with PAH. [30] Fractalkine (CX3CL1), a chemokine that facilitates leukocyte recruitment, has been found to be unregulated in the T lymphocytes of PAH patients. CC chemokine ligand 2 also known as monocyte-chemotacticprotein 1 (MCP-1) was detected at high concentrations in the plasma as well as in lung tissues of patients with IPAH as compared with healthy controls. Interestingly, this chemokine was overproduced by pulmonary endothelial cells (EC) and has been shown to increase migration of monocytes. [31-32] CXC-chemokine ligand 10 (CXCL10) is also involved in the pathogenesis of PAH. Thus, higher levels of CXCL10 were detected in patients with IPAH than in healthy controls. Since

CXCL10 inhibits angiogenesis, its increased activity could lead to decreased vascular remodeling and to an improvement of outcome in IPAH through the recruitment of T cells and the inhibition of angiogenesis. [33] A number of circulating autoantibodies including antifibroblast antibodies and anti-endothelial antibodies are also present in the peripheral blood of patients with PAH. [34] (Figure 1)

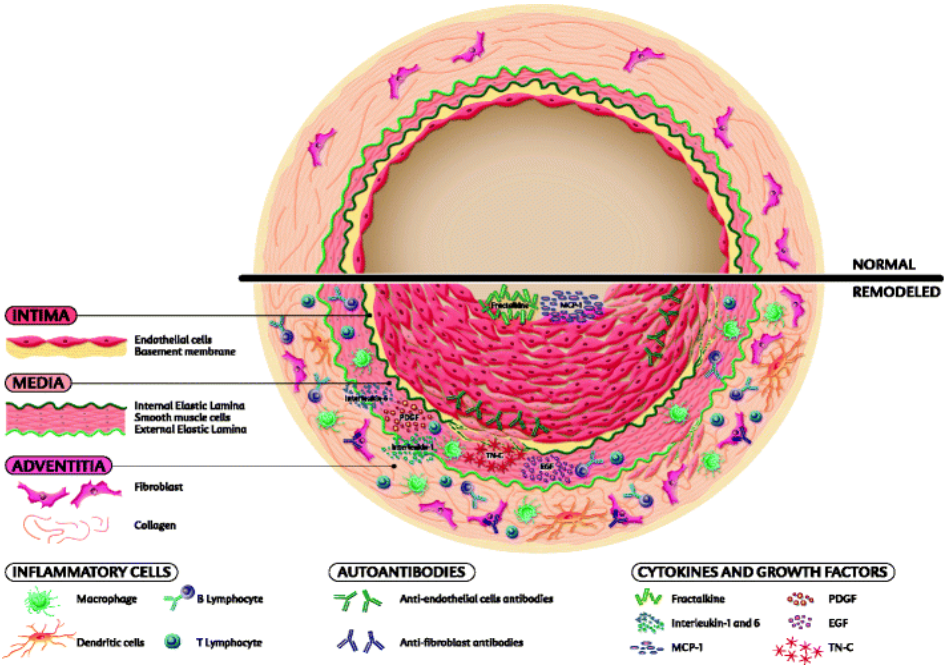

Figure 1: Pulmonary vascular remodeling in pulmonary arterial hypertension (PAH)
Inflammatory mediators, cells, and mechanisms involved in the pulmonary vascular remodeling of PAH. Vascular changes affect all three layers, i.e., intima, media, adventitia, of the pulmonary vessel and involve endothelial cell activation and proliferation leading to vessel obliteration, with evidence of inflammatory cell recruitment and increased production of chemokines such as fractalkine and monocyte-chemotactic-protein 1 (*MCP-1*). A number of circulating autoantibodies including anti-fibroblast antibodies and anti-endothelial antibodies are present in the peripheral blood of patients with PAH. Finally, an increased number of cytokines, growth factors, and extracellular matrix proteins are also present within the vascular wall: interleukin-1 and interleukin-6, platelet-derived growth factor (*PDGF*), epidermal growth factor (*EGF*) as well as tenascin-C (*TN-C*) (Kherbeck et al.)[34]

Furthermore a number of growth factors are found to contribute to the pathogenesis of PAH such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). They modulate the proliferation and migration of pulmonary artery cells including pulmonary artery smooth muscle cell (PASMC), fibroblasts, and endothelial cell (EC). [25] PDGF is released by vascular SMC, EC, and macrophages. [35] It is believed to play a critical role in vascular remodeling leading to PAH, as it promotes the proliferation, contraction and migration of vascular SMC and fibroblasts. [36] (Figure 2)

In addition, some studies have shown a correlation between levels of circulating inflammatory mediators and patients' survival in the idiopathic and the familial pulmonary arterial hypertension. [37]

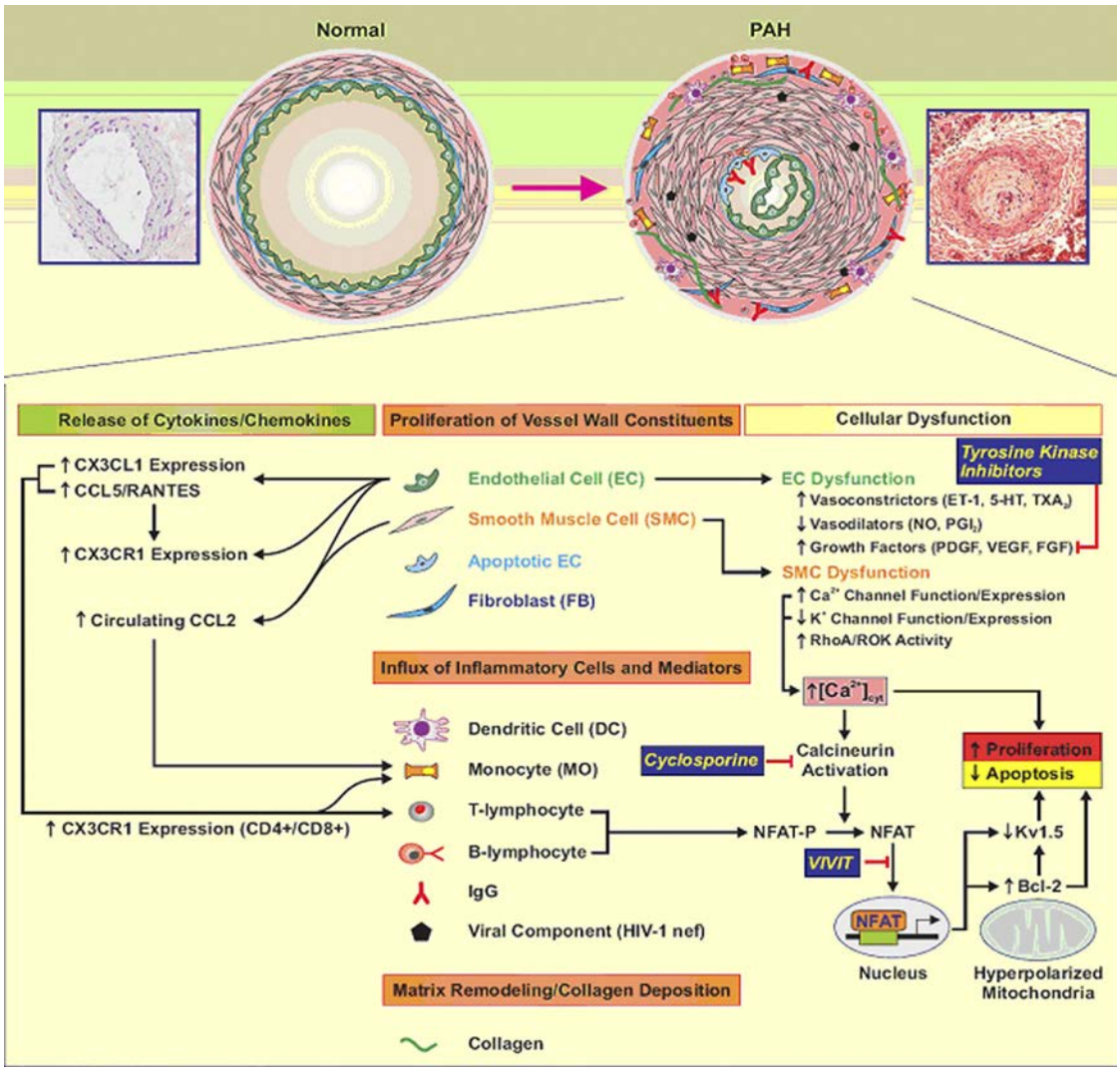

Figure 2: Mechanisms of Inflammation-Mediated Remodeling
Inflammatory mediators, cells, and mechanisms involved in pulmonary vascular remodeling as well as potential therapeutic targets. Release of cytokines and chemokines in remodeled vessels (e.g., plexiform lesions) or in the circulation, from activated endothelial cells (ECs) and smooth muscle cells (SMCs), mediate the influx of inflammatory cells (e.g., monocytes, T and B lymphocytes). Cellular dysfunction (particularly involving EC and SMC) contributes to release of vasomotor and growth mediators, activation of transcriptional factors (e.g., nuclear factor of activated T lymphocytes [NFAT]), influx of calcium, and mitochondrial dysfunction. The net effect is a shift of balance in favor of cell proliferation and decreased apoptosis, leading to remodeling and narrowing of the pulmonary vascular lumen. bcl2 = B-cell lymphoma 2; CCL2 = chemokine (C-C motif) ligand 2; CCL5 = chemokine (C-C motif) ligand 5 or RANTES (Regulated upon Activation, Normal T cell expressed and secreted); CX3CL1 = chemokine (C-X3-C motif) ligand 1 (fractalkine); CX3CR1 = chemokine (C-X3-C motif) receptor 1; DC = dendritic cells; ET1 = endothelin 1; FB _ fibroblasts; FGF = fibroblast growth factor; 5-HT = serotonin; HIV-1 = human immunodeficiency virus 1; IgG = immunoglobulin G; MO = monocyte; NO = nitric oxide; PAH = pulmonary arterial hypertension; PDGF = platelet-derived growth factor; PGI2 = prostacyclin; ROK = Rho kinase; VEGF = vascular endothelial growth factor (Hassoun et al.)[25]

Hypoxia-induced pulmonary hypertension

Several animal models of PH are now available to researchers. The most commonly used animal models of PH are the chronic hypoxic model and the monocrotaline injury model. [38] These animal models have been used for quite some time and have undoubtedly contributed to a better understanding of the pulmonary hypertensive process.

Long-term exposure of mice to hypoxia leads to muscularization of small pulmonary arteries – increase in the expression of smooth muscle actin in cells - and recapitulates the elevation of right ventricular systolic pressure and the pathology of human disease. [39] Right ventricular systolic pressure, the Fulton Index, and the medial wall thickness index become significantly elevated as early as 7 days of exposure to hypoxia in both bitransgenic (CC77) and control (CCTA) mice. The Fulton Index, the ratio of right ventricle weight to left ventricle plus septum weight, represents a hallmark of right ventricular hypertrophy resulting from increased right ventricle pressure afterload. In the same study conducted by our lab it was demonstrated that hypoxia provokes an accumulation of alternatively activated alveolar macrophages (M2) that precedes the development of pulmonary hypertension and appears to play a critical role in the pathogenesis of disease. Furthermore overexpression of HO-1 induced a switch in macrophage polarity toward an anti-inflammatory phenotype, and this effect was associated with protection from PH. [40]

Moreover, vascular leakage, accumulations of inflammatory cells in multiple organs, and elevated serum levels of cytokines occur in mice after short-term exposure to low oxygen concentrations.[41-42] These responses are mediated by complex intracellular cascades leading to altered gene expression and cell–cell interaction. [43] In addition, in some strains of rats it was reported that hypoxia induced an early and persistent pulmonary artery-specific vascular inflammatory response. [44]

Studies of the hypoxia signaling pathway have further verified the fact that hypoxia can lead to inflammation. This becomes evident in individuals with mountain sickness whose levels of circulating proinflammatory cytokines increase, and leakage of fluid (“vascular leakage”) causes pulmonary or cerebral edema. [45-46] Increased serum levels of interleukin-6, the interleukin-6 receptor, and C-reactive protein — all markers of inflammation — were increased in healthy volunteers who spent 3 nights

at an elevation higher than 3400 m. [47]

Hypoxia-inducible factor (HIF) is a transcription factor that plays a central role in cellular and systemic homeostatic responses to hypoxia. HIF is inactive when oxygen is abundant but is activated in hypoxic conditions. [48-49] Oxygen-dependent hydroxylation of prolyl residues in HIF-1α or HIF-2α in the HIF heterodimer by PHDs creates a binding site for the von Hippel–Lindau (VHL) gene product, which is a component of the E3 ubiquitin ligase complex; the binding of the VHL gene product to HIF-1α (or HIF-2α) culminates in the destruction of the α subunit in proteasomes. In addition, hydroxylation of asparagyl residues in HIF-1α (or HIF-2α) by factor-inhibiting HIF — an oxygen-dependent asparagyl hydroxylase — reduces the transcriptional activity of HIF. The functions of both hydroxylases (PHDs and factor-inhibiting HIF) depend on oxygen. [48] Members of the nuclear factor κ B (NF-κB) family of transcription factors regulate inflammation, immune responses and tissue homeostasis. [50-52] The link between inflammation and hypoxia becomes evident with the interaction between members of the NF-κB family and the PHD–HIF pathway. (Figure 3)

The role of HIF in the development of pulmonary hypertension associated with hypoxemia has been explored using murine models, where partial deficiency of either HIF-1a (Hif1a$^{+/-}$ mice) or HIF-2a (Hif2a$^{+/-}$ mice) markedly attenuated the increase in pulmonary arterial pressure and right ventricular hypertrophy. [53-54] The reduction in pulmonary hypertension was partly due to the reduced pulmonary vascular remodeling observed in these animals. [55]

MCP-1 (CCL2), a potent monocyte attractant is a member of the CC subfamily. It was the first discovered human CC chemokine. Mouse monocyte chemoattractant protein-5 (MCP-5), known as chemokine (C-C motif) ligand 12 (Ccl12) or small inducible cytokine A12 (Scya12), is also a potent monocyte chemokine homologous to human MCP-1 with 66% amino acid identity. Sequence analyses identified HIF-1-binding sites in the promoters of MCP-1 and MCP-5 genes. CCL2 is produced by a variety of cell types (including epithelial, endothelial, smooth muscle, fibroblasts, astrocytes, monocytes and microglial cells), either constitutively or after induction by oxidative stress, and recruits monocytes, memory T-cells, and dendritic cells to sites of tissue injury and infection. However, major source of MCP-1 is monocytes and macrophages and their activity is controlled by IFN-γ, IL-4, IL-10,

and IL-13. IL-4, IL-10, and IL-13 are secreted from T-helper cell type 2 (Th2) lymphocytes. [81] Astrocytes subjected to in vitro ischemia/hypoxia produce a large amount of chemoattractant MCP-1 which is 30-time higher than the secreted by human brain endothelial cells subjected to the same treatment. Recent findings suggest that HIF-1α is involved in transcriptional induction of these two chemokines by hypoxia. [56]

Hypoxia-induced mitogenic factor (HIMF/FIZZ1), also known as found in inflammatory zone 1 and resistin-like molecule a, belongs to a novel class of cysteine-rich secreted proteins. It exhibits mitogenic and chemotactic properties during pulmonary hypertension associated vascular remodeling, as well as fibrogenic properties during pulmonary fibrosis. HIMF expression in the lung was reported to be regulated by Th2 cytokines (IL-4 and IL-13) via the transcription factor STAT6 pathway in a bleomycin-induced pulmonary fibrosis model. In addition, HIMF-induced production of angiogenic factors/chemokines, such as vascular endothelial growth factor, MCP-1, and stromal-derived factor-1, in the lung resident cells. [57]

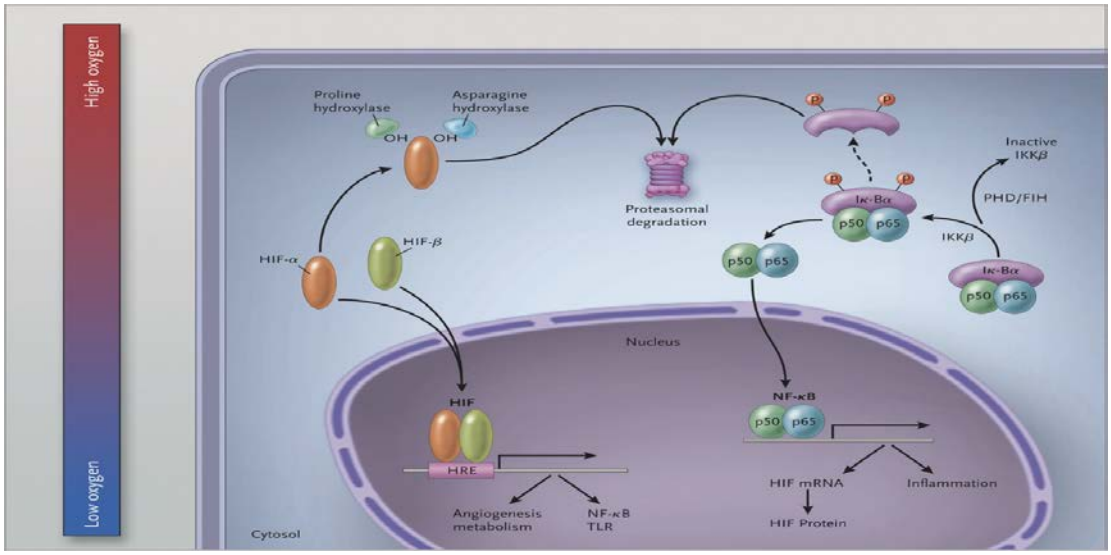

Figure 3. Schematic Overview of the Molecular Interaction between the HIF and (Canonical) NF-κB Pathways.
In hypoxic conditions (left), hypoxia-inducible factor (HIF) α and HIF-β subunits translocate to the nucleus, where they bind as heterodimers to a hypoxia response promoter element (HRE), inducing transcription of numerous genes, including those of nuclear factor κB (NF-κB) and toll-like receptors (TLRs). In normoxia, HIF-α is hydroxylated by prolyl hydroxylases (PHDs) and factor-inhibiting HIF (FIH) and is thereby targeted for proteasomal degradation. In resting cells (right), NF-κB, a heterodimer consisting of p50 and p65 subunits, is inactive in the cytosol because it is associated with nuclear factor of kappa light polypeptide gene enhancer in B cells alpha (IκBα), a regulatory component of NF-κB. At the time of cellular activation, the beta subunit of the IκB kinase complex (IKKβ) phosphorylates the inhibitor IκBα, which thereby becomes degraded and liberates NF-κB for translocation in the nucleus, where it can activate the transcription of inflammatory genes as well as of HIF. PHDs and FIH regulate NF-κB activation by controlling the activity of IKKβ. (Eltzschig et al.) [58]

The protection of heme oxygenase-1 in pulmonary hypertension

Heme oxygenases (HO) are the rate-limiting enzymes that catalyze the conversion of heme into biliverdin, carbon monoxide (CO), and free iron. [59-60] Biliverdin is rapidly converted to bilirubin, a potent endogenous antioxidant. [61] Three isoforms of HO have been reported, the inducible HO-1 and the constitutively expressed HO-2 and HO-3. [62] Under normal physiological conditions, HO-1 and HO-2 are both expressed at low levels in vascular smooth muscle cells (VSMCs) and cardiomyocytes. However, under pathophysiological conditions, only HO-1 is induced in the heart and blood vessels. [63-64] The function of HO-3 is unclear, and the current thinking is that it represents a pseudo-gene.

HO-1 is widely distributed in tissues and induced by its substrate heme [65] and by numerous stressful stimuli such as ultraviolet radiation, heat shock, inflammation, hypoxia, and various oxidative agents. [63] An increasing number of studies implicate HO-1 in the regulation of inflammation. A protective effect of HO-1 has been shown in cardiac xenograft rejection endotoxin challenge, as well as in hyperoxic lung injury and ischemia–reperfusion in the liver. HO-1 deficiency, on the other hand, is associated with a chronically inflamed state and increased leukocyte recruitment as has been reported in studies with humans and mice, null for the HO-1 gene. [67]

Multiple sources of evidence from our lab support the idea that biliverdin, bilirubin, and CO contribute to the physiologic functions of HO-1. [43] High levels of HO-1 expression in the lung (Treatment With NiCl2 or Hemin) protected rats from developing pulmonary hypertension and prevented the accompanying structural remodeling of pulmonary arterioles. [66] Genetically engineered mice that express high levels of a human cDNA HO-1 transgene in lung epithelium were used to assess the effect of HO-1 in lung inflammation. Two separate models of inflammation were studied; hypoxic exposure and lipopolysaccharide (LPS) challenge. Both mRNA and protein levels of specific cytokines and chemokines (IL-1β, IL-6,) were significantly elevated in response to hypoxia in the lungs of wild-type mice after 2 and 5 days of exposure. While at the same time they were significantly suppressed in the hypoxic lungs of transgenic mice implying that overexpression of HO-1 leads to inhibition of these cytokines. [67] The HO-1 transgenic mice (SHO1) were also protected from the development of both pulmonary inflammation as well as hypertension and vessel wall hypertrophy induced by hypoxia. [70]

Biliverdin treatment protects the HO-1 $^{-/-}$ mice from right ventricle (RV) injury and an exaggerated increase in RV weight after seven weeks of chronic hypoxia without diminishing pulmonary hypertension. In contrast, HO-1 $^{-/-}$ mice treated with inhaled CO are protected from pulmonary vascular remodeling, however, they still develop RV failure and thrombus with significant mortality, despite normal right ventricular pressures. The divergent effects of two enzymatic products of HO-1 in the same disease model are highlighting the complexity of HO-1's protective actions in the cardiovascular system. Moreover, the finding that CO protected from pulmonary hypertension but failed to protect from RV injury indicates that hypoxia has a direct effect on the right ventricle that is not mediated by pulmonary vascular constriction or remodeling. [68-69]

b. MiRNA and pulmonary hypertension

The discovery of MicroRNAs (miRNAs) is considered one of the major breakthroughs of the last decade. However they were probably first mentioned in the 1960's when Britten and Davidson proposed the existence of "activator" RNAs transcribed from redundant genomic regions. In 1993 the first miRNA lin-4 was discovered in C.elegans and eight years later the highly conserved let-7 was also found in C.elegans, which was crucial for developmental timing. [71] MicroRNAs have been shown to play important biological roles in various contexts; during development, cell differentiation, and immune regulation and also in pathologies such as cancer. [72,82]

MiRNAs are small noncoding transcripts of 16 to 29 nucleotide RNAs that regulate gene expression posttranscriptionally by targeting mRNAs. Animal miRNAs are processed from longer primary transcripts (primary miRNAs) that can contain multiple miRNAs. [74] This precursor is then processed in 3 steps. First, a complex comprising the nuclear RNase III enzyme Drosha and the double strand (ds) RNA-binding protein Pasha/DGCR8 cleaves the primary miRNA in approximately 60 nucleotides (nt) precursor miRNA hairpin. After this a shorter precursor is actively transported to the cytoplasm by Exportin-5 [75] where the precursor miRNA undergoes further processing into an approximately 21- to 22-nt duplex (the mature miRNA) by the multi-domain ribonuclease Dicer and cofactors. [76] One strand of the duplex is preferentially selected for entry into a silencing complex that includes an argonaute

protein. This mature miRNA guides the argonaute complex to complementary sites on target transcripts. [77]

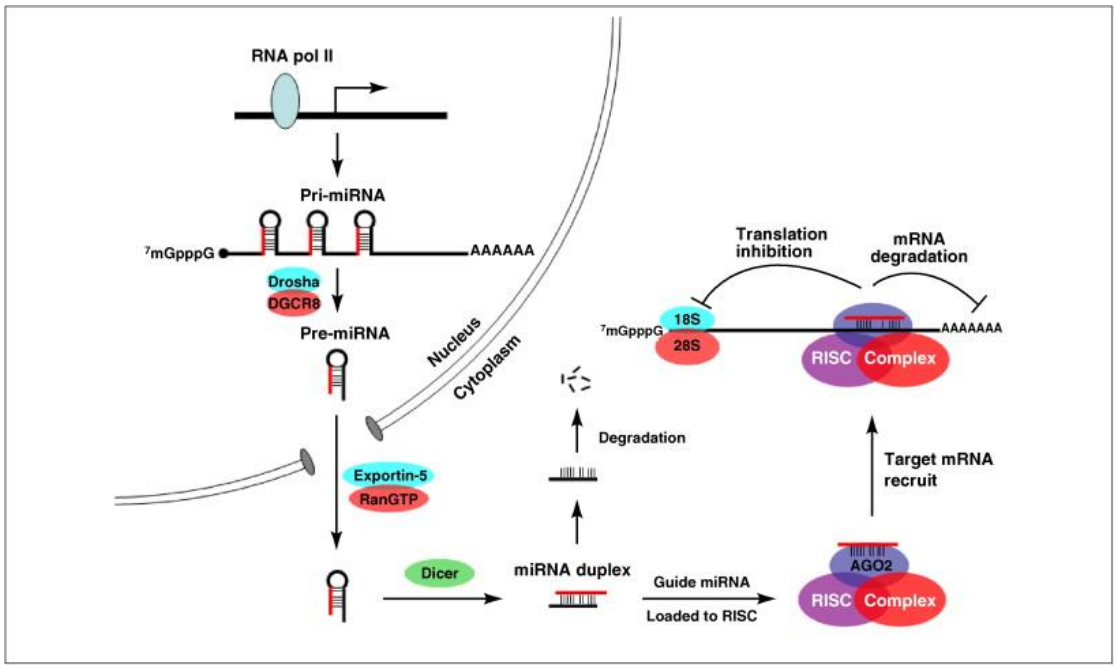

Figure 4. Schematic view of miRNA biogenesis and functioning pathways.
Genes encoding miRNAs are transcribed into pri-miRNAs by RNA polymerase II (RNA Pol II). The pri-miRNA is first processed by the type III RNA endonuclease Drosha into pre-miRNAs that are 60–70 nucleotides in length and have a stem–loop structure. The pre-miRNA is exported out of the nucleus by exportin-5 and is further processed by another type III RNA endonuclease Dicer to generate a mature miRNA duplex 22 nucleotides in length. The sense strand of the miRNA duplex is then loaded into the RISC, whereas the complementary strand of the miRNA duplex is degraded. The RISC regulates gene expression through the inhibition of RNA translation or degradation of target mRNA by base pairing the ‘‘seed region’’ of a miRNA to the 3’ UTR of target mRNA. (Huang et al.) [83]

The selectivity of miRNA action is given by the nucleotides 2–7 at their 5’end (the “seed region”) that pairs to its complementary site in the targeted 3’-UTR by Watson-Crick interactions directing the RNA-induced silencing complex action. [78] The inhibitory activity of microRNAs ensues by blocking the target mRNA translation into protein and/or the degradation of the mRNA. [79]

Functionally, an individual miRNA can regulate the expression of multiple target genes. More than 700 different miRNAs are found in the mouse genome (miRBase http://microrna.sanger.ac.uk; Sources of predicted miRNA target http://microrna.org), and each miRNA may act on hundreds of target mRNAs. Almost one third of genes are fine-tuned by miRNAs. [79] Micro RNA regulation and expression has been the focus of many studies with multiple levels of control being identified recently. O’Connell et al describe three levels of control, namely at the stages of (i) transcription, (ii) processing and (iii) subcellular localization**.** [80]

Given the diverse roles that miRNAs play in numerous aspects of cellular functions, it is not surprising that they also play a role in hypoxia gene regulation. [83] During the past 5 years, various studies have determined miRNA expression profiles, or signatures, from a variety of different organisms, cell types, and disease states in relation to hypoxia. These studies have described more than 90 hypoxia-regulated miRNAs (HRMs); however, many are only regulated in certain cellular contexts. Differences in detection methods, in the period and severity of oxygen deprivation, and the cellular and organismal context have all lead to different HRM profiles. [86] Recently, miRNAs have been implicated in regulating both upstream and downstream signaling of the HIF pathways: miR-199a, miR-17-92 clusters and miR-20b regulate HIF1a under hypoxia, whereas miR-23, miR-24, miR-26, miR- 107, miR-210 and miR-373 have been shown to be induced by HIFs. [83]

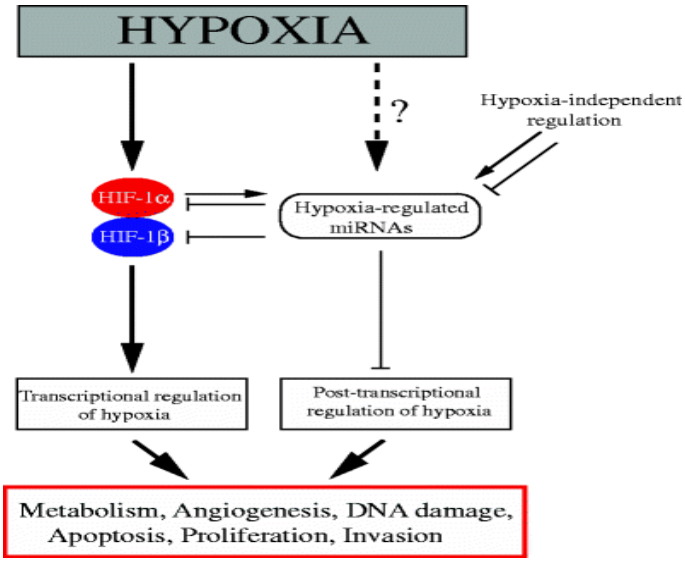

Figure 5. Transcriptional and post-transcriptional hypoxia pathways
The HIF-1 transcriptional pathway mediates canonical hypoxic responses. miRNA pathways integrate hypoxia-dependent and -independent signals and act on HIF-1 pathway components (HIF-1α and HIF-1β) and possibly on non-HIF-1-regulated pathways. (Roger et al.) [82]

MiR-210 induction has been found in the literature to be a feature of the hypoxic response in both normal and transformed cells and its overexpression has been detected in a variety of cardiovascular diseases and solid tumors. High levels of miR-210 were found to be related to an in vivo hypoxic signature and associated with adverse prognosis in cancer patients. [84] Furthermore, overexpression of miR-210 results in increased HIF-1α accumulation during hypoxia. The enzyme glycerol-3-phosphate dehydrogenase 1-like (GPD1L) has been found to be a novel regulator of

HIF-1α stability and a direct target of miR-210. MiR-210 expression results in stabilization of HIF-1α due to decreased levels of GPD1L resulting in an increase in HIF-1α target genes. [85]

Through bioinformatics analysis, it was found that miR-155 is predicted to target IL-13 receptor α 1(IL13Rα1). This suggested that miR-155 might be involved in the regulation of the M1/M2 balance in macrophages by modulating IL-13 effects. In human macrophages, miR-155 directly targets IL13Rα1 and reduces the levels of IL13Rα1 protein, leading to diminished activation of STAT6. [73] MiR-155 affects the IL-13-dependent regulation of several genes involved in the establishment of an M2/pro-Th2 phenotype in macrophages, which demonstrates a possible role of miR-155 in determining the M2 phenotype in human macrophages.

c. Biological significance - Objectives

The primary goal of this study is to examine the effects of the hypoxic inflammatory response on the development of pulmonary hypertension. Recognition of miRNAs as the main contributors of the inflammatory cytokines expression may play a significant role in treatment options of pulmonary hypertension. The bi-transgenic mouse model will be used with inducible HO-1 expression to assess the pathways by which HO-1 exerts its anti-inflammatory properties. One of HO-1's main products, CO will also be used to further investigate the anti-inflammatory pathways. The effects of HO-1 and CO on miRNA expression will be studied to shed light on the molecular mechanism by which hypoxia induced inflammation leads to pulmonary hypertension.

B. Materials and Methods

Generation of bitransgenic mice (CC77)

In our laboratory, bitransgenic mice were generated by the crossing of Balb/c transgenic mice that harbor the tetracycline transcriptional activator (rtTA , tet-on system) under the control of the Clara Cell secreted protein (CCSP) promoter (CC10) (CCTA line: a kind gift from DR J.A Whitsett) [87] with FVB transgenic mice that carry the human HO-1 transgene under the control of the tetracycline response element (TH77 line: CC10-rTTA x TRE-hHO1xSV40 polyA). The latter was generated by microinjection of a (TetO)7-CMV-hHO-1 transgene that consists of seven copies of the tet operator DNA binding sequence linked to a minimal CMV promoter, the human HO-1 cDNA, and SV40 polyadenylation signals (Figure 6 A, B). The result was a bitransgenic mouse line (CC77 is our working designation) that overexpresses human HO -1 in an inducible lung-specific way.

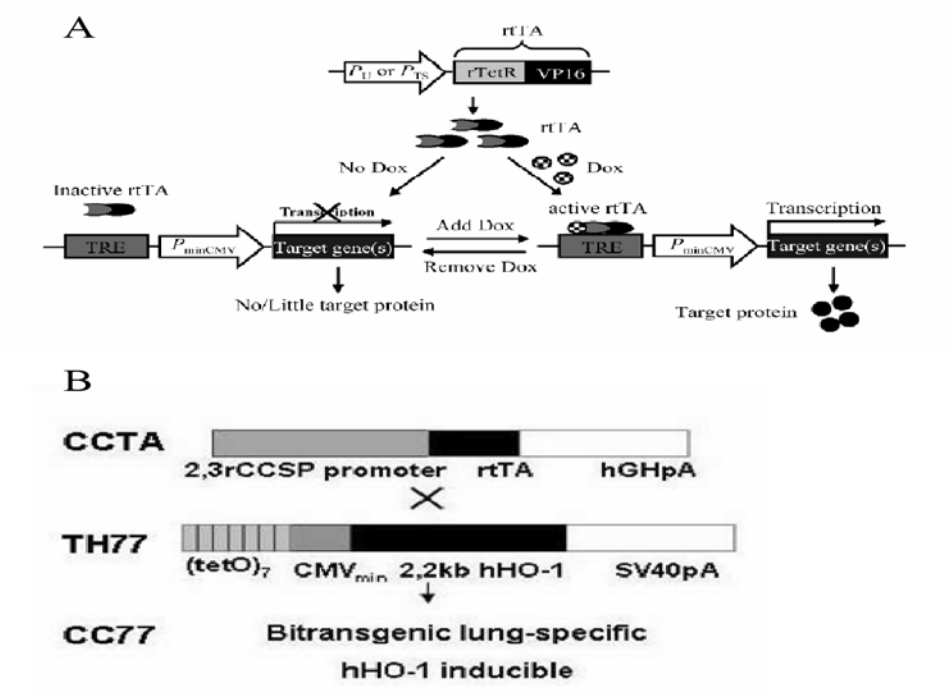

Figure 6. Inducible HO-1 expression by a bitransgenic mouse line A, The tetON System for inducible tissue specific expression of the gene of interest. Only upon the presence of dox, the tetracycline transactivator initiates transcription. B. Outline of the constructs designed. Bitransgenic mice (CC77) were produced by crossing of mice bearing the reverse tetracycline transactivator (rtTA) under the control of the 2.3-kb rat CCSP (Clara cells secreted protein) promoter (CC10) (CCTA mouse line) with transgenic mouse line (TH77) generated by microinjection of a (TetO)7-cmv-hHO-1-polyA transgene

Transgenic mice (SHO1)

A plasmid containing a 3.7-kb genomic region encompassing the promoter of the human surfactant protein C (SP-C) gene was a kind gift of Jeffrey A. Whitsett (Children's Hospital Medical Center, Cincinnati). The SP-C promoter–human HO-1 cDNA transgene was constructed by standard cloning methods, and microinjection was performed in FVB/N strain pronuclei by the Brigham & Women's Hospital Core Transgenic Mouse Facility (Boston). Founder mice were identified by Southern blot analysis of genomic DNA isolated from mouse-tail biopsies.

Mice treatments

Expression of human HO-1 in the lung was achieved by the addition of 1mg/ml dox in the drinking water. After 2 days pretreatment with Dox, animals were introduced to normobaric hypoxia (8.5% oxygen) inside a chamber where oxygen was tightly regulated by an oxycycler controller (Biospherix, Redfield, NY). Nitrogen was automatically introduced as required to maintain the proper FiO2 and ventilation was adjusted to keep CO2 levels less than 8,000ppm (0.8%). Ammonia was removed by charcoal filtration by an electric air purifier. Dox administration was continued for the entire duration of the hypoxic exposure. Age and sex-matched littermates were exposed to identical conditions in hypoxia or normoxia and served as controls.

CO treated mice inhaled CO intermittently: 250 ppm for 1h prior to hypoxic exposure and then received 250 ppm for 1h twice daily, inside the hypoxic chamber for a total of 48 hours. Control mice inhaled room air or hypoxic air (8.5% oxygen) without CO.

Animals were maintained in a pathogen-free environment in the Children’s Hospital Animal Care facility and all animal experiments were approved by the Children’s Hospital Boston Animal Care and Use Committee.

Hemodynamic and ventricular weight measurements

After hypoxic exposure at the indicated time periods, mice were anesthetized and hemodynamic and ventricular weight measurements were performed. Right ventricular systolic pressure (RVSP) was measured through a trans-thoracic route: a pressure transducer (ADI Instruments, Inc., Colorado Springs, CO) attached to a 23G

needle was used and data were collected and analyzed using the PowerLab Software (ADI Instruments, Inc., Colorado Springs, CO).[88] Right ventricular (RV) hypertrophy was assessed by harvesting hearts, removing atria, dissecting the RV and deriving Fulton's Index, i.e the weight ratio of (right ventricle)/ (left ventricle and septum) [(RV)/(LV+S)].

Isolation of alveolar macrophages

Animals were anesthetized with 2,2,2-tribromoethanol (avertin, Sigma-Aldrich, Inc., St. Louis, MO) after exposure for the indicated time periods in hypoxia. Bronchoalveolar lavage fluid (BALF) was obtained through intratracheal instillation of 3.3 ml PBS (0.8, 0.8, 0.8, 0.9) (Ca^{+2} and Mg^{+2} free, supplemented with 10 mM EDTA) and filtered via a 35 μm cell strainer to exclude contamination from epithelial cells that appeared in clusters. Cell suspension was cytocentrifuged in 400g for 10min. Red blood cells were lysed using ammonium chloride lysis buffer (Sigma-Aldrich, Inc., St. Louis, MO). More than ninety percent of the cells isolated this way appeared to be of the monocyte/macrophage lineage and this was confirmed by cell-specific markers in flow cytometry. Isolated cells were used for RNA extraction.

Primary alveolar macrophage culture

For cell culture experiments, BALF was obtained through intratracheal instillation of 4 x 1 ml Hank's Balanced Salt Solution (without calcium and magnesium) supplemented with 10 mM EDTA and 1 mM HEPES and filtered twice via a 35 μm cell strainer to exclude contamination of epithelial cells. 300.000 macrophages per dish were seeded in 60 x 15 mm tissue-culture dishes in a volume of 3ml macrophage complete medium (DMEM/10: Dulbecco's Modified Eagle Medium) (GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% (v/v) FBS, 10 mM L-glutamine, 100 IU/ml penicillin and 100 ug/ml streptomycin). Cells were incubated at 37°C for 6-12 hours before the hypoxic exposure.

Immunohistochemical analysis

Lungs were initially perfused with PBS through the right ventricle. The perfusion flow was kept at approximately 1ml/min by the use of a peristaltic pump

with Platinum L/S 13 Masterflex silicone tubing. Lungs were then intratracheally inflated with 4% paraformaldehyde, fixed overnight at 4°C, then stored in 70% ethanol before embedding inparaffin. Lung tissue sections were deparaffinized and rehydrated. Immunohistochemical assessment of vascular remodeling was performed by staining for alpha-smooth muscle actin (anti-α-SMA antibody, Sigma-Aldrich, Inc., St. Louis, MO), a marker of smooth muscle cells. [89] Endogenous peroxidase activity was inhibited with 3% H2O2 (Sigma- Aldrich, Inc., St. Louis, MO) in methanol. Next, the sections were incubated with a biotinylated horse antimouse IgG (Vector Laboratories, Inc., Burlingame, CA), treated with the avidin–biotin complex (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA), and stained with 3,3'-diaminobenzidine substrate (KPL, Inc., Gaithersburg, MD). Slides were counterstained with 1% Methyl Green (Sigma-Aldrich, Inc., St. Louis, MO).

Quantitative real time PCR assays for mRNA

RNA from total lung or from alveolar macrophages was isolated using the Qiagen RNAeasy mini and micro extraction kit, respectively (Qiagen, Hilden, Germany). One μg of total DNAdigested RNA was used for cDNA synthesis (Superscript III oligo dT primer kit, Invitrogen Corporation, Carlsbad, CA). The following primers were used in the PCR reaction: human HO-1; fwd: 5'-GCAGTCAGGCAGAGGGTGATA-3', rev: 5'- AGCCTGGGAGCGGGTGTTGAG-3', Fizz1; fwd: 5'-GCTGATGGTCCCAGTGAATAC-3', rev: 5'-CCAGTAGCAGTCATCCCAGC-3', Ribosomal Protein S9 (Rps9) with forward primer 5'-GCTAGACGAGAAGGATCCCC-3' and reverse primer 5'-. CAGGCCCAGCTTAAAGACCT -3' served as housekeeping gene. Annealing was carried out at 60˚C for 30 sec, extension at 72˚C for 30 sec, and denaturation at 95˚C for 30 sec for 40 cycles.

Ccl12, Ccl2, Fam181b, Foxs1, Ntrk2, Tiam1, Sox17, Ptprr, and Ankrd37 were quantified using Applied Biosystems Gene Array TaqMan® primers with the TaqMan® Gene expression PCR Master Mix (Cat# 4370048), using the Applied Biosystems StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). Reactions were performed at 52 C for 2 min, 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec, 60 C for 1 min. Ribosomal Protein S9 (Rps9) served as

housekeeping gene. Analysis of the fold change was performed based on the Pfaffl method. [90]

Quantitative real time PCR assays for miRNA

In order to measure miRa-199-3p, miR-155 and miR-210 expression the mirVana kit (Applied Biosystems) was used to extract total RNA from lungs according to the manufacturer's instructions. Absorbance of the RNA samples was quantified at 260 and 280 nm, and the 260/280 ratio was calculated. The samples showed a 260/280 ratio ≥ 1.9, which was assumed as an indicator of RNA purity. cDNA was synthesized from total RNA using stem-loop reverse transcription primers according to the TaqMan MicroRNA Assay protocol (Applied Biosystems, Foster city, CA, USA).Ten nanograms of total RNA was reverse transcribed into cDNA. Real-time PCR was performed using a standard TaqMan PCR kit protocol on an Applied Biosystems StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers and probes for miRNAs were provided in TaqMan Gene Expression Assay kits (hsa-miRa-199-3p, mmu-miR-155, hsa-miR-210, small nucleolar RNA for the internal control, snoRNA202; Applied Biosystems, Foster City, CA). A 20ul reaction mixture containing cDNA, specific primers, probe, and TaqMan Fast Universal PCR Master Mix was used in the PCR. Reactions were performed at 52 C for 2 min, 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec, 60 C for 1 min. Results were normalised to the expression of the snoRNA202.

Western blot analysis

Frozen lung tissues were placed into a 2ml flat-bottomed tube. 400 ul of dissociation Solution was added and the samples were kept on ice for 3 minutes. The tissues were homogenized using polytron with for 3 to 5 seconds and were subsequently placed back on ice. The sample was then centrifuged at 4000 rpm for 3 minutes. The supernatant was aspirated and 1ml of dissociation buffer was added to the sample. The procedure was repeated twice. Following that 150 ul of Lysis buffer was added and the mixture was centrifugred at 13,000 rpm for 15 minutes. For the protein measurement 2 ul were taken and mixed with 38 ul of PBS (1/20 dilution of samples in RIPA is suitable to use Bradford assay).

Bronchoalveolar lavage fluid (BALF) samples were concentrated with 20% trichloroacetic acid (TCA, Sigma Aldrich, Inc., St. Louis, MO) overnight, washed with ice-cold acetone and resuspended in SDS-containing loading dye.

Twenty μg of protein was electrophoresed on 13.3% denaturing polyacrylamide gel prior to wet transfer to 0.2 μm PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Briefly, after blocking with 5% bovine serum albumin (BSA) in phosphate buffered saline (pH 7.4) containing 0.1% Tween 20 (PBST) for an hour at room temperature, the membranes were incubated with rabbit polyclonal anti-mouse Fizz1 antibody (Abcam, Cambridge, MA), rabbit polyclonal anti-human HO-1 antibody (Enzo Life Sciences International, Inc., Plymouth Meeting, PA), Plymouth Meeting, PA) at 4°C overnight. The membranes were then incubated with 40 ng/ml of peroxidase-conjugated anti-rabbit for 30 min at room temperature followed by reaction with Lumi-Light ECL substrate (Thermo Fisher Scientific, Inc., Waltham, MA).

Statistical analysis

All values are expressed as mean +/-SD. Comparison of results between different groups was performed by 1-way ANOVA or Mann-Whitney U test when appropriate with GraphPad InStat (GraphPad Software, San Diego, CA). Values of $P<0.05$ were considered significant.

C. Results

Lung-specific, inducible expression of human Heme Oxygenase-1

Based on the design of the bitransgenic model (Figure 6A, B), the hHO-1 transgene is under the control of both doxycycline and the Clara cell secretory protein promoter and therefore it is induced and expressed in the lung epithelium. Quantitative polymerase chain reaction analysis on total lung mRNA with hHO1-specific primers indicates that hHO-1 mRNA levels were up-regulated more than 20-fold upon 1mg/ml doxycycline administration but remained undetectable in the absence of doxycycline (Figure 7A). Using an antibody that detects human HO-1, we detected profoundly elevated protein levels of HO-1 in the lungs of doxycycline-treated CC77 mice compared to the lungs of regular water (without doxycycline) treated CC77 mice (Figure 7B).

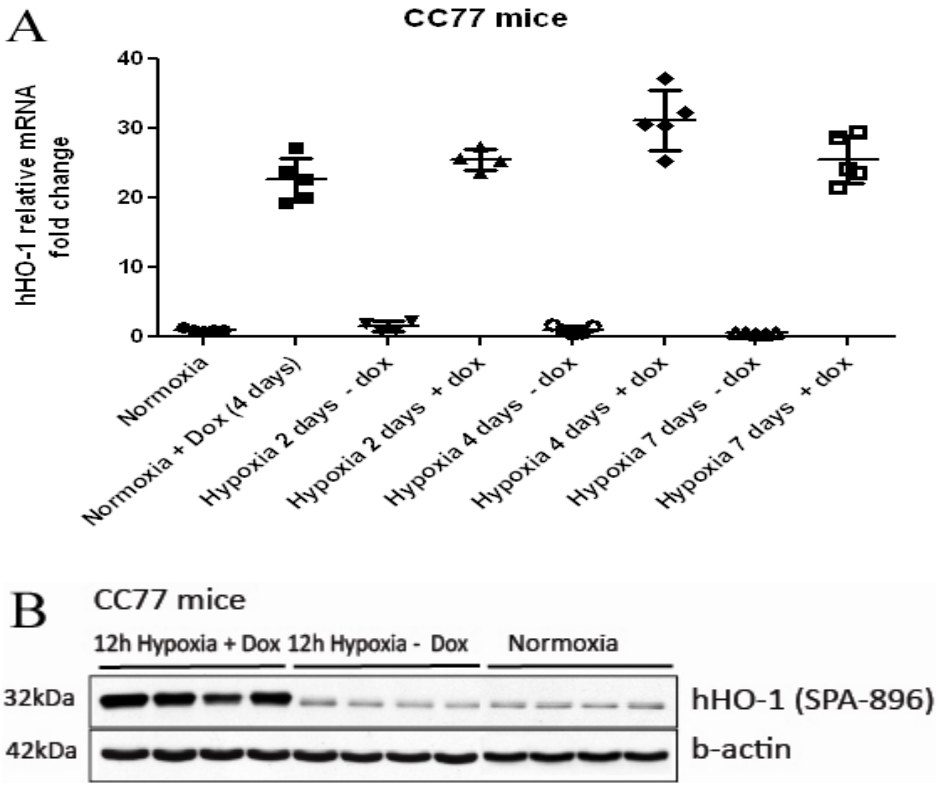

Figure 7. Lung-specific, doxycycline (dox) regulated expression of human heme oxygenase-1 (HO-1). (A) Bitransgenic mice (CC77) were treated with 1 mg/mL dox in the drinking water for 2, 4 and 7 days follow by quantitative polymerase chain reaction analysis on total lung RNA for hHO1. Values were normalized with RPS9 housekeeping gene. The primers used target a divergent region on HO-1 mRNA and do not amplify endogenous murine HO-1 transcripts. (B) Western blot analysis of HO-1 protein in total lung extracts of CC77 mice. Note that the antibody used detects the dox-regulated human HO-1 and the endogenous murine HO-1.

Sustained induction of Heme Oxygenase-1 prevents hypoxia-induced pulmonary hypertension

The development of PAH in our model was assessed by the measurement of right ventricular systolic pressure, the Fulton Index, and the medial wall thickness index. The Fulton Index, the ratio of right ventricle weight to left ventricle plus septum weight, represents a hallmark of right ventricular hypertrophy resulting from increased right ventricle pressure afterload. The medial wall thickness index was estimated from the histological sections of pulmonary arterioles stained with α-smooth muscle actin (adopted from the work of Dr. Eleni Vergadi). Right ventricular systolic pressure, the Fulton Index, and the medial wall thickness index were significantly elevated after 21 days of hypoxia in bitransgenic mice (CC77) (Figure 8A through 8C). Doxycycline administration for the entire course of hypoxia prevented the increase in right ventricular systolic pressure, the Fulton Index, and the medial wall thickness index in the bitransgenic mice but not in the water treaded control CC77 mice. Immunostaining of pulmonary arterioles for α-smooth muscle actin revealed thickened and remodeled medial vascular walls in lung sections of hypoxic mice. This pathology was absent in hypoxic mice treated with doxycycline (Figure 8D).

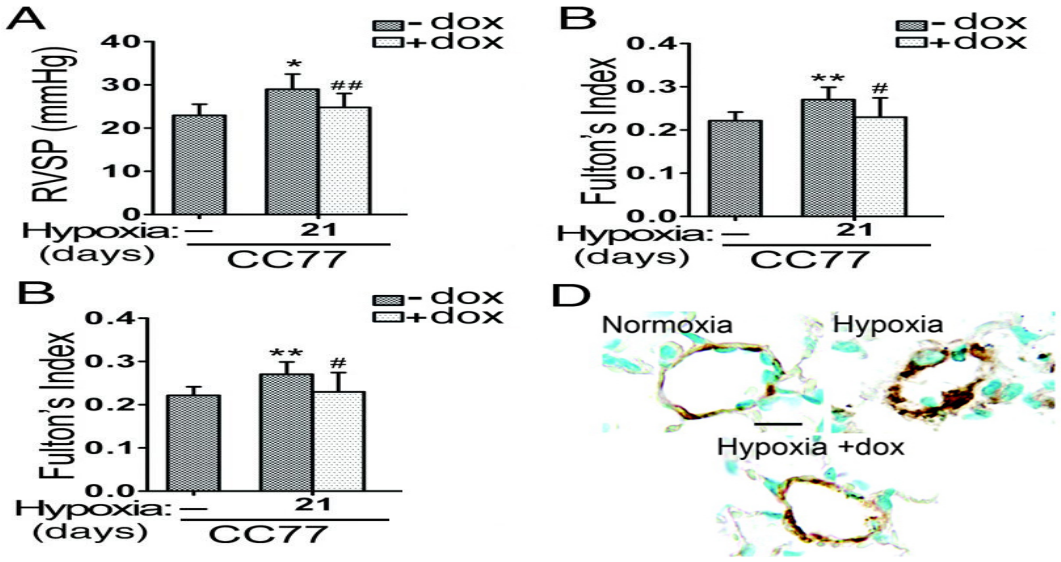

Figure 8. Sustained expression of heme oxygenase-1 (HO-1) prevents hypoxia-induced pulmonary hypertension. Right ventricular systolic pressure (RVSP; A), the Fulton Index (B), and the medial wall thickness index (C) were determined 21 days after hypoxia exposure in the presence or absence of 1 mg/mL doxycycline (dox) in the drinking water of CC77 and mice. (D) Representative images of vascular remodeling in lung sections of mice exposed to hypoxia for 21 days and stained for α-smooth muscle actin. Numbers represent mean +/-SD; n=6 per group *P<0.05, **P<0.01, relative to normoxia; #P<0.05, ##P<0.01 relative to hypoxia - dox.

Hypoxia induction of genes that are involved in cell differentiation and vessel remodeling in the lung of CC77 and FVBs mice

In order to assess the hypoxic inflammatory response at the initial stages of hypoxic exposure and before the development of pulmonary hypertension, CC77 bitransgenic and FVB mice were exposed to hypoxia (8.5%O_2). The expression levels of several genes were measured and found to be up-regulated in the lung in as early as two days of hypoxia. Up-regulation of the forkhead box S1 (Foxs1) and SRY box 17 (Sox17) transcription factors which are found to be involved in regulation of endothelial cell differentiation and vessel remodeling was observed to be 3.3and 2.9, fold respectively (Figure 9A,B). The T-cell lymphoma invasion and metastasis 1 (Tiam1), a guanine nucleotide exchange factor protein that specifically activates the Rho family GTPase Rac1 and is important for the integrity of adherens junctions, tight junctions, and cell-matrix interactions was also found to have a 3.3 fold increase. (Figure 9C) Furthermore, the neurotrophic tyrosine kinase, receptor type 2 (Ntrk2), a membrane-bound receptor that upon neurotrophin binding, phosphorylates itself and members of the Mitogen-activated protein kinases (MAPK) pathway, was increased by 22 fold. (Figure 9D) Interestingly, a striking increase by 17-fold of Ankrd37 a novel HIF-1-target gene was also observed in both FVB and CC77 mice. (Figure 9E)

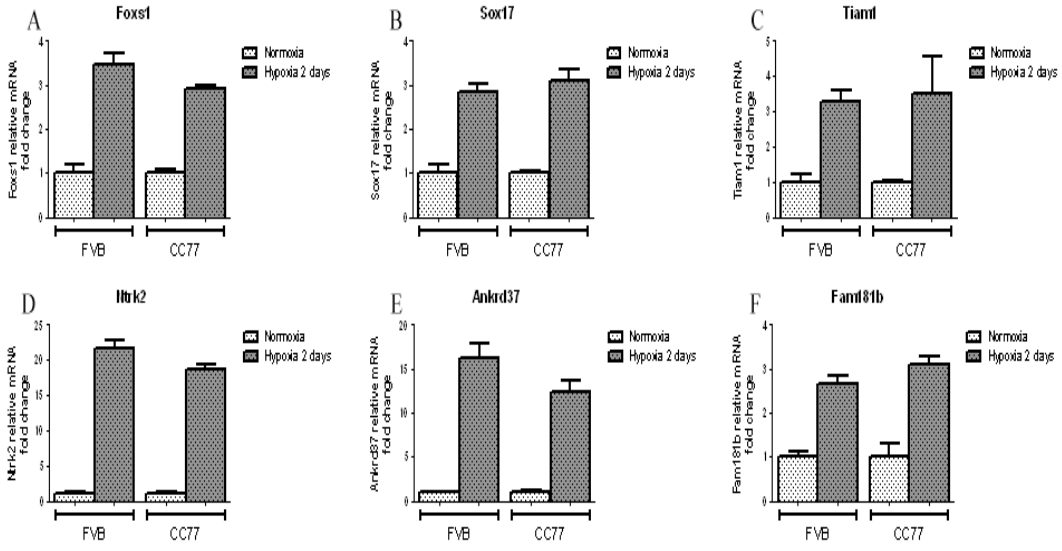

Figure 9. Hypoxia induced lung gene expression in CC77 and FVBs mice. Quantitative PCR analysis of mRNA levels of the transcription factors Foxs1 (A) and Sox 17 (B), Tiam1 (C) which is implicated in the adherens junctions and cell-matrix interactions, a gene involved in the MAPK pathways Ntrk2 (D), and a novel HIF-1 target gene Ankrd37 (D) were up-regulated in hypoxia. Fam18b (F) is an unknown functions gene. Values were normalized with RPS9 housekeeping gene. Numbers represent mean +/-SD; n=4-5 animals per group and animal strain.

Hypoxia induction in the lung of the monocyte chemo-attractant protein-1 (CCL1) and chemokine (C-C motif) ligand 12 (CCL12) is ameliorated by carbon monoxide (CO)

To examine the effects of CO (an enzymatic product of HO-1) on the expression of the pro-inflammatory cytokines CCL2 and CCL12 in the lung, FVB mice were exposed to hypoxia for 2 days. In the same experiment another group of mice received inhaled CO 250 ppm for 1 hour twice a day as stated above. Quantitative polymerase chain reaction analysis of total lung from FVB mice revealed an increase of CCL2 and CCL12 (non-canonical inducers of M2 polarization of the macrophages) by 6-fold and 2-fold respectively. This result corresponds with previous results from our lab indicating that the numbers of monocytes/macrophages were significantly increased in the BALF of hypoxic mice, reaching a peak at 2 days of hypoxia compared with normoxic animals. Administration of CO effectively suppressed CCL2 and CCL12 (Figure 10A,B) suggesting that M2 polarization, which is induced by hypoxia, can be reversed with CO administration.

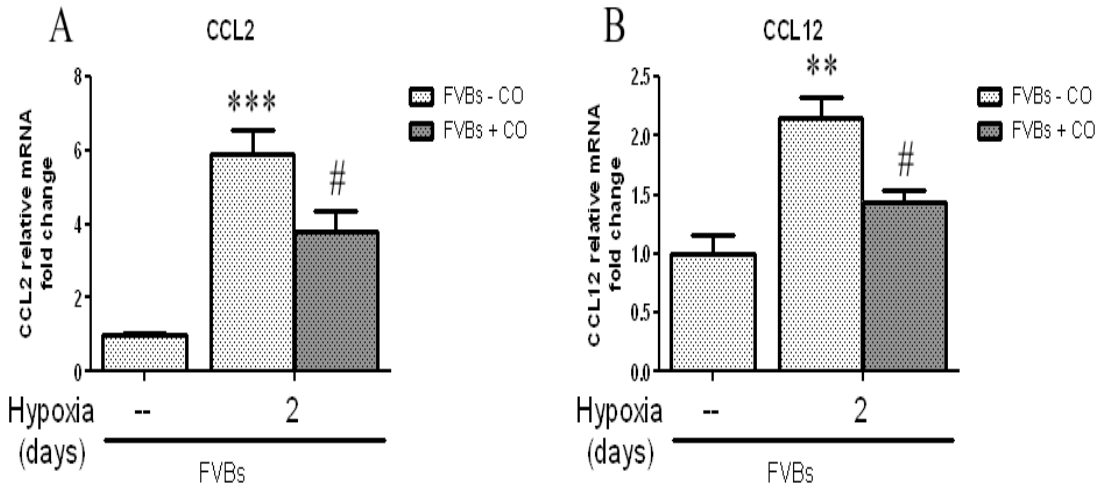

Figure 10. CCL2 and CCL12 mRNA up regulation in hypoxic lungs suppressed by CO administration. Mice were exposed in hypoxia for 2 days and mRNA levels of CCL2 (A) and CCL12 (B) were assessed in total lung extracts by quantitative PCR. The suppressive effect of carbon monoxide (CO) is also indicated. Numbers represent mean +/-SD; n= 5-6 animals per time point or treatment group. Values were normalized with RPS9 housekeeping gene. n=4-5 animals per group *p<0.05, **p<0.01, ***p<0.001 relative to Normoxia; #p<0.05, ##p<0.01, ###p<0.001 relative to hypoxia (-CO).

Hypoxia induction of HIMF (Fizz1) expression was suppressed by constitutive expression of HO-1 in SHO1 transgenic mice and CO treatment

In an effort to evaluate the effects of HO-1 and CO on macrophage phenotype, mRNA from total lung and alveolar macrophages as well as protein from BAL fluid was examined. Messenger-RNA levels of Fizz1 a well-defined marker of M2 macrophages were found to be increased by 26-fold and 11-fold respectively in the lung of FVB mice after 2 and 4 days of hypoxia exposure compared to the normoxic control group. (Figure 11A) On the other hand, constitutive expression of HO-1 in SHO-1 transgenic mice suppressed the induction of Fizz1 in total lung seen in FVB mice. This effect was also detected in freshly isolated alveolar macrophages from FVB and SHO-1 mice that were exposed to hypoxia for 3 days. (Figure 11B) Fizz1 protein levels as measured in BALF were found to be increased after 2 days of hypoxia. This effect was ameliorated by the administration of CO. (Figure 11C) These results suggest a potential reversing effect of HO-1 and CO on the alternative activation of macrophages (M2).

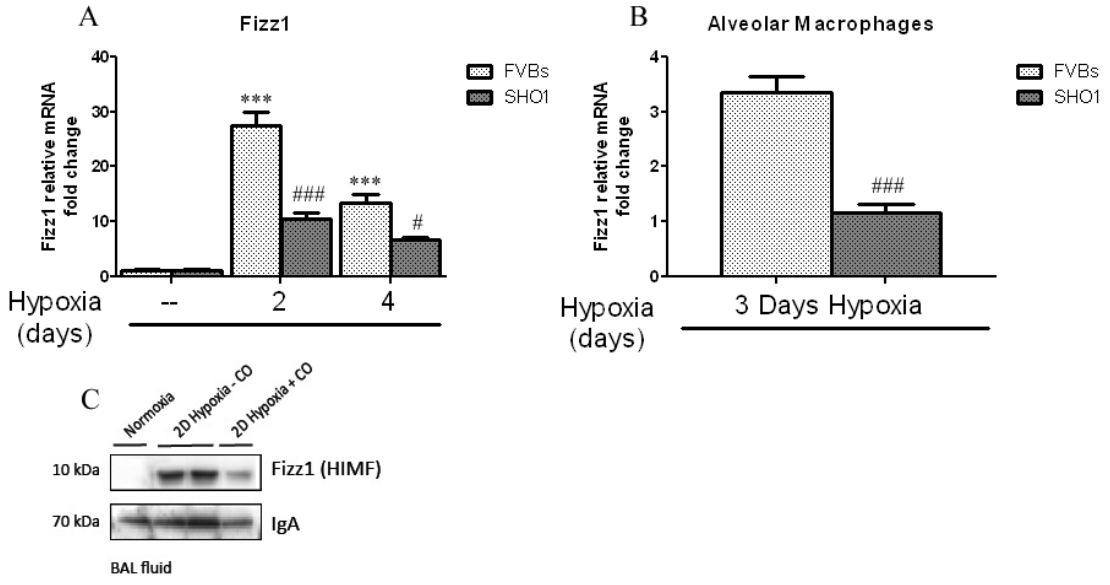

Figure 11. Constitutive expression of the Heme Oxygenase-1 (SHO1 mice) ameliorated the induction of Fizz1 in total lung and in isolated alveolar macrophages. Suppressive effect of the CO in the BALF. (A, B) Quantitative PCR analysis of Fizz1 mRNA levels in total lung and alveolar macrophages from FVBs and SHO1 mice. Fizz1 mRNA levels were up-regulated in hypoxia (2 and 4 days total lung; 3 days alveolar macrophages). Constitutive expression of HO-1 in SHO-1 transgenic mice suppressed the induction of Fizz1 compare with the FVBs control mice (C) Western blot analysis for Fizz1 on bronchoalveolar lavage fluid (BALF) from normoxic mice (Normoxia) and mice exposed to hypoxia for 2 days) with or without CO treatment (2D Hypoxia +/- CO). IgA served as internal control. Numbers represent mean +/-SD; n=4-5 animals per group *P<0.05, **P<0.01, ***P<0.001 relative to normoxia; #P<0.05, ###P<0.001 relative to FVB

MiR-155and miR-210 hypoxia expression profile. CO administration promotes the induction of miR-155 in the hypoxic lung

The observation that the M2 macrophage marker, Fizz1, was reduced in response to HO-1 and CO lead us to further investigate the potential role of miRNAs in the macrophage alternative activation. MiR-155 was reported to target IL-13 receptor alpha 1 (IL13Ralpha1) and thus potentially having a role in the regulation of M1/M2 balance in macrophages by modulating IL-13 effects. The 2-fold hypoxic down-regulation of miR-155 (which leads to up-regulation of IL13Ralpha1 and potential expression of the M2 phenotype) was documented in isolated BAL alveolar macrophages, which were exposed to hypoxia (1 % O_2) for 6 hours. (Figure 12C) CO administration increased the levels of miR-155 in total lung of FVB mice thus reversing the down-regulation seen under hypoxia conditions (Figure 12A). Another miRNA of interest, miR-210, which is induced by HIF-1, was found to also be increased 3-fold during hypoxic conditions, however CO administration did not have the same reversing effect. (Figure 12B) In the in vitro experiments miR-210 levels were also found to be up-regulated by hypoxia.

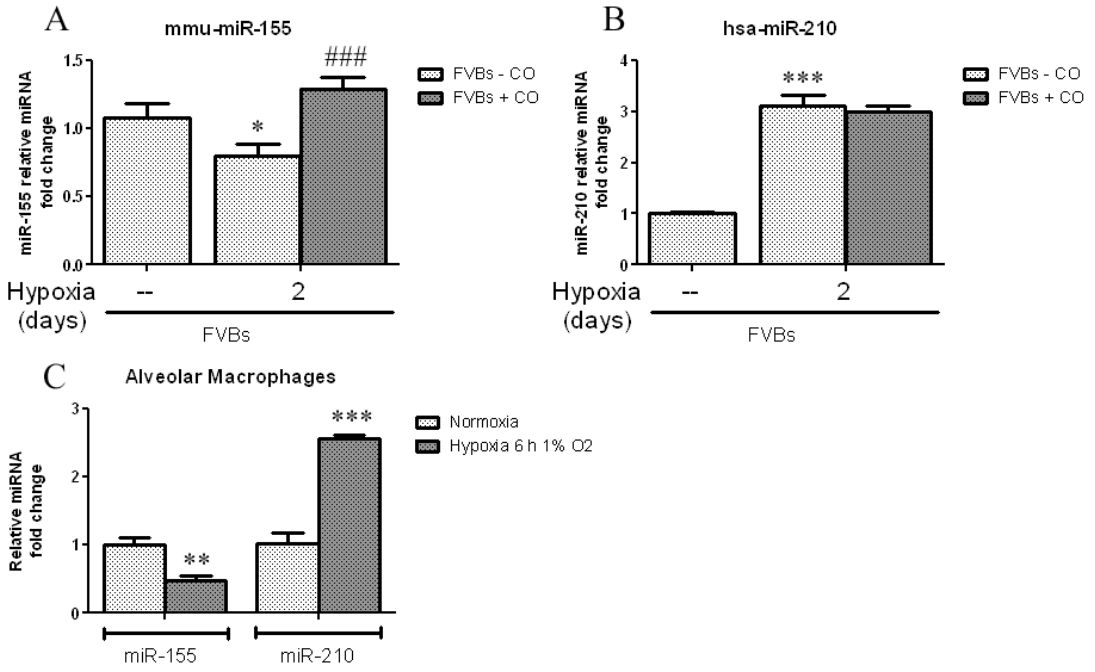

Figure 12. MiR-155 and miR-210 hypoxia expression profile. (A,B) Mice were exposed in hypoxia for 2 days and the levels of miR-155 and mir-210 were assessed in total lung extracts by quantitative PCR (TaqMan MicroRNA Assays). Effect of carbon monoxide (CO) is also indicated (C) Alveolar macrophages were isolated from FVBs mice and exposed to hypoxia 1 % O_2 for 6 hours; Values were normalized with small nucleolar RNA (snoRNA202) housekeeping gene. Numbers represent mean +/- SD; n=4-5 animals per group *P<0.05, **P<0.01, ***P<0.001 relative to normoxia; #P<0.05, ###P<0.001 relative to hypoxia (-CO)

Global miRNA profiling of the lungs of CC77 mice exposed to 8.5 % O2 for 2 days with or without doxycycline treatment

The expression level of 96 miRNAs in the lung was investigated using a MAM-001: Mouse miFinder - miRNA PCR Array (SABiosciences), allowing us to identify their expression pattern induced by HO-1 during the first stages of PAH pathogenesis (Figure 13A). The expression of miR-322, miR-451, miR-21, miR-22, miR-30c, let-7f, and let-7a was the most significantly altered in the presence of doxycycline. More specifically miR-138, miR-124, miR-9 miR-196b were increased more than 1.3 fold compared to the group without doxycycline. Doxycycline administration lowered the levels of miR-106b, miR-18a, miR-54 1.6 fold, 1.5 fold, 1.4 fold respectively. (Figure 13B)

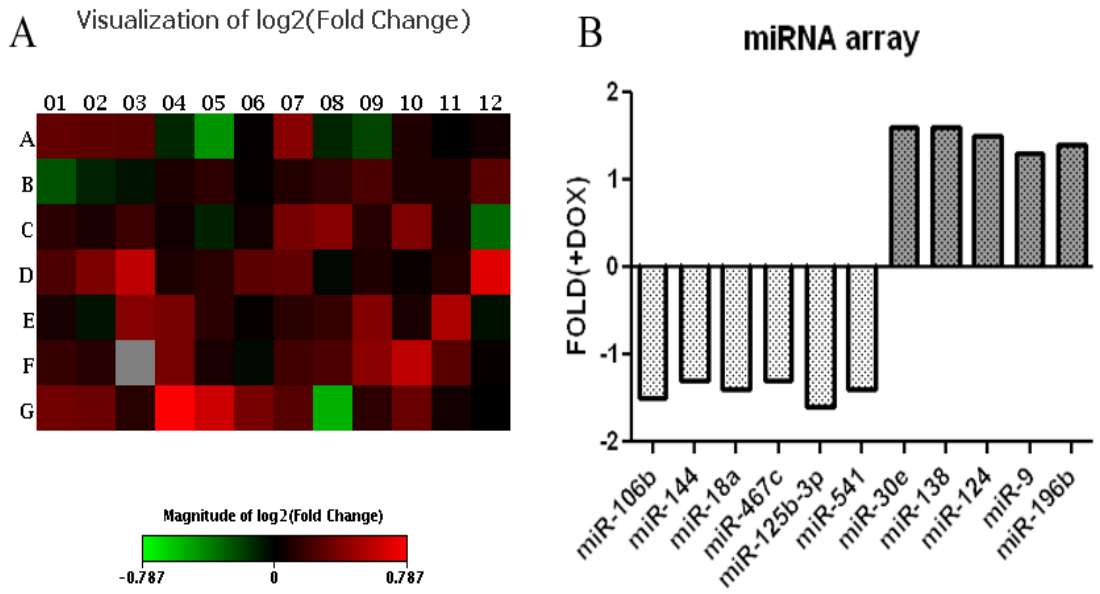

Figure 13. (A, B) Global miRNA Profiling of the Lungs of CC77 mice exposed to 8.5 % O2 for 2 days with or without doxycycline treatment

Discussion

Pulmonary hypertension (PH) is a disease characterized by elevated pulmonary artery pressure. The prognosis of PH is variable and depends on its severity and the underlying disease causing it. The pathological findings of PH include intimal hyperplasia, and fibrosis, medial hypertrophy and in situ thrombi of small pulmonary arteries and arterioles. Early identification and treatment of pulmonary hypertension is highly recommended as advanced disease may be less responsive to therapy.[5-7] Symptomatic patients with idiopathic pulmonary arterial hypertension (IPAH) who do not receive treatment have an average survival of approximately three years.

It has been well established that lung inflammation has an important role in the initiation and maintenance of vascular remodeling in pulmonary hypertension. Inflammation has been studied via long-term exposure of mice to hypoxia, which subsequently leads to muscularization of small pulmonary arteries – an increase in the expression of smooth muscle actin in cells - and recapitulates the elevation of right ventricular systolic pressure and the pathology of human disease. [39]

In our bitransgenic model we demonstrated that right ventricular systolic pressure, the Fulton Index, and the medial wall thickness index were significantly elevated after 21 days of hypoxia exposure. Doxycycline, which results in increased expression of HO-1 in the lungs, when administered for the entire course of hypoxia prevented the increase in right ventricular systolic pressure, the Fulton Index, and the medial wall thickness index in our model.

Foxs1 and Sox17, two transcription factors that are involved in the regulation of endothelial cell differentiation and vessel remodeling, were found to be up-regulated in CC77 bitransgenic and FVB mice exposed to hypoxia (8.5%O_2). This might represent a link between hypoxia exposure and the development of pulmonary hypertension through vascular wall remodeling. Another interesting gene that was up-regulated is the Ntrk2 gene. Ntrk2 is a membrane-bound receptor that upon neurotrophin binding phosphorylates itself and members of the MAPK, which in turn, leads to cell differentiation. A future direction in the study of Fox1, Sox17 and Ntrk2 would be to determine the possible effects of HO-1 and the CO on their expression levels.

In terms of the possible protective effect that CO treatment may have on the development of PH we determined that the administration of CO, 250 ppm twice a day, down-regulated the expression level of CCL12 and CCL2 in hypoxia exposed mice. CCL2 and CCL12 are involved in the recruitment of macrophages in the hypoxic lung a result, which has been previously documented by our lab to be involved in the development of PH. Furthermore, Vergadi et al [40] documented that the overexpression of HO-1 in a lung specific way was found to block this macrophage infiltration and induced a switch in macrophage polarity toward an anti-inflammatory phenotype, thus conferring protective effects against PH.

In order to assess the mechanism by which the alternative activation of macrophages was reduced by HO-1 we started to examine miR-155, which was found to be related to IL13Ralpha1 expression levels. IL-13, IL-4 and IL-5 are well known inducers of the M2 phenotype in macrophages. We have demonstrated that CO administration increased the levels of miR-155 in total lung of FVB mice exposed to hypoxia and reversed the down-regulation seen under the hypoxia conditions. Further evidence supporting the idea of the reversal of the macrophage phenotype either by HO-1 or by its enzymatic product, CO, is provided by the fact that Fizz1 (marker of M2 phenotype) protein levels as measured in BALF are decreased after administration of CO. Finally our HO-1 transgenic mice showed low expression levels of Fizz1 in total lung after two or four days of hypoxia compared to FVB control mice.

HO-1 induced miRNA expression during the first stages of pulmonary hypertension was studied using a miRNA array. Among the miRNAs studied we have found five miRNAs that up-regulated by the administration of doxycycline and six miRNAs that are down-regulated, and these were the most significantly altered in the presence of doxycycline. Further verification and investigation of these specific miRNAs is needed.

On the basis of our findings further clarification of the mechanisms by which miRNAs control the alternative activation of macrophages is needed. Further understanding of the cellular and molecular pathways that contribute to the progression of pulmonary hypertension is necessary, in order to lead to the development of targeted therapies, which may raise hope for the cure.

References:

1. Badesch DB, Champion HC, Sanchez MA, et al. Diagnosis and assessment of pulmonary arterial hypertension. *J Am Coll Cardiol.* Jun 30 2009;54(1 Suppl):S55-66.

2. Barst RJ, McGoon M, Torbicki A, et al. Diagnosis and differential assessment of pulmonary arterial hypertension. *J Am Coll Cardiol.* Jun 16 2004;43(12 Suppl S):40S-47S.

3. Simonneau G, Galie N, Rubin LJ, et al. Clinical classification of pulmonary hypertension. *J Am Coll Cardiol.* Jun 16 2004;43(12 Suppl S):5S-12S.

4. Simonneau G, Robbins IM, Beghetti M, et al. Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol.* Jun 30 2009;54(1 Suppl):S43-54.

5. McLaughlin VV, Archer SL, Badesch DB, et al. ACCF/AHA 2009 expert consensus document on pulmonary hypertension a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents and the American Heart Association developed in collaboration with the American College of Chest Physicians; American Thoracic Society, Inc.; and the Pulmonary Hypertension Association. *J Am Coll Cardiol.* Apr 28 2009;53(17):1573-1619.

6. Galiè N, Rubin LJ, Hoeper MM, et al. Treatment of patients with mildly symptomatic pulmonary arterial hypertension with bosentan (EARLY study): a double-blind, randomised controlled trial. *The Lancet.* 2008;371(9630):2093-2100.

7. Barst RJ, Gibbs JS, Ghofrani HA, et al. Updated evidence-based treatment algorithm in pulmonary arterial hypertension. *J Am Coll Cardiol.* Jun 30 2009;54(1 Suppl):S78-84.

8. Mahapatra S, Nishimura RA, Sorajja P, Cha S, McGoon MD. Relationship of pulmonary arterial capacitance and mortality in idiopathic pulmonary arterial hypertension. *J Am Coll Cardiol.* Feb 21 2006;47(4):799-803.

9. Olivier Sitbon M, Marc Humbert, MD, Hilario Nunes, MD, Florence Parent, MD, Gilles Garcia, MD, Philippe Herve´, MD, Maurizio Rainisio, PHD, Ge´rald Simonneau, MD. Long-Term Intravenous Epoprostenol Infusion in Primary Pulmonary Hypertension Prognostic Factors and Survival. *J Am Coll Cardiol.* 2002;40(4).

10. Benza RL, Miller DP, Gomberg-Maitland M, et al. Predicting survival in pulmonary arterial hypertension: insights from the Registry to Evaluate Early and Long-Term Pulmonary Arterial Hypertension Disease Management (REVEAL). *Circulation.* Jul 13 2010;122(2):164-172.

11. Yildiz P. Molecular mechanisms of pulmonary hypertension. *Clin Chim Acta.* May 2009;403(1-2):9-16.

12. Marc Humbert M, PHD, Nicholas W. Morrell, MD, Stephen L. Archer, MD,‡ Kurt R. Stenmark, MD, Margaret R. MacLean, BSC, PHD, Irene M. Lang, MD,Brian W. Christman, MD,E. Kenneth Weir, MD,Oliver Eickelberg, MD, Norbert F. Voelkel, MD, Marlene Rabinovitch, MD. Cellular and Molecular Pathobiology of Pulmonary Arterial Hypertension. *J Am Coll Cardiol.* 2004;43(12).

13. Pietra GG, Capron F, Stewart S, et al. Pathologic assessment of vasculopathies in pulmonary hypertension. *J Am Coll Cardiol.* Jun 16 2004;43(12 Suppl S):25S-32S.
14. Yuan JX, Rubin LJ. Pathogenesis of pulmonary arterial hypertension: the need for multiple hits. *Circulation.* Feb 8 2005;111(5):534-538.
15. Austin ED, Loyd JE. Genetics and mediators in pulmonary arterial hypertension. *Clin Chest Med.* Mar 2007;28(1):43-57, vii-viii.
16. Deng Z, Morse JH, Slager SL, et al. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet.* Sep 2000;67(3):737-744.
17. Kimura N, Matsuo R, Shibuya H, Nakashima K, Taga T. BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6. *J Biol Chem.* Jun 9 2000;275(23):17647-17652.
18. Wu X, Chang MS, Mitsialis SA, Kourembanas S. Hypoxia regulates bone morphogenetic protein signaling through C-terminal-binding protein 1. *Circ Res.* Aug 4 2006;99(3):240-247.
19. West J, Fagan K, Steudel W, et al. Pulmonary hypertension in transgenic mice expressing a dominant-negative BMPRII gene in smooth muscle. *Circ Res.* Apr 30 2004;94(8):1109-1114.
20. Trembath RC TJ, Machado RD, Morgan NV, Atkinson C, Winship I, Simonneau G, Galie N, Loyd JE, Humbert M, Nichols WC, Morrell NW, Berg J, Manes A, McGaughran J, Pauciulo M, Wheeler L.. Clinical and molecular genetic features of pulmonary hypertension in patients with hereditary hemorrhagic telangiectasia. *The New England Journal of Medicine.* 2001;345(5).
21. Marcos E, Fadel E, Sanchez O, et al. Serotonin-induced smooth muscle hyperplasia in various forms of human pulmonary hypertension. *Circ Res.* May 14 2004;94(9):1263-1270.
22. Chang Y CE, Pessin MS, Lee F, Culpepper J, Knowles DM, Moore PS. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science.* 1994;266.
23. Mehta NJ. HIV-Related Pulmonary Hypertension : Analytic Review of 131 Cases. *Chest.* 2000;118(4):1133-1141.
24. Yu Y, Fantozzi I, Remillard CV, et al. Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. *Proc Natl Acad Sci U S A.* Sep 21 2004;101(38):13861-13866.
25. Hassoun PM, Mouthon L, Barbera JA, et al. Inflammation, growth factors, and pulmonary vascular remodeling. *J Am Coll Cardiol.* Jun 30 2009;54(1 Suppl):S10-19.
26. Tuder RM GB, Badesch DB, Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol.* 1994;144(2):275
27. Pinto RF, Higuchi Mde L, Aiello VD. Decreased numbers of T-lymphocytes and predominance of recently recruited macrophages in the walls of peripheral pulmonary arteries from 26 patients with pulmonary hypertension secondary to congenital cardiac shunts. *Cardiovasc Pathol.* Sep-Oct 2004;13(5):268-275.

28. Perros F, Dorfmuller P, Souza R, et al. Dendritic cell recruitment in lesions of human and experimental pulmonary hypertension. *Eur Respir J.* Mar 2007;29(3):462-468.
29. Pullamsetti SS SR, Janssen W, Dahal BK, Seeger W, Grimminger F, Ghofrani HA, Weissmann N, Schermuly RT. Inflammation, immunological reaction and role of infection in pulmonary hypertension. *Clin Microbiol Infect.* 2011;17(1).
30. Dorfmuller P, Perros F, Balabanian K, Humbert M. Inflammation in pulmonary arterial hypertension. *European Respiratory Journal.* 2003;22(2):358-363.
31. Morrell NW, Adnot S, Archer SL, et al. Cellular and molecular basis of pulmonary arterial hypertension. *J Am Coll Cardiol.* Jun 30 2009;54(1 Suppl):S20-31.
32. Sanchez O, Marcos E, Perros F, et al. Role of endothelium-derived CC chemokine ligand 2 in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med.* Nov 15 2007;176(10):1041-1047.
33. Heresi GA, Aytekin M, Newman J, Dweik RA. CXC-chemokine ligand 10 in idiopathic pulmonary arterial hypertension: marker of improved survival. *Lung.* Jun 2010;188(3):191-197.
34. Kherbeck N, Tamby MC, Bussone G, et al. The Role of Inflammation and Autoimmunity in the Pathophysiology of Pulmonary Arterial Hypertension. *Clin Rev Allergy Immunol.* Mar 11 2011.
35. Heldin CH WB. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev.* 1999;79(4).
36. Gunaje JJ, Bahrami AJ, Schwartz SM, Daum G, Mahoney WM, Jr. PDGF-dependent regulation of Regulator of G-protein Signaling-5 expression and vascular smooth muscle cell functionality. *Am J Physiol Cell Physiol.* May 18 2011.
37. Soon E, Holmes AM, Treacy CM, et al. Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension. *Circulation.* Aug 31 2010;122(9):920-927.
38. Naeije R, Dewachter L. Animal models of pulmonary arterial hypertension. *Rev Mal Respir.* Apr 2007;24(4 Pt 1):481-496.
39. Stenmark KR, Meyrick B, Galie N, Mooi WJ, McMurtry IF. Animal models of pulmonary arterial hypertension: the hope for etiological discovery and pharmacological cure. *Am J Physiol Lung Cell Mol Physiol.* Dec 2009;297(6):L1013-1032.
40. Vergadi E, Chang MS, Lee C, et al. Early macrophage recruitment and alternative activation are critical for the later development of hypoxia-induced pulmonary hypertension. *Circulation.* May 10 2011;123(18):1986-1995.
41. Eckle T, Faigle M, Grenz A, Laucher S, Thompson LF, Eltzschig HK. A2B adenosine receptor dampens hypoxia-induced vascular leak. *Blood.* Feb 15 2008;111(4):2024-2035.
42. Thompson LF, Eltzschig HK, Ibla JC, et al. Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. *J Exp Med.* Dec 6 2004;200(11):1395-1405.
43. Kourembanas S. Hypoxia and carbon monoxide in the vasculature. *Antioxid Redox Signal.* Apr 2002;4(2):291-299.

44. Burke DL, Frid MG, Kunrath CL, et al. Sustained hypoxia promotes the development of a pulmonary artery-specific chronic inflammatory microenvironment. *Am J Physiol Lung Cell Mol Physiol.* Aug 2009;297(2):L238-250.
45. Grocott MP, Martin DS, Levett DZ, McMorrow R, Windsor J, Montgomery HE. Arterial blood gases and oxygen content in climbers on Mount Everest. *N Engl J Med.* Jan 8 2009;360(2):140-149.
46. O'Brien B. High-altitude illness. *N Engl J Med.* Oct 25 2001;345(17):1280; author reply 1280-1281.
47. Hartmann G, Tschop M, Fischer R, et al. High altitude increases circulating interleukin-6, interleukin-1 receptor antagonist and C-reactive protein. *Cytokine.* Mar 2000;12(3):246-252.
48. Semenza GL. Life with oxygen. *Science.* Oct 5 2007;318(5847):62-64.
49. Kaelin WG, Jr., Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol Cell.* May 23 2008;30(4):393-402.
50. Naugler WE, Karin M. NF-kappaB and cancer-identifying targets and mechanisms. *Curr Opin Genet Dev.* Feb 2008;18(1):19-26.
51. Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol.* 2009;27:693-733.
52. Pasparakis M. IKK/NF-kappaB signaling in intestinal epithelial cells controls immune homeostasis in the gut. *Mucosal Immunol.* Nov 2008;1 Suppl 1:S54-57.
53. Brusselmans K. Heterozygous deficiency of hypoxia-inducible factor-2alpha protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. *Journal of Clinical Investigation.* 2003;111(10):1519-1527.
54. Yu AY, Shimoda LA, Iyer NV, et al. Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. *J Clin Invest.* Mar 1999;103(5):691-696.
55. Shimoda LA, Semenza GL. HIF and the lung: role of hypoxia-inducible factors in pulmonary development and disease. *Am J Respir Crit Care Med.* Jan 15 2011;183(2):152-156.
56. Mojsilovic-Petrovic J, Callaghan D, Cui H, Dean C, Stanimirovic DB, Zhang W. Hypoxia-inducible factor-1 (HIF-1) is involved in the regulation of hypoxia-stimulated expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) and MCP-5 (Ccl12) in astrocytes. *J Neuroinflammation.* 2007;4:12.
57. Yamaji-Kegan K, Su Q, Angelini DJ, Myers AC, Cheadle C, Johns RA. Hypoxia-induced mitogenic factor (HIMF/FIZZ1/RELMalpha) increases lung inflammation and activates pulmonary microvascular endothelial cells via an IL-4-dependent mechanism. *J Immunol.* Nov 1 2010;185(9):5539-5548.
58. Eltzschig HK, Carmeliet P. Hypoxia and inflammation. *N Engl J Med.* Feb 17 2011;364(7):656-665.
59. Abraham NG, Kappas A. Pharmacological and clinical aspects of heme oxygenase. *Pharmacol Rev.* Mar 2008;60(1):79-127.

60. Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev.* Apr 2006;86(2):583-650.
61. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. *Science.* Feb 27 1987;235(4792):1043-1046.
62. McCoubrey WK, Jr., Huang TJ, Maines MD. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem.* Jul 15 1997;247(2):725-732.
63. Loboda A, Jazwa A, Grochot-Przeczek A, et al. Heme oxygenase-1 and the vascular bed: from molecular mechanisms to therapeutic opportunities. *Antioxidants & redox signaling.* Oct 2008;10(10):1767-1812.
64. Leopold JA, Loscalzo J. Oxidative enzymopathies and vascular disease. *Arterioscler Thromb Vasc Biol.* Jul 2005;25(7):1332-1340.
65. Shan Y, Pepe J, Lu TH, Elbirt KK, Lambrecht RW, Bonkovsky HL. Induction of the heme oxygenase-1 gene by metalloporphyrins. *Arch Biochem Biophys.* Aug 15 2000;380(2):219-227.
66. Christou H, Morita T, Hsieh CM, et al. Prevention of hypoxia-induced pulmonary hypertension by enhancement of endogenous heme oxygenase-1 in the rat. *Circ Res.* Jun 23 2000;86(12):1224-1229.
67. Zampetaki A, Minamino T, Mitsialis SA, Kourembanas S. Effect of heme oxygenase-1 overexpression in two models of lung inflammation. *Exp Biol Med (Maywood).* May 2003;228(5):442-446.
68. Vitali SH, Mitsialis SA, Christou H, Fernandez-Gonzalez A, Liu X, Kourembanas S. Mechanisms of heme oxygenase-1-mediated cardiac and pulmonary vascular protection in chronic hypoxia: roles of carbon monoxide and bilirubin. *Chest.* Dec 2005;128(6 Suppl):578S-579S.
69. Vitali SH, Mitsialis SA, Liang OD, et al. Divergent cardiopulmonary actions of heme oxygenase enzymatic products in chronic hypoxia. *PLoS One.* 2009;4(6):e5978.
70. Minamino T, Christou H, Hsieh CM, et al. Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. *Proc Natl Acad Sci U S A.* Jul 17 2001;98(15):8798-8803.
71. Oglesby IK, McElvaney NG, Greene CM. MicroRNAs in inflammatory lung disease--master regulators or target practice? *Respir Res.* 2010;11:148.
72. Jiang S, Zhang HW, Lu MH, et al. MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene. *Cancer Res.* Apr 15 2010;70(8):3119-3127.
73. Martinez-Nunez RT, Louafi F, Sanchez-Elsner T. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). *J Biol Chem.* Jan 21 2011;286(3):1786-1794.
74. Altuvia Y, Landgraf P, Lithwick G, et al. Clustering and conservation patterns of human microRNAs. *Nucleic Acids Res.* 2005;33(8):2697-2706.
75. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. *Science.* Jan 2 2004;303(5654):95-98.
76. Macrae IJ, Zhou K, Li F, et al. Structural basis for double-stranded RNA processing by Dicer. *Science.* Jan 13 2006;311(5758):195-198.

77. Hammell CM. The microRNA-argonaute complex: a platform for mRNA modulation. *RNA Biol.* Jul-Sep 2008;5(3):123-127.
78. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* Jan 23 2004;116(2):281-297.
79. Chang CL, Au LC, Huang SW, Fai Kwok C, Ho LT, Juan CC. Insulin up-regulates heme oxygenase-1 expression in 3T3-L1 adipocytes via PI3-kinase- and PKC-dependent pathways and heme oxygenase-1-associated microRNA downregulation. *Endocrinology.* Feb 2011;152(2):384-393.
80. O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D. Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol.* Feb 2010;10(2):111-122.
81. Yadav A, Saini V, Arora S. MCP-1: chemoattractant with a role beyond immunity: a review. *Clin Chim Acta.* Nov 11 2010;411(21-22):1570-1579.
82. Pocock R. Invited review: decoding the microRNA response to hypoxia. *Pflugers Arch.* Mar 2011;461(3):307-315.
83. Huang X, Le QT, Giaccia AJ. MiR-210--micromanager of the hypoxia pathway. *Trends Mol Med.* May 2010;16(5):230-237.
84. Devlin C, Greco S, Martelli F, Ivan M. miR-210: More than a silent player in hypoxia. *IUBMB Life.* Feb 2011;63(2):94-100.
85. Kelly TJ, Souza AL, Clish CB, Puigserver P. A Hypoxia-Induced Positive Feedback Loop Promotes Hypoxia-Inducible Factor 1{alpha} Stability through miR-210 Suppression of Glycerol-3-Phosphate Dehydrogenase 1-Like. *Mol Cell Biol.* Jul 2011;31(13):2696-2706.
86. Caruso P, MacLean MR, Khanin R, et al. Dynamic changes in lung microRNA profiles during the development of pulmonary hypertension due to chronic hypoxia and monocrotaline. *Arterioscler Thromb Vasc Biol.* Apr 2010;30(4):716-723.
87. Perl AK, Tichelaar JW, Whitsett JA. Conditional gene expression in the respiratory epithelium of the mouse. *Transgenic Res.* Feb 2002;11(1):21-29.
88. Fagan KA, Fouty BW, Tyler RC, et al. The pulmonary circulation of homozygous or heterozygous eNOS-null mice is hyperresponsive to mild hypoxia. *J Clin Invest.* Jan 1999;103(2):291-299.
89. Jones R, Jacobson M, Steudel W. alpha-smooth-muscle actin and microvascular precursor smooth-muscle cells in pulmonary hypertension. *Am J Respir Cell Mol Biol.* Apr 1999;20(4):582-594.
90. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* May 1 2001;29(9):e45.