

# The 2011 Grover Conference

## Risk Factors in Pulmonary Hypertension: Gender, Sex Hormones, Obesity, Novel Genetic Influences and Risk Factors Sedalia, Colorado, USA 7-11 September, 2011

## 1.1

**Apelin and pulmonary hypertension***Andersen CU*

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The peptide apelin and the G-protein-coupled apelin receptor (APLNR) are expressed in several tissues throughout the organism, and are highly expressed in the lungs. In accordance, apelin and the APLNR have proven to influence many physiological functions such as food and water intake and regulation of insulin in obesity. However, some of the first established effects of apelin and the APLNR were regulatory roles in vascular and cardiac tissues. In the cardiovascular system, apelin is localized in endothelial cells in agreement with an endothelial synthesis, while the APLNR is localized in both endothelial and smooth muscle cells in the heart and vessels. Apelin plays a role in angiogenesis, and there is evidence from experimental and human studies that apelin exerts nitric oxide (NO)-dependent vasodilatation in the systemic circulation and modulates the expression and activity of endothelial NO synthase (eNOS). In addition, apelin has proven to be a potent positive inotropic agent. In patients with heart failure, including patients with pulmonary hypertension (PH), plasma levels of apelin are altered. On the basis of apelin's localization and effects in the systemic circulation, interest in the role of the peptide in PH has emerged. Apelin has subsequently been investigated as a potential lung-derived biomarker and a possible new therapeutic for PH. A study has shown that apelin levels in the lungs are decreased in animals with PH. This decrease was, however, not reflected in plasma, which does not support that plasma apelin measurements give information about changes in the pulmonary vascular endothelium in the setting of PH. Apelin modulates vasoconstriction in isolated rat pulmonary arteries, and chronic treatment with apelin has been demonstrated to reduce pulmonary pressure in experimental models of monocrotaline and hypoxia induced PH. In agreement, a recent study shows that apelin deficiency worsens the development of PH. The mechanisms involved were downregulation of eNOS and loss of small-size arteries. The existing literature thus renders APLNR a potential new therapeutic target for PH that needs more investigation.

## 1.2

**Alterations in estrogen metabolism: Implications for higher penetrance of FPAH in females***Austin ED, West JD, Hamid R, Hemnes AR, Cogan JD, Parl FF, Phillips JA III, and Loyd JE*

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Female gender is the strongest and best-established risk factor for pulmonary arterial hypertension (PAH), with a female to male ratio of ~2 to 4:1 in many etiologies of PAH, including the idiopathic (IPAH) and heritable (HPAH) forms. However, until recently mechanistic details behind the female predominance were unknown. Complicating the gender dimorphism, recent reports from the two large epidemiological registries

of PAH in France and in the United States found improved survival among females, with males over 60 years perhaps the most disadvantaged. The female predominant incidence and prevalence suggest a role for sex hormones in disease pathogenesis, implicating estrogens, androgens, and their metabolic products as a potential cause. However, sex hormones' impact upon the pulmonary vasculature is complex, operating by both acute and chronic effects: acutely protective, some estrogens may be detrimental on a chronic basis. In fact, we and others have hypothesized that while absolute sex hormone levels are important, it is the relative ratio of their metabolites that influence disease risk—especially among those with a genetic predisposition due to a BMPR2 gene mutation. Subjects within families affected by PAH associated with BMPR2 gene mutations (BMPR2-PAH) provide the opportunity to study patients, as well as those at genetic risk of disease, to determine factors which may influence risk. Parent compound estrogens (e.g., estradiol, E2) in both men and women are predominantly metabolized through hydroxylation at the 2- or 16- position. In vitro, 16-estrogens are more mitogenic and may also be more genotoxic via the formation of unstable DNA adducts, although in most women the precise ratio 2- to 16-estrogens is of minimal concern. However, in the context of a BMPR2 mutation perturbed estrogen metabolism may influence disease penetrance: Women who have a BMPR2 mutation and preferentially metabolize E2 into 16-estrogens may be at higher risk of disease, while those who preferentially metabolize E2 into 2-estrogens may be protected. CYP1B1 is a cytochrome P450 enzyme critical to estrogen metabolism, although its activity generates 2-estrogens and 4-estrogens preferentially over the 16-estrogens from E2. An association between low CYP1B1 mRNA expression and disease penetrance has been demonstrated, and it was subsequently shown that functional promoter polymorphisms in CYP1B1 are associated with risk of PAH among female BMPR2 mutation carriers, which both imply causation. Furthermore, female BMPR2-PAH patients have a low ratio of 2-estrogens to 16-estrogens in their urine compared to matched- unaffected BMPR2 mutation carriers. These findings suggest that a low 2-estrogen:16-estrogen ratio is a risk factor for BMPR2-PAH penetrance and perhaps an exacerbating factor in established PAH of other types. Thus, factors that restore the 2-estrogens, or those that block 16-estrogens, may prevent disease penetrance and progression among BMPR2 mutation carriers and perhaps other subjects at risk. Interestingly, 2-estrogens have been used to prevent and treat monocrotaline-induced rodent pulmonary hypertension, and 16-estrogens appear to exacerbate pulmonary vascular resistance in a rodent model of BMPR2-PAH. Although much work remains to fully understand the complex influences of estrogens, estrogen metabolites, and other sex hormones on the pulmonary vasculature, mounting data suggest that they are relevant to PAH pathogenesis.

## 1.3

**Etiologies and comorbidities in PH: Insight from the REVEAL Registry***Badesch DB, Raskob GE, Elliott CG, Krichman AM, Farber HW, Frost AE, Barst RJ, Benza RL, Liou TG, Turner M, Giles S, Feldkircher K, Miller DP, and McGoon MD*

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The Registry to Evaluate Early And Long-term pulmonary arterial hypertension disease management (REVEAL Registry) was established to provide updated characteristics of patients with pulmonary arterial hypertension (PAH) and to improve diagnosis, treatment, and management. Fifty-four US centers enrolled consecutively screened patients with World Health Organization group I PAH who met expanded hemodynamic criteria of mean pulmonary arterial pressure (PAP) >25 mmHg at rest (30 mmHg with exercise), pulmonary capillary wedge pressure (PCWP) ≤18 mmHg, and pulmonary vascular resistance ≥240 dynes · s · cm<sup>-5</sup>. Patients meeting the traditional hemodynamic definition (PCWP ≤15 mmHg) were compared with those with a PCWP of 16-18 mmHg. Between March 2006 and September 2007, 2,967 patients enrolled. Among 2,525 adults meeting traditional hemodynamic criteria, the mean age was 53 ± 14 years, and 2,007 (79.5%) were women. Forty-six percent had idiopathic pulmonary arterial hypertension (IPAH), and 51% associated PAH. Of those with associated PAH, 50% had connective tissue disease (CTD), 20% had congenital heart disease, and 10% had drug- and toxin-related PAH. The mean duration between symptom onset and diagnostic catheterization was 2.8 years, and 1,008 (41.3%) patients were treated with more than one pulmonary vascular-targeted medication. Comorbidities included systemic hypertension, obesity, depression and thyroid disease. Compared with patients meeting the traditional hemodynamic definition of PAH, patients with a PCWP of 16 to 18 mmHg were older, more obese, had a lower 6-minute walk distance, and had a higher incidence of systemic hypertension, sleep apnea, renal insufficiency, and diabetes. Patients in the REVEAL Registry are older and more often female than in previous descriptions. Delays between symptom onset and diagnostic catheterization persist. Many treatment regimens are fundamentally empirical, and data will be required to determine outcomes, improve risk stratification, and develop and validate more precise prognostic tools. Patients with PCWP of 16-18 mmHg differ in a number of important respects from those meeting the traditional hemodynamic definition of PAH.

#### 1.4

### Persistent pulmonary hypertension of the newborn: Serotonin as a risk factor

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Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome characterized by the failure to achieve or sustain the normal decrease in pulmonary vascular resistance (PVR) after birth. Recently, epidemiological studies have linked the use of selective serotonin reuptake inhibitors (SSRIs) for the treatment of maternal depression to a 6-fold increase in the incidence of PPHN when taken during the second half of gestation, suggesting that altered serotonin (5-HT) signaling may contribute to the risk for PPHN. However, very little is known about the roles of 5-HT in the normal development of lungs or in the pathobiology of PPHN. In addition, the direct effects of SSRIs on the fetal pulmonary circulation are unknown, and insights into these observations may lead to a greater understanding of the pathophysiology and treatment of PPHN. In the adult pulmonary circulation, 5-HT is a potent vasoconstrictor and stimulates smooth muscle cell growth and proliferation. Pharmacological or genetic strategies that augment or inhibit 5-HT signaling worsen or improve the severity of pulmonary hypertension in adult animal models, respectively. In striking contrast to the effects of maternal SSRI use on the fetal lung, recent studies suggest that SSRI therapy reduces pulmonary hypertension in adult animal models and in the clinical setting. This apparent disparity between the effects of SSRI further suggests striking developmental differences in 5-HT signaling; however, data on mechanisms of 5-HT signaling pathways in the developing lung or in models of PPHN are limited. We hypothesize that 5-HT plays a key role in maintaining high pulmonary vascular resistance (PVR) in the fetus, and that fetal exposure to SSRIs increases 5-HT activity and causes pulmonary hypertension. We studied the hemodynamic effects of 5-HT,

5-HT receptor antagonists, and SSRIs in both chronically prepared fetal sheep and in an ovine model of PPHN. In the chronically prepared ovine fetus, brief infusions of 5-HT (3-20 µg) increased PVR in a dose-related fashion. Ketanserin, a 5-HT 2A receptor antagonist, caused pulmonary vasodilation and inhibited 5-HT induced pulmonary vasoconstriction. In contrast, intrapulmonary infusions of GR127945 and SB206553, 5HT 1B and 5HT 2B receptor antagonists, respectively, had no effect on basal PVR or 5HT-induced vasoconstriction. Pretreatment with fasudil, a Rho kinase inhibitor, blunted the effects of 5-HT infusion. Brief infusions of the SSRIs, sertraline and fluoxetine, caused potent and sustained elevations of PVR. SSRI-induced pulmonary vasoconstriction was reversed by infusion of ketanserin, and did not affect the acute vasodilator effects of acetylcholine. In the lamb with experimental PPHN, SSRIs and 5-HT cause further elevation of pulmonary vascular without any evidence for vasodilation. We conclude that 5-HT causes pulmonary vasoconstriction and contributes to maintenance of high PVR in the normal fetus through stimulation of 5-HT 2A receptors and Rho kinase activation, and mediates the hypertensive effects of SSRIs. The effect of SSRIs on the developing pulmonary circulation in neonatal pulmonary hypertension differs from their effect in adult models of pulmonary hypertension. This may be due to developmental differences in 5-HT receptor or transporter expression, or unique effects on utero-placental circulation.

#### 1.5

### microRNAs-control of essential genes: Implications for pulmonary vascular disease

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During lung development and disease pathogenesis structural cells in the lungs adapt to permit changes in lung function. Fibroblasts, myofibroblasts, smooth muscle, epithelial cells, and various progenitor cells can all undergo phenotypic modulation. In the pulmonary vasculature occlusive vascular lesions occurring in severe pulmonary arterial hypertension are multifocal, polyclonal lesions containing cells presumed to have undergone phenotypic transition resulting in altered proliferation, cell lifespan or contractility. Dynamic changes in gene expression and protein composition that underlie processes responsible for such cellular plasticity are not fully defined. Advances in molecular biology have shown that multiple classes of RNA collaborate to establish the set of proteins expressed in a cell. Both coding (mRNA, rRNA) and noncoding RNAs (snoRNA, snRNA, miRNA and lncRNA) act via multiple parallel signaling pathways to regulate transcription, mRNA processing, mRNA stability, translation and possibly protein lifespan. Rapid progress has been made in describing dynamic features of miRNA expression and miRNA function in some vascular tissues. However posttranscriptional gene silencing by microRNA-mediated mRNA degradation and translational blockade is not as well defined in the pulmonary vasculature. Recent progress in defining miRNAs that modulate vascular cell phenotypes will be reviewed to illustrate both functional and therapeutic significance of small noncoding RNAs in pulmonary arterial hypertension.

#### 1.6

### The role of medical therapy in non-PAH pulmonary hypertension

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The somewhat copious term non-pulmonary arterial hypertension (PAH) pulmonary hypertension (non-PAH PH) is used to subsume groups 2, 3, and 4 of the current classification of PH, i.e., PH due to left heart disease, PH due to lung disease and/or hypoxia, and

chronic thromboembolic pulmonary hypertension (CTEPH). Among these, CTEPH stands out as the only entity where even severe PH may be potentially curable. Surgical pulmonary endarterectomy is the treatment of choice for these patients. Medical therapy with "PAH drugs," i.e., prostanoids, endothelin receptor antagonists (ETRA), or phosphodiesterase-5 (PDE-5) inhibitors, can be considered for inoperable patients but the evidence to support this recommendation remains limited and none of these drugs have received FDA approval for treating CTEPH. PH due to left heart disease or lung disease is much more common than PAH or CTEPH, respectively, but they have been less well evaluated. For both groups there is considerable evidence showing that the development of PH has profound effects on exercise capacity and survival. The pathophysiology of these forms of PH, however, differs substantially from that of PAH and it is unclear whether these patients (or a subpopulation) may benefit from treatment with "PAH drugs." Several randomized, controlled clinical trials with ETRA have been performed in patients with left heart diseases but have failed to show beneficial effects in these patients. The same is true for patients with chronic obstructive pulmonary disease. ETRA were also investigated in patients with pulmonary fibrosis as experimental data have suggested anti-fibrotic effects of endothelin receptor blockade. However, four randomized clinical trials with ETRA in patients with pulmonary fibrosis were negative and another clinical trial with an ETRA in patients with idiopathic pulmonary fibrosis (IPF) and pulmonary hypertension was early terminated after a futility analysis. PDE-5 inhibitors have also not yet proven to have clinical value in patients with chronic lung disease and PH. A recent randomized controlled clinical trial with sildenafil in patients with IPF did not meet the primary endpoint but gave some positive signals on secondary endpoints, such as changes in oxygen saturation and change in DLCO. The clinical implications of these findings are uncertain. In patients with left heart disease and PH, PDE-5 inhibitors hold promise as experimental data suggest that these substances may not only act as pulmonary vasodilators but may also improve the systolic and diastolic function of the left ventricle. Taken together, for all forms of non-PAH PH there is a lack of robust evidence to support any form of targeted medical therapy and much more research is needed to define which therapeutic approach will be best suited for which patient.

### 1.7

#### Epigenetics, sex, and cardiovascular function with a focus on exchange

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Epigenetics, in its purest form, investigates heritable changes in gene expression caused by mechanisms other than alterations in DNA sequence. Those mechanisms fall into three categories: the first being an environmental signal; the second being a cellular response that localizes to the affected chromosomal location; and the third being a signal that results in a chromatin change that transmits phenotypic changes in subsequent generations. These changes result in a wide variety of cell phenotypes that help in accounting for the wider variety of normal and disease states than can be accounted for by genetics alone. It is currently being further appreciated that genomic sex (XX or XY), independent from the actions of the reproductive hormones, is a factor requiring consideration in the study of cardiovascular function in health and disease. The interactions of epigenetics and sex have received attention in the fields of neuroscience, cancer, and mental health; much less focus on the combination of factors has been paid in the areas of cardiovascular and pulmonary function or disease. Drawing on examples from recent research into how males and females regulate vascular exchange similarly and differently, this talk should focus on why epigenetics and sex requires consideration in the clinical and laboratory settings.

### 1.8

#### Update and overview of PAH genetics

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Pulmonary arterial hypertension (PAH) in families (FPAH) has been known for most of the last century, but progress in understanding its molecular basis is just beginning. Heritable PAH (HPAH) is a useful term adopted to encompass either FPAH or idiopathic (IPAH) patients who are found to carry a responsible mutation (10-40%) despite negative family history. HPAH should not replace FPAH, but should instead expand our PAH vocabulary. One PAH family in Tennessee has suffered 18 patients from 1950 to 2000, and another 18 patients from 2000 to 2011. Another six American families have each experienced 10 or more PAH patients. Familial PAH is also reported from Western Europe (France, Germany, Italy, UK) and Asia (Japan, China). Heterozygous mutation in *BMPR2* accounts for the majority of HPAH (75%), but is confounded by variable age of onset and decreased penetrance. The lifetime penetrance of symptomatic PAH in *BMPR2* mutation carriers is about 20%, so only a small minority ever develop PAH, and it may begin at any age. PAH patients with *BMPR2* mutation have a more severe hemodynamic profile at the time of diagnosis, but it remains unproven whether survival differs. Mutation in other TGF- $\beta$  family members, including *ACVRL1* or *ALK1*, *endoglin*, and *SMAD 8*, are rarely the basis of HPAH. Prospective studies of PAH outcome related to mutation status are few, but one such study in France suggested similar PAH survival of *BMPR2* mutation versus not, while *ALK1*-mutant patients showed far worse survival. Some genetic modifiers for clinical expression of *BMPR2* mutation were identified by a functional candidate approach (TGF- $\beta$ ) and others by comparison of expression arrays in extremes of phenotype (*CYP1B1*). The latter finding led to follow-up studies which demonstrate a role for estrogen and its metabolism in the female disparity. Studies in *BMPR2* mutations which have nonsense-mediated decay demonstrate that the expression level of the normal allele relates to the clinical development of disease. Novel linkage studies have identified 4 loci which hold promise for identification of modifiers for clinical expression of *BMPR2* mutation. Next generation sequencing including whole exome sequencing is expected to identify other genes responsible for PAH families without *BMPR2* mutation (>40 families known in the US). Importantly for the several thousand asymptomatic mutation carriers in the 120 families in the US with *BMPR2* mutation, early detection of disease is not currently possible. No tests are known to detect the vascular disease itself, and extensive pulmonary vascular loss (~60%) occurs unrecognized prior to onset of symptoms, thus the disease process is already far advanced when the first symptom occurs. The translation of current knowledge to develop new diagnostic strategies and new therapies is drastically needed for this tragic disease.

### 1.9

#### The influence of serotonin and estrogen in the development of pulmonary arterial hypertension

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Serotonin has been implicated in the development of clinical and experimental pulmonary arterial hypertension (PAH). The development of hypoxia- and dexfenfluramine-induced is ablated in mice devoid of tryptophan hydroxylase 1 (TPH1) and hence requires peripheral serotonin. Mice that over-express the human gene for the serotonin transporter (SERT+mice) develop elevated pulmonary pressures and exaggerated hypoxia-induced PAH. We recently demonstrated that only female SERT+ mice demonstrate this phenotype and only at 5-6 months of age. Ovariectomy of these mice abolishes the PAH phenotype and

subsequent replacement of 17 $\beta$ -estradiol restores the PAH phenotype. Dexamfluramine-induced PAH is also only observed in female mice and abolished by ovariectomy. In hPASCs, 17 $\beta$ -estradiol-induced proliferation is abolished by inhibitors of the serotonin system and 17 $\beta$ -estradiol can increase expression of TPH1, SERT and the 5-HT1B receptor. Microarray studies show that several genes are elevated in female SERT+ mice that may account for the PAH phenotype. For example, CEBPB, CYP1B1 and FOS are increased in SERT+ female mice. Serotonin and 17 $\beta$  estradiol increased CEBPB, CYP1B1 and FOS protein expression in hPASCs and in addition, CEBPB, CYP1B1 and FOS mRNA and protein expression are increased in PASCs derived from IPAH patients. As the activity of CYP1B1 has been shown to be altered in patients with IPAH, we examined the development of PAH in CYP1B1 $^{-/-}$  mice and demonstrated that PAH ablated in these mice. Likewise the CYP1B1 inhibitor TMS ablated the development of hypoxia-induced PAH. Of the estrogen metabolites examined, 16-hydroxyestrone induced profound proliferation in hPASCs. We also showed that 1.5 mg/kg 16-hydroxyestrone I.P. for 21 days can induce PAH in mice. Hence we propose that serotonin and estrogens interact to facilitate the development of PAH. We propose that the activity of CYP1B1 and estrogen metabolites play a key role in influencing the PAH phenotype. This work was funded by the British Heart Foundation and the BBSRC, UK.

### 1.10

#### **Fat, fire and muscle: The effects of adiponectin on pulmonary vascular inflammation and remodeling**

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Accumulating evidence demonstrates that obesity can increase both the incidence and severity of inflammatory diseases such as atherosclerosis, diabetes, and pulmonary hypertension (PH). PH results from a heterogeneous group of diseases, many of which demonstrate characteristic pathologic changes of pulmonary vascular inflammation and remodeling. Adipocytes secrete multiple bioactive mediators that can influence inflammation and tissue remodeling, suggesting that adipose tissue may directly influence the pathogenesis of PH. One of these mediators is adiponectin, a protein with a wide range of metabolic, anti-inflammatory, and anti-proliferative activities. Interestingly, individuals with obesity have low plasma adiponectin levels, suggesting that decreased adiponectin may contribute to the increased prevalence of disease seen in obesity. We recently reported that deficiency of adiponectin exacerbated PH in a mouse model, and was associated with enhanced eosinophil recruitment and pulmonary artery smooth muscle cell (PASMC) proliferation. In more recent experiments, we utilized eosinophil-deficient mice and adiponectin-overexpressing mice in a murine model of PH induced by allergic inflammation to demonstrate that adiponectin suppresses pulmonary vascular remodeling via effects on eosinophil recruitment and PASMC proliferation. In support of a pathogenic role of eosinophil recruitment, elimination of eosinophils in the lung significantly attenuated pulmonary arterial muscularization and reduced right ventricular systolic pressure (RVSP) in this model of PH. In addition, we found that the extracts of eosinophil granules were able to promote the proliferation of PASMCs in vitro and induce mitogenic signaling in these cells. Furthermore, expression levels of the eosinophil-specific chemokines CCL11 and CCL24 were increased in macrophages isolated from adiponectin-deficient mice in the model compared to macrophages from wild-type mice. These data suggest that adiponectin may modulate PH in this model in part by affecting eosinophil recruitment into the lung. In separate experiments, we demonstrated that transgenic mice that overexpress adiponectin had reduced PH and pulmonary vascular remodeling compared to wild-type

mice, but developed similar levels of pulmonary vascular inflammation in the model. Consistent with this result, adiponectin directly suppressed pulmonary artery smooth muscle cell proliferation in vitro, and reduced activity of several pro-proliferative molecular pathways in these cells. Overall, these data suggest that eosinophilic inflammation is necessary for pulmonary vascular remodeling in this model, and that adiponectin regulates remodeling through effects on eosinophil accumulation and direct effects on pulmonary artery smooth muscle cells.

### 1.11

#### **Progenitor cells in pulmonary arterial hypertension**

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Recent interest has focused on the potential role of endothelial progenitor cells (EPCs) in pulmonary arterial hypertension (PAH). Circulating cytokines stimulate the bone marrow to release progenitor cells, which home to areas of vascular injury. Within the circulation, EPCs can be identified by the expression of cell-surface markers such as CD34, CD133, cKit, and vascular endothelial growth factor receptor (VEGFR). The true endogenous role of these progenitor cells is currently unknown. Recent studies have measured the levels of putative endothelial progenitor cells (EPCs) in the circulation of individuals with PAH. EPCs, as defined by the expression of CD133, CD34, and VEGFR2, were elevated in the peripheral circulation of individuals with idiopathic PAH and those with the heritable form of pulmonary hypertension with mutations in BMPR2. While another group has reported similar results, others found decreases in these markers, and the role of EPCs in pulmonary vascular repair remains unclear. In addition to counting cells in the peripheral circulation, the researchers have looked for the expression of EPC markers in the tissues from patients with pulmonary hypertension. CD133 was faintly expressed in normal peripheral pulmonary arteries, more highly expressed in concentric intimal lesions, and very highly expressed in plexiform lesions. In addition, the major homing signal for EPCs is upregulated in plexiform lesions, raising the possibility that cells that express EPC markers may be involved in the formation of these lesions. Therapeutically, EPCs, particularly early (E)-EPCs, have been reported to both prevent and reverse the pulmonary hypertensive phenotype in the monocrotaline rat model. These findings led to an ongoing trial in humans, which is producing encouraging preliminary data. Further research using human cells in an athymic nude rat model has indicated that late outgrowth (L-EPCs) do not have the same effect as E-EPCs in rescuing pulmonary hypertensive phenotypes. This research also found that E-EPCs maintain this therapeutic action despite being retained within the lung for only a short period of time (<24 h). A repetition of this study in nude rats in which natural killer cells had been ablated resulted in a longer retention time for EPCs. However, removing the natural killer cells removed the therapeutic benefit of E-EPCs, suggesting an important immune interaction between these cell types. Further studies are required to elucidate the role of these cells as therapeutic agents in PAH.

### 1.12

#### **Gene expression profiling of peripheral blood mononuclear cells from cattle with high altitude pulmonary hypertension (Brisket disease)**

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High altitude pulmonary hypertension (HAPH) is a consequence of chronic alveolar hypoxia, leading to hypoxic vasoconstriction and remodeling of the pulmonary circulation. HAPH in cattle is a naturally occurring animal model of hypoxic pulmonary hypertension. Genetically

susceptible cattle develop severe pulmonary hypertension and right heart failure at altitudes >7,000 ft. No information currently exists regarding the identity of the pathway(s) and gene(s) responsible for HAPH or influencing severity. We hypothesized that initial insights into the pathogenesis of the disease could be discovered by a strategy of gene expression profiling of affected cattle compared to altitude-matched normal controls, combined with gene set enrichment analysis and Ingenuity Pathway analysis. We isolated blood from a single herd of Black Angus cattle of both genders; age 12-18 months, by jugular vein puncture. Mean PA pressures were  $85.6 \pm 13$  mmHg STD in the 10 affected and  $35.3 \pm 1.2$  mmHg STD in 10 resistant cattle,  $P < 0.001$ . RNA was isolated from peripheral blood mononuclear cells and used to probe an Affymetrix Bovine genome array. Data were analyzed by the Partek software package to identify sets of genes with expression that was statistically different between the two groups. Gene Set Enrichment (GSEA) and Ingenuity Pathways analyses were then conducted on the refined expression data to identify key cellular pathways and to generate networks and conduct functional analyses of the pathways and networks. A 60-gene signature was identified that differentiated affected from unaffected cattle. Forty-six genes were overexpressed in affected and 14 genes were downregulated in the affected cattle by at least 20%. GSEA and Ingenuity analysis identified respiratory diseases, inflammatory diseases and pathways as the top diseases and disorders ( $P < 5.14 \times 10^{-14}$ ), cell development and cell signaling as the top cellular functions ( $P < 1.20 \times 10^{-08}$ ), and IL6, TREM, PPAR, NFkB cell signaling ( $P < 8.69 \times 10^{-09}$ ) as the top canonical pathways associated with this gene signature. This study provided insights into the pathogenesis of HAPH at a molecular level and suggests that HAPH shares cellular pathways and processes with pulmonary hypertension in humans. Further studies are needed to validate and refine these preliminary findings and to determine their role in the development of human pulmonary hypertension.

### 1.13

#### Dehydroepiandrosterone and experimental pulmonary hypertension

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Dehydroepiandrosterone (DHEA), a C-19 steroid synthesized from cholesterol mainly by the adrenal cortex, circulates primarily in its sulfated form, DHEAS. DHEA and DHEAS are the most abundant steroids in human the bloodstream and are precursors to both androgens and estrogens. Epidemiological observations indicate that DHEA has a wide variety of beneficial effects, including prevention of obesity, diabetes, cancer, and cardiovascular diseases. However, little is known about its role in patients with pulmonary hypertension. Plasma levels of DHEA are very low in rodents, but pharmacological doses of exogenous DHEA exert a broad range of vasculoprotective activities, including vasodilatory, antiproliferative, anti-inflammatory, and antioxidant effects. DHEA or DHEAS effectively prevents and reverses chronic hypoxia-induced pulmonary hypertension in rats by upregulating  $K^+$  channels and improving endothelial function. In addition, DHEA treatment of left-pneumonectomized monocrotaline-injected rats inhibits the upregulation and activation of lung tissue RhoA/Rho kinase signaling, completely arrests progression of pulmonary hypertension, and increases survival from 30% to 100%. How DHEA treatment suppresses activation of Rho kinase is unclear, but possibilities include inhibition of HMG-CoA reductase and upregulation of soluble guanylate cyclase, both of which can lead to inhibition of RhoA-mediated activation of Rho kinase. Its antioxidant/anti-inflammatory effects and conversion to estrogen may also play a role. We have recently observed that oral treatment with DHEA arrests progression of severe pulmonary arterial hypertension and normalizes cardiac output in SU5416/hypoxia/normoxia-exposed rats. Considering that DHEA is a relatively safe and orally active drug, and that it or one or more of its metabolites modulate various cellular/molecular signaling pathways that

are involved in the pathogenesis of pulmonary hypertension, DHEA or a metabolite might be useful for the treatment of pulmonary hypertension.

### 1.14

#### Genetic risk factors for portopulmonary hypertension in patients with advanced liver disease

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Portopulmonary hypertension occurs in 6% of liver transplant candidates. The pathogenesis of this complication of portal hypertension is poorly understood. To identify genetic risk factors for portopulmonary hypertension in patients with advanced liver disease, we performed a multicenter case-control study of patients with portal hypertension. Cases had a mean pulmonary artery pressure >25 mmHg, pulmonary vascular resistance >240 dynes $\cdot$ s $\cdot$ cm $^{-5}$ , and pulmonary capillary wedge pressure  $\leq$ 15 mmHg. Controls had a right ventricular systolic pressure <40 mmHg (if estimated) and normal right-sided cardiac morphology by transthoracic echocardiography. We genotyped 1,086 common single nucleotide polymorphisms in 94 candidate genes in each patient. The study sample included 31 cases and 104 controls. Twenty-nine single nucleotide polymorphisms in 15 candidate genes were associated with the risk of portopulmonary hypertension ( $P < 0.05$ ). Multiple single nucleotide polymorphisms in the genes coding for estrogen receptor 1, aromatase, phosphodiesterase 5, angiotensin 1, and S100A4 were associated with the risk of portopulmonary hypertension. The biological relevance of one of the aromatase single nucleotide polymorphisms was supported by an association with plasma estradiol levels. Genetic variation in estrogen signaling and cell growth regulators is associated with the risk of portopulmonary hypertension. These biologic pathways may elucidate the mechanism for the development of portopulmonary hypertension in certain patients with severe liver disease.

### 1.15

#### VIP is a physiological modulator of pulmonary arterial hypertension

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Pulmonary arterial hypertension (PAH) is characterized by a combination of structural and functional abnormalities in the pulmonary circulation: (1) Pulmonary vasoconstriction; (2) pulmonary vascular thickening; (3) lung inflammation; and (4) RV hypertrophy (RVH). As a means of exploring the physiological role of the VIP gene in the pulmonary circulation, we examined the effects of targeted deletion of the VIP gene in mice. There were two sets of complementary findings, both largely unexpected. First: a spontaneous expression of a PAH phenotype, in the absence of hypoxia or any other "second hit." This phenotype included: PAH, pulmonary vascular thickening, lung inflammation, and RVH. Second: gene array analysis of lungs from these mice confirmed the absence of the VIP gene, and showed a wide range of additional gene expression alteration: overexpression of most vasoconstrictor, pro-proliferative and pro-inflammatory genes, and underexpression of most vasodilator, antiproliferative genes. These results demonstrate that: (1) the mere lack of the VIP gene alone is sufficient to generate a PAH-like phenotype in mice; and (2) the VIP gene exerts a powerful, wide-ranging influence on the expression of a majority of genes and transcription factors controlling the structure and function of the pulmonary circulation. Having identified the multiple genes and pathways working in concert with VIP to modulate PAH, we searched for the dominant

pathway that might explain this regulatory influence of VIP. Evidence so far strongly suggests that VIP modulates PAH pathogenesis mainly by inhibiting the calcineurin-NFAT pathway (Nuclear Factor of Activated T cells). Activation of this transcription factor can elicit all features of PAH: vascular smooth muscle proliferation, inflammation, and cardiac myocyte proliferation. Calcineurin-NFAT activation, as evidenced by nuclear localization, has been demonstrated in VIP<sup>-/-</sup> mice and two other experimental models of PH, as well as in IPAH patients. In all three PH models, treatment with VIP suppressed this activation, much like VIVIT, the selective NFAT inhibitor.

### 1.16

#### Risk factors and responsiveness to therapies in the modern era of PAH treatment

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The last decade has witnessed a remarkable increase in the number of effective treatment options available for the management of patients with pulmonary arterial hypertension (PAH). The advent of PAH-specific pharmacological treatments has offered hope to patients with a debilitating, progressive disease and a poor prognosis. In this regard, agents belonging to the therapeutic classes that specifically target the prostacyclin, endothelin and nitric oxide pathways have shown the greatest efficacy in clinical studies to date. These various drug treatments have individually been shown to confer improvements in symptoms, exercise capacity, pulmonary hemodynamics and possibly survival in different patient subgroups. However, despite these substantial improvements, long-term survival remains poor in patients with PAH, particularly in those with the most severe functional impairment (New York Heart Association (NYHA)/World Health Organization (WHO) functional class (FC) III-IV). With greater availability of therapies, strategies aimed at optimizing patient outcomes have also evolved. Among them, combination therapy and a “treat-to-target” approach are two current treatment strategies that are strongly linked. PAH is characterized by dysregulation of a variety of pathways. As a consequence, there is increasing interest in the use of treatment combinations in order to target multiple targets with the aim of restoring normal pulmonary vascular function in order to improve clinical status. Combined drug treatment offers improved benefits over monotherapy, and current European treatment guidelines for PAH (*European Respiratory Society and European Society of Cardiology*) recommend a sequential add-on approach to combination therapy for patients in New York Heart NYHA/WHO FC II-IV. Alternatively, first-line combination therapy could be considered in FC III-IV PAH patients. Recent data from our center suggest that upfront triple combination therapy with epoprostenol, bosentan and sildenafil dramatically improve symptoms and hemodynamics in particularly severe patients (fall in pulmonary vascular resistance by 70% after 4-month triple combination therapy). For PAH patients who continue to show inadequate clinical response despite optimal medical therapy, or where medical treatments are unavailable, balloon atrial septostomy and/or lung transplantation can be indicated. “Treat-to-target” or “goal-oriented” approaches, in which appropriate treatment goals are established for individual patients, are crucial in maximizing the benefits of pharmacotherapy in PAH. These strategies involve continual monitoring and assessment in order to evaluate patients’ response to therapy. With respect to prespecified treatment targets, decisions can be made based on whether the response to therapy is satisfactory or inadequate. Goal-oriented therapy determines the timing of treatment escalation by inadequate response to known prognostic indicators. These include NYHA/WHO FC 6-minute walk distance (6MWD) as well as a number of hemodynamic variables (right atrial pressure, cardiac output, pulmonary vascular resistance). Based on the measurement of such parameters, patients can be classified as “stable and satisfactory,” “stable and not satisfactory” or “unstable and deteriorating.” Patients described

as “stable and not satisfactory” or “unstable and deteriorating” are considered to have an inadequate response to therapy, and an escalation of their treatment should be considered. Close monitoring of patients (every 3-6 months) aids the early identification of inadequate response, so that treatment can be escalated promptly and before the patient’s condition can further deteriorate. Existing treatment goals are based on baseline values of prognostic indicators, but it is vital to identify risk factors that are both relevant during treatment and can be assessed during follow-up appointments. Data from different PAH etiologies indicate that NYHA/WHO FC is the most appropriate prognostic marker, with 6-minute walk distance and several hemodynamic parameters representing alternatives. Future refinement of goal-oriented therapy could include the use of multiple prognostic markers, while additional large clinical trials will answer questions concerning choice and combination of treatment goals.

### 1.17

#### Chronic hypoxia leads to emergence of proinflammatory pulmonary adventitial fibroblasts capable of inducing proinflammatory and profibrogenic activation of monocytes/macrophages: Amelioration by HDAC inhibitors

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A persistent accumulation of monocytes/macrophages in the adventitia of pulmonary arteries of animals and humans with pulmonary hypertension (PH) is well recognized. Chronic inflammation correlates positively with fibro-proliferative changes in the vessel wall. However, the cellular mechanisms contributing to chronic inflammatory responses remain unclear. Profibrotic cells can originate from circulating CD14<sup>+</sup> monocytes and include fibrocytes and activated macrophages. Although accumulation of fibrocytes has been implicated in the pulmonary vascular remodeling process, little information exists regarding activation of macrophages by adventitial fibroblasts. We hypothesized that the pathogenesis of hypoxic PH involves emergence of adventitial fibroblasts with a persistently activated proinflammatory phenotype capable of recruiting and activating macrophages towards a proinflammatory and profibrogenic phenotype. We further hypothesized that the proinflammatory fibroblast phenotype is the result of epigenetic changes and particularly abnormal activity of histone-modifying enzymes, specifically, class I histone deacetylases (HDACs). Our data demonstrate that, compared to control fibroblasts, PH-Fibs expressed an activated pro-inflammatory phenotype characterized by significantly increased mRNA and protein levels of CCL2/MCP-1, CXCL12/SDF-1, GM-SCF, RANTES, IL-6, and IL-1, but not TNF- $\beta$  or IL-4 or IL-13. The proinflammatory phenotype of PH-Fibs was associated with epigenetic alterations as evidenced by increased activity of type I HDACs, and the findings that class I HDAC inhibitors markedly decreased cytokine/chemokine mRNA expression and protein levels in these cells. PH-Fibs also induced increased adhesion of THP-1 monocytes, and produced soluble factors that induced increased migration of THP-1 and murine bone marrow-derived macrophages (BMDMs). Further, since increased numbers of CD163<sup>+</sup> macrophages were observed in vivo in the pulmonary vascular adventitia of chronically hypoxic animals we examined the effect of PH-Fibs on unstimulated monocytes/macrophages. Supernatants from PH-fibs, but not CO-Fibs, stimulated increases in mRNA levels of TLR-signaling dependent (IL-6) and inflammasome signaling dependent (IL-1, IL-18) pro-inflammatory cytokines as well as NF- $\kappa$ B dependent profibrogenic mediators (MMP2,9, Col-1a, TIMP1, 3) in both THP-1 monocytes and BMDMs. Intriguingly, expression of canonical markers for classical M1 activation of macrophages was low (NOS2) or undetectable (IL-23, IRF5). Class I HDAC inhibitors markedly reduced the ability of PH-Fibs to induce proinflammatory activation. BMDMs incubated with supernatants from PH-Fibs, but not CO-Fibs, also exhibited mRNA expression of STAT3 dependent functional markers of alternative

(M2) activation: Arginase-1 and IL-4RA. Importantly, expression of STAT6 dependent M2 markers YM1 and FIZZ1 was not detected. Expression of above markers was unaltered in MyD88<sup>-/-</sup> and TLR4<sup>-/-</sup> BMDMs. The in vivo relevance of these findings was confirmed in vivo where administration of Class I HDAC inhibitors both inhibited and reversed hypoxic-induced PH and vascular remodeling. In conclusion, we propose a novel concept for the pathogenesis of chronic hypoxic PH, in which persistently activated proinflammatory adventitial fibroblasts play a key role in promoting TLR, inflammasome and STAT3-dependent signaling pathways leading to the induction of a functionally distinct monocyte and macrophage phenotype tailored to initiate and propagate vascular inflammation and fibrosis.

### 1.18

#### Novel loci interacting epistatically with BMP2 cause FPAH

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The genetic locus for familial pulmonary hypertension (FPAH) was first identified in 1997. Then, 3 years later, BMP2 was positionally cloned and shown unequivocally to increase risk for FPAH. Thereafter, the vast majority of all known carriers were shown experimentally (or are expected on the basis of exonic sequence) to have mutations that dramatically reduce BMP2 gene expression. However, it is estimated that only 20-30% of all carriers go on to develop FPAH. As a result, we hypothesize that some of the reduced penetrance of BMP2 may be due to genetic variation at interacting loci (aka *modifiers*). To increase our power to detect modifiers, we analyzed the genomes of 197 carriers obtained from 37 FPAH families. The families ranged in size from 2 to 5 generations with 4 to 23 members per family with the carrier status of most individuals determined experimentally by exon sequencing. To further increase power and precision we used EAGLET—our fast and flexible new method of analysis, to extract the maximum amount of information for association and linkage from the available dense single nucleotide polymorphism (SNP) data. Relative to several competing methods, EAGLET has been shown to increase power and to yield accurate and narrow confidence intervals for disease gene location. Our results are consistent with the established fact that BMP2 influences FPAH in Caucasians (maximum eLOD=6.44), but our results also suggest that there may only be a small number of modifier genes with moderate genetic effects. Furthermore, these modifiers may differ across Caucasian subpopulations. For example, in 18 families that originate from an ongoing study at Vanderbilt University, we found strong evidence for a modifier locus on chromosome 5 (P<0.01). Similarly, in 19 families that originate from a Columbia University study, we found suggestive evidence for a modifier locus on chromosome 3 (P=0.11). Moreover, the only genomic location where both studies showed strong evidence for linkage was BMP2. This suggests that there are both shared and distinct genetic factors influencing FPAH, with BMP2 being the shared factor that potentially interacts with distinct factors on chromosomes 3 and 5. Undoubtedly a complete genetic description of FPAH remains unclear, but the success of our powerful new approach may inspire others to unite data sets with new methods of analysis to advance our overall understanding of the genetics of this life-threatening disease.

### 1.19

#### Aerobic capacity and pulmonary arterial hypertension

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Despite modern therapies, pulmonary arterial hypertension (PAH) remains a progressive, fatal disease where mortality is closely associated with right ventricular hemodynamic function, and exercise limitation. The potential benefit of exercise to increase aerobic capacity as an adjunct to conventional PAH therapies remains controversial. Here, we review clinical and preclinical literature around exercise, aerobic capacity and PAH within the context of emerging data revealing the role of altered cardiomyocyte metabolism in the failing right ventricle. These principles are further explored in novel studies comparing chronic hypoxia-induced PAH pathologies in rats selectively bred for divergent aerobic capacity - might the demonstrated linkage between low aerobic capacity and susceptibility to disease also feature in PAH, and what mechanisms govern such an association? We exposed high capacity runner (HCR) and low capacity runner (LCR) rats to a 10% O<sub>2</sub> hypoxic environment for 21 days. While all rats exposed to 10% oxygen developed hypertensive-like changes relative to normoxic controls, hypoxia-exposed LCR rats developed more severe changes than their HCR counterparts, as measured by right ventricular pressure, Fulton index, echocardiographic indices and vessel remodeling. Lung microarray analysis of the lungs revealed perturbed expression of elements in the BMP2/TGF- $\beta$  axis in hypoxic LCR animals. Biomarker analysis revealed a ~250% increase in plasma serotonin levels in the hypoxic LCR group, but remained unchanged in all other groups, suggesting that the mechanism of enhanced responsiveness to chronic hypoxia may be via a dysregulated serotonin pathway. Daily treatment with an inhibitor of tryptophan hydroxylase (pCPA), the rate-limiting step in 5-HT synthesis, ablated 5-HT levels and reduced all PAH pathologies in all groups. However, subtractive analysis revealed no impact on the exacerbated vessel remodeling observed in LCR animals, and only a partial effect on RV pressure. RV mass and echocardiographic measures of RV function; however, were fully reversed by pCPA treatment. Gene array analysis in the hearts of hypoxic LCR versus HCR rats  $\pm$  5-HT ablation offer clues to the mechanisms by which aerobic capacity influences PAH susceptibility. The impact of altered oxidative metabolism, aerobic capacity and the serotonin pathway on PAH therapies are discussed.

### 1.20

#### Estradiol metabolites and progestins in experimental pulmonary hypertension

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Pulmonary arterial hypertension (PAH) occurs more frequently in women, yet animal studies in classical models of PAH and limited clinical data suggest protective effects of estrogens (*the estrogen paradox in PAH*). This contradiction may be explained by the complexity of estradiol metabolism and the influential balance between estradiol and its metabolites on pulmonary vascular homeostasis. One line of evidence strongly suggests that the vascular protective effects of 17 $\beta$ -estradiol (E2) are mediated largely by its downstream metabolites. E2 is metabolized to 2-hydroxyestradiol (2HE) by CYP1A1/CYP1B1, and 2HE is converted to 2-methoxyestradiol (2ME) by catechol-O-methyl transferase. 2ME is extensively metabolized to 2-methoxyestrone (2ME1), a metabolite that lacks biologic activity but may be converted back to 2ME by 17 $\beta$ -hydroxysteroid-dehydrogenase (17 $\beta$ -HSD-1). Previously, we have shown that in male rats 2ME, 2HE and synthetic analog 2-ethoxyestradiol attenuate monocrotaline (MCT)-induced PAH. In female rats with MCT- or bleomycin-induced PAH, 2ME attenuates both the exacerbation of disease and the increased mortality due to ovariectomy (OVX). Also, 2ME1, a largely biologically inactive E2 metabolite, has preventive effects in male MCT-PAH rats, and 2ME and all-trans retinoic acid (an inducer of 17 $\beta$ -HSD-1 and 2ME1-to-2ME conversion) have synergistic effects in ameliorating MCT-induced PAH. This suggests that 2ME-2ME1 interconversion and the 17 $\beta$ -HSD pathway may play a role in the development of PAH. Whereas E2

and 2ME exert similar effects in other vascular cells, they have divergent effects in endothelium and therefore we have proposed that unbalanced E2 metabolism may lead to the development of PAH. Because the effects of E2 and 2ME in severe PAH, as characterized by obliterative proliferation of endothelial cells, are currently unknown, recently we examined the effects of gender, estrogen deficiency and treatment with 2ME or E2 on the development and progression of disease in rats with severe occlusive/angioproliferative PAH. Adult male, intact female, and OVX female rats were given the VEGF receptor inhibitor SU 5416 (100 mg/kg) and exposed to hypoxia for 21 days. Subsets of F and OVX rats were treated in a preventive (Day 0-21) or therapeutic (Day 10-21) manner with 2ME and E2 (10 µg/kg/h and 1 µg/kg/h respectively, *via* osmotic minipumps). Male rats developed less severe PAH and lung injury compared to intact females, and 2ME had therapeutic effects in female, but not male PAH rats. Estrogen deficiency (OVX) did not exacerbate the disease. In OVX-PAH rats, 2ME, but not E2, reduced PAH and lung injury, while both 2ME and E2 reduced isolated RV hypertrophy. The effects of progestins in experimental PAH have been poorly studied, despite vascular effects of progesterone and presence of its receptors in intact endothelium and plexiform lesions in PAH patients. Therefore, we examined the effects of progesterone, medroxyprogesterone and tibolone on MCT-induced PAH in ovariectomized female rats. Progesterone attenuated development of PAH and right ventricular hypertrophy and inhibited pulmonary vascular remodeling; medroxyprogesterone showed effects similar to progesterone; and notably, tibolone, a combined progestin/estrogen compound, prevented development of disease and eliminated MCT-induced mortality. These studies warrant further investigation of the role of estradiol metabolism in pulmonary vascular disease and the development of PAH.

### 1.21

#### Adipose tissue expandability, lipotoxicity and the metabolic syndrome

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The link between obesity and Type 2 diabetes is clear on an epidemiological level; however, the mechanism linking these two common disorders is not well defined. One hypothesis linking obesity to Type 2 diabetes is the adipose tissue expandability hypothesis. The adipose tissue expandability hypothesis states that a failure in the capacity for adipose tissue expansion, rather than obesity per se is the key factor linking positive energy balance and Type 2 diabetes. All individuals possess a maximum capacity for adipose expansion which is determined by both genetic and environmental factors. Once the adipose tissue expansion limit is reached, adipose tissue ceases to store energy efficiently and lipids begin to accumulate in other tissues. Ectopic lipid accumulation in nonadipocyte cells causes lipotoxic insults including insulin resistance, apoptosis and inflammation. This article discusses the links between adipokines, inflammation, adipose tissue expandability and lipotoxicity. Finally, we will discuss how considering the concept of allostasis may enable a better understanding of how diabetes develops and allow the rational design of new anti diabetic treatments.

### 1.22

#### Endocrine determinants of severe pulmonary arterial hypertension

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Idiopathic pulmonary arterial hypertension (PAH) and also secondary

forms of severe PAH occur predominantly in women, and clinically associations with immunological disorders and thyroid diseases are well recognized. Very likely a disordered immune system, female and endocrine factors contribute to the pathobiology of pulmonary vascular remodeling, but very little is known. We postulate that all of these factors participate in inflammation and angiogenesis which are both mechanisms involved in pulmonary vascular remodeling. Because in excess of 80% of patients with Graves disease (hyperthyroidism) develop PAH and because thyroid hormones (T3 and T4) are angiogenic, we hypothesized that thyroid hormones would participate in the development of angioproliferative pulmonary remodeling in the rat Sugen5416/chronic hypoxia model of PAH. We found that thyroidectomy prevented the development of severe angioproliferative PAH, while T4 replacement of thyroidectomized rats treated with Sugen5416 and exposed to hypoxia led to development of severe PAH. When animals with established severe angioobliterative PAH were treated with propylthiouracil (PTU) a decrease in the RVSP and the number of obliterated vessels was observed. Because treatment of rats with Sugen5416 plus T4 did not result in PAH. We conclude that the T4 contributes (is permissive) in the setting of pulmonary hypertension. In this angiogenic action T4 may signal via the integrin  $\alpha\beta3$  and FGF<sub>2</sub>. Both of these proteins are increased in expression in the lungs from Sugen5416/chronic hypoxia rat lung tissues.

### 1.23

#### Adiponectin and pulmonary hypertension

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Pulmonary hypertension is a life-threatening condition that develops in association with various medical diseases. Recent clinical studies indicate obesity to be a risk factor for development of pulmonary hypertension; however, the mechanisms leading to this association are unknown. Adiponectin is a circulating factor derived from adipose tissue that is present in high concentration in serum of lean healthy individuals but decreases in obesity. Recent animal studies implicate adiponectin in the pathogenesis of pulmonary hypertension. Most notably, mice deficient in adiponectin develop a spontaneous lung vascular phenotype characterized by upregulation of E-selectin on endothelium, age-dependent increases in perivascular inflammatory cells and elevated pulmonary artery pressures. Moreover, experimental studies showed adiponectin to ameliorate lung vascular remodeling due to hypoxia and to chronic allergic airway inflammation. Emerging evidence indicates adiponectin's effects are mediated through anti-inflammatory and antiproliferative actions on cells in the pulmonary circulation. This review aims to synthesize the existing data related to adiponectin's effects on pulmonary vascular cells and to discuss how alterations in adiponectin signaling might contribute to the development of pulmonary hypertension in human patients.

### 1.24

#### Sildenafil-cGMP-PKG-PPAR- $\gamma$ signaling pathway inhibits angiotensin-II-induced transient receptor potential canonical 6 expression in pulmonary arterial smooth muscles cells

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Sildenafil, a specific type V phosphodiesterase inhibitor that increases cellular cGMP, is recently identified as a promising agent for treatment of

pulmonary hypertension; however, the underlying mechanisms are not fully understood. In pulmonary arterial smooth muscle cells (PASMCs),  $Ca^{2+}$  influx through store-operated  $Ca^{2+}$  channels thought to be composed of transient receptor potential canonical (TRPC) proteins is an important determinant of intracellular free calcium concentration ( $[Ca^{2+}]_i$ ) and pulmonary vascular tone. We previously demonstrated that sildenafil inhibits chronic hypoxic upregulation of TRPC expression in PASMCs. In this study, we further examined the regulatory effect and signaling of sildenafil on angiotensin-II-induced TRPC6 expression in rat distal PASMCs. Treatment with angiotensin-II-induced TRPC6 mRNA expression about 4.5-fold, which was individually blocked by pretreatment with sildenafil (1  $\mu$ M), CPT-cGMP (1 mM) or PPAR- $\gamma$  agonist GW1929 (1  $\mu$ M). The blocking effects of sildenafil and CPT-cGMP was attenuated by cotreatment with PKG inhibitor Rp8 (1  $\mu$ M), KT5823 (0.5  $\mu$ M), or PPAR- $\gamma$  antagonist T0070907. Treatment with sildenafil or CPT-cGMP alone increased PPAR- $\gamma$  activation, which was blocked by pretreatment with PKG inhibitor Rp8, or KT5823. These results provide novel evidence indicating that sildenafil negatively regulates TRPC6 expression via cGMP-PKG-PPAR- $\gamma$  signaling pathway, which may contribute to the mechanism of its therapeutic effect on pulmonary hypertension.

### 1.25

#### The estrogen metabolite 16-OHE exacerbates BMPR2-related PAH, associated with defects in receptor trafficking

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We have previously shown that decreased CYP1B1 expression and the resulting increase in the estrogen metabolite 16-OHE are associated with disease penetrance in pulmonary arterial hypertension (PAH) patients. The goal of this study was to determine whether this was correlation or causation, and why estrogen, which is beneficial to many models of pulmonary vascular disease, is harmful in the context of PAH. 16-OHE or vehicle were delivered in osmotic pumps to BMPR2 mutant or control mice chronically, followed by detailed hemodynamic, histological, and molecular phenotyping. Estrogen receptor (ER) signaling was examined in BMPR2 mutant or control pulmonary microvascular endothelial cells (PMVEC) through immunohistochemistry and ER sensitive luciferase reporter constructs. Adding 16OHE to BMPR2 mutant mice more than doubles pulmonary vascular resistance, associated with dropout or structural narrowing of resistance level arteries. This accelerates the course of PAH, but has little effect in control mice. BMPR2 mutation causes defects in ER trafficking; addition of exogenous estrogen no longer causes translocation to the nucleus, instead resulting in accumulation of the ER on the cell surface and in a perinuclear structure. This defect in ER trafficking appears to be common to other steroid hormone receptors, although with a different time course. 16-OHE is causative of PAH in the context of BMPR2 mutations. BMPR2 mutation alters steroid hormone receptor trafficking, likely through cytoplasmic tail domain (LIMK or TCTEX-1)-dependent signaling.

### 1.26

#### Biomarkers for pulmonary arterial hypertension— Can they be used to predict response to treatment?

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Biomarker is an inclusive term that refers to any objective measurement that informs on a person's state of health. It includes physical measurements such as hemodynamics as well as circulating molecules and cells, genetic markers and imaging findings. A biomarker may be used to detect disease susceptibility, diagnose overt disease, categorize

disease severity and monitor disease history or therapy response. In the management of pulmonary arterial hypertension (PAH), genotype (e.g., BMPR2 sequence) can inform risk of developing PAH. For diagnosis, hemodynamic measurements are the gold standard. Circulating brain natriuretic peptide (BNP) levels are used in the clinic to follow patients although interindividual variation in levels reduces their value and data have to be interpreted in the context of the sum of available clinical information on each patient. There is a particular interest in using biomarkers to stratify patients and target treatment to individuals. There is considerable potential for this approach in pulmonary arterial hypertension (PAH). The current classification of patients with pulmonary hypertension is based on clinical and pathological criteria but this is a blunt instrument. It is well recognized that WHO Group 1 PAH is a heterogeneous collection of diseases. This is reflected in the broad dynamic plasma concentration range seen in many circulating biomarkers. It is also seen in the response to treatment. Less than 10% of patients respond to a calcium antagonist. Not all patients with idiopathic PAH respond to PDE5 inhibitors and for reasons that are not entirely clear, patients with sickle cell disease seem to be poorly tolerant of this drug class. And then there is the differential response of the right ventricle to a pressure-load, allowing some patients to tolerate PAH better than others. The response of the pulmonary circulation to an acute vasodilator challenge, e.g., with nitric oxide, is helpful in predicting the long-term response to an oral calcium antagonist. In this sense, the hemodynamic measurements are acting as a predictive biomarker. While clinically useful, a less invasive test for identifying patients sensitive to calcium channel blockers would be preferred. Careful molecular dissection of well-phenotyped patients on calcium antagonist treatment is overdue. Genomic biomarkers are proving valuable in patient stratification in oncology. Obtaining disease tissue from patients with PAH, except at transplantation, is not possible but the pulmonary vasculature provides an extensive surface area and damaged tissue will leak cells, proteins and genomic material into the circulation. This could be a rich source of material for molecular phenotyping. Detailed cardiac and molecular imaging provides another potentially powerful approach to understanding cardiac and pulmonary vascular pathology at the individual level. The use of 3-D myocardial imaging and positron emitting tracers to track disease/drug targets is so far underexplored in PAH. The validation of biomarkers is not an insignificant challenge. This is best addressed by embedding candidate biomarkers in clinical trials. To date, this has only been done routinely with BNP.

### 1.27

#### Pathological role of TRPC channels in idiopathic pulmonary arterial hypertension

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Idiopathic pulmonary arterial hypertension (IPAH), a fatal and progressive disease that predominantly affects young women, is associated with profound pathobiological alternations in small pulmonary arteries. Regardless of the initial etiology, sustained vasoconstriction, excessive vascular remodeling, in situ thrombosis and increased vascular wall stiffness are the major courses for the elevated pulmonary vascular resistance in patients with IPAH and animals with pulmonary hypertension. An increase in cytosolic free  $Ca^{2+}$  concentration ( $[Ca^{2+}]_{cyt}$ ) in pulmonary artery smooth muscle cells (PASMC) is a major trigger for pulmonary vasoconstriction and an important stimulus for PASMC proliferation that leads to medial hypertrophy and vascular remodeling. A novel class of nonselective cation channels, the transient receptor potential (TRP) channels, have emerged at the forefront of research into cardiovascular diseases. The canonical TRP channels, TRPC channels, are identified as molecular correlates for both store-operated and receptor-operated cation channels in different cell and tissue types (1-3),

including the pulmonary vasculature. Many TRP isoforms are identified at the mRNA and protein expression levels in human lung tissues and pulmonary vasculature. Upregulated TRPC channel expression and increased  $\text{Ca}^{2+}$  influx through TRPC channels are involved in growth factor-mediated PASMC proliferation. In lung tissues and PASMC from patients with IPAH, we previously found that the mRNA and protein expression of TRPC channels (e.g., TRPC3 and TRPC6) was significantly greater than in lung tissues and PASMC from normal subjects and normotensive patients. Using DNA samples from normal subjects and more than 250 patients with IPAH, we identified a polymorphism (SNP) in the promoter region of the TRPC6 gene (-245C-to-G). The allele frequency of the -254(C-to-G) SNP in IPAH patients (12%) is significantly higher than in normal subjects (6%;  $P < 0.01$ ). Genotype data showed that the percentage of -254(C-to-G) homozygotes in IPAH patients was 2.85 times that of normal subjects. Interestingly, the -254(C-to-G) SNP creates a binding sequence for nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ). Functional analyses revealed that the -254(C-to-G) SNP-enhanced NF- $\kappa\text{B}$ -mediate promoter activity and stimulated TRPC6 expression in PASMC. Inhibition of NF- $\kappa\text{B}$  activity attenuated TRPC6 expression and decreased agonist-mediated  $\text{Ca}^{2+}$  influx in PASMC of IPAH patients harboring the -254G allele. These data implicate upregulation of specific TRPC isoforms to be associated with increased  $\text{Ca}^{2+}$  influx in PASMC from IPAH patients, which subsequently causes pulmonary vasoconstriction and vascular remodeling by stimulate PASMC contraction and proliferation, respectively. The -254(C-to-G) SNP in the TRPC6 gene may predispose individuals to an increased risk of developing IPAH by linking abnormal TRPC6 transcription and expression to NF- $\kappa\text{B}$ , an inflammatory transcription factor. Targeting TRPC6 transcription, expression and function may lead to the development of novel and effective therapeutic approaches for IPAH.

### 1.28

#### Cardiac sympathetic activity evaluated by $^{123}\text{I}$ -MIBG myocardial scintigraphy in patients with right ventricular dysfunction associated with pulmonary arterial hypertension

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Most patients with pulmonary arterial hypertension (PAH) die from right ventricular (RV) heart failure. Recently, it has been reported that patients with severe RV dysfunction associated with PAH might have autonomic nerve dysfunction evaluated by heart rate variability and muscle sympathetic nerve activity. However, noninvasive direct methods to measure the RV sympathetic nerve activity have not been established yet. In this study, we examined whether  $^{123}\text{I}$ -metaiodobenzylguanidine ( $^{123}\text{I}$ -MIBG) myocardial imaging is useful to assess the RV sympathetic nerve activity in patients with RV dysfunction associated with PAH. Patients underwent right heart catheterization and echocardiography to determine RV function. SPECT was performed in the resting state 15 min. (early imaging) and 4 hr. (delayed imaging) after the injection of  $^{123}\text{I}$ -MIBG. RV sympathetic nervous function was assessed by the washout ratio of  $^{123}\text{I}$ -MIBG in RV-free wall. Patients with severe RV dysfunction showed higher washout ratios and the more extensions of the scintigraphic defects in the RV-free walls compared with the patients with mild RV dysfunction. The washout ratio of  $^{123}\text{I}$ -MIBG in RV-free wall was also positively correlated with the plasma level of brain natriuretic peptide (BNP). Our results suggest that  $^{123}\text{I}$ -MIBG imaging may be useful to evaluate the degree of RV sympathetic activities in the PAH patients with RV dysfunction. In a future study, it remains to be examined whether  $^{123}\text{I}$ -MIBG imaging will also be useful as a prognostic method in the PAH patients with RV dysfunction.

### 1.29

#### Somatic chromosome abnormalities in PAH lungs—Modifier or bystander?

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Vascular remodeling in pulmonary arterial hypertension (PAH) has been likened to a neoplastic process, with evidence of monoclonal expansion of endothelial cells within plexiform lesions, microsatellite instability, hyperproliferation, apoptosis resistance and mitochondrial abnormalities. Recently we reported somatic chromosome abnormalities in endothelial cells from explant lungs in 5 of 9 PAH patients. All were mosaic abnormalities, present in 10-50% of cells. Many outstanding questions remain, including the timing of these events in the course of the disease process and their role (if any) in disease pathogenesis. We have now extended our analysis and identified abnormalities in 10 of 30 cases, including whole or partial deletion of chromosome 13 (2 cases) and X-chromosome deletions in 4 of 24 females. Two cases harbor more than one abnormality. Only one case has a germline *BMPR2* mutation. No abnormalities were identified among 16 controls. One case of particular interest was a large interstitial deletion of chromosome 13, including *SMAD9*, present in approximately 70% of endothelial cells. This high level of abnormality has enabled functional studies. The cells proliferated significantly faster than controls and were indistinguishable from cells with a germline *BMPR2* or *SMAD9* mutation. They also showed significant attenuation of non-canonical microRNA processing, analogous to the 3 germline mutation cases. In contrast, cytogenetically normal pulmonary artery smooth muscle cells from the same patient showed normal proliferation rates and microRNA processing. While it remains difficult to determine how early these changes arose in the course of the disease, these results suggest that somatic chromosome changes may act as disease modifiers and contribute to the altered phenotype of lung vascular cells in PAH patients.

### 1.30

#### Large conductance calcium-activated potassium (BK) channel activation causes enhanced vasodilatation in hypoxic lung microvasculature

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Pulmonary vasculature endothelial cells expresses BK channels that upon activation can cause endothelial cell hyperpolarization and vasodilation. However, the effect of BK channel activation on macro- and microvasculature in chronically hypoxic lungs is not known. We assessed the effect of BK channel activation in pulmonary arterial (PA) rings and microvasculature ex vivo in lungs isolated from rats exposed to 3 weeks of normoxia (N) or hypoxia (H; 10%  $\text{FiO}_2$ ). BK channel  $\alpha$ - and  $\beta 4$ -subunit expression was assessed in pulmonary artery (PAEC) and microvascular (PMVEC) endothelial cells cultured for 10 days in N/H (1%  $\text{FiO}_2$ ). Phenylephrine-constricted PA rings from N/H animals were used to assess the dose-dependent effects of acetylcholine on vessel relaxation in the presence or absence of BK channel activator (NS1619) and various inhibitors. In isolated ventilated-perfused rat lungs from N/H animals precontracted with U46619, we monitored PA pressures upon exposure to NS1619 in presence/absence of various inhibitors (L-NAME, tetraethylammonium-TEA, iberiotoxin). Hypoxia resulted in increased BK  $\beta 4$ -subunit expression in PAEC. Increased endothelium-dependent vasodilation, which was attenuated by iberiotoxin and TEA, was noted in the presence of NS1619 in PA rings from normoxic but not in hypoxic rats. In contrast, hypoxia was associated with decreased expression of BK  $\alpha$ - and  $\beta 4$ -subunits in PMVEC. NS1619 caused enhanced vasodilation in lungs isolated from hypoxic compared

to normoxic rats. The vasodilation in both normoxic and hypoxic-isolated lungs was attenuated by eNOS inhibition, iberiotoxin and TEA. Enhanced vasodilation is noted in hypoxic lungs and not in PA in response to BK channel activation. This may be related to differential effects of hypoxia on the expression of BK channel subunits across the lung vasculature.

### 1.31

#### Compensatory regulation of the BMP-targets ID1 and ID3 in hypoxic pulmonary hypertension

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Bone morphogenetic protein (BMP) signaling has been linked to the development of pulmonary hypertension (PH). In previous studies we have shown chronic hypoxia up-regulates BMP2 and BMP4 ligand expression in and around the pulmonary vasculature, and this is associated with increased activating phosphorylation of the downstream mediators of BMP signaling, Smad1/5 and 8. In this study we have explored the downstream effects of BMP signal activation by chronic hypoxia on the regulation and function of inhibitors of differentiation proteins (ID1-4). The ID family proteins are basic helix-loop-helix (bHLH) transcription factors that are downstream targets of the BMP signaling pathway, but the role that ID proteins play in the development of PH is unknown. To address this, we evaluated pulmonary expression of ID proteins in a mouse model of hypoxia-induced PH. There is selective induction of ID1 and ID3 in hypoxic pulmonary vascular smooth muscle cells (VSMCs) in vivo, and ID1 and ID3 expression are increased by hypoxia in cultured pulmonary VSMCs in a BMP-dependent fashion. ID4 protein is barely detectable in the mouse lung, and while ID2 is induced in hypoxic peripheral VSMCs in vivo, it is not increased by hypoxia or BMP signaling in cultured pulmonary VSMCs. In addition, the PH response to chronic hypoxia is indistinguishable between wild type and *Id1 null* mice. This is associated with a compensatory increase in ID3 but not ID2 expression in pulmonary VSMCs of *Id1 null* mice. These findings indicate that ID1 is dispensable for mounting a normal pulmonary vascular response to hypoxia, but suggest that ID3 may compensate for loss of ID1 expression in pulmonary VSMCs. Taken together, these findings indicate that ID1 and ID3 expression are regulated in a BMP-dependent fashion in hypoxic pulmonary VSMCs, and that suggest that ID1 and ID3 may play a cooperative role in regulating BMP-dependent VSMC responses to chronic hypoxia.

### 1.32

#### Effects of NMD status on susceptibility to pulmonary hypertension and endothelial dysfunction in mice carrying different germ line BMPR2 mutations

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The majority of HPAH patients have heterozygous mutations in the Bone Morphogenetic Type 2 receptor (BMPR2). Experimental and clinical evidence indicate that these patients have a primary defect in pulmonary endothelial cells (PECs), and that germ line BMPR2 mutations subject to nonsense-mediated mRNA decay (NMD+ mutations) promote less severe pulmonary hypertension (PH) than BMPR2 mutations that escape NMD (NMD- mutations). However, the mechanisms by which NMD+ and NMD- BMPR2 mutations exert distinct effects on the pulmonary vasculature are unknown. We are addressing this question by studying the biology and processing of endogenously expressed Bmpr2 mutations in PECs derived from

mouse mutants that harbor known NMD+ (Bmpr2<sup>+/-</sup>) and NMD- Bmpr2 mutations (Bmpr2<sup>Δex2/+</sup>, in which there is an in-frame deletion of Bmpr2 exon 2) since we have shown that Bmpr2<sup>+/-</sup> mice have less severe PH than Bmpr2<sup>Δex2/+</sup> mice. PECs from Bmpr2<sup>Δex2/+</sup> and wild type mice both express the expected 150 kDa wild type Bmpr2 protein product. However, the Bmpr2<sup>Δex2/+</sup> mice also express high levels of a 125 kDa product that is undetectable when probed with an antibody raised against exon 2. This 125 kDa product is also observed in skin fibroblasts from HPAH patients with carrying the same BMPR2 EXON2 deletion mutation, indicating that these mutations are able to escape the NMD pathway and express a mutant protein product. Smad 1/5/8 are activated and phosphorylated in response to BMP ligands in wild-type PECs but this response is markedly reduced in BMPR2<sup>Δex2/+</sup> PECs. These findings suggest that the BMPR2<sup>Δex2</sup> product is exerting dominant inhibitory effects on BMP signaling. To determine how this occurs we have evaluated the subcellular localization of wild-type and BMPR2<sup>Δex2</sup> products in these cells. Cell-surface biotinylation studies indicate that unlike the wild-type allelic product, BMPR2<sup>Δex2</sup> does not reach the cell surface. Furthermore, the BMPR2<sup>Δex2</sup> mutant band is sensitive to Endo-H endoglycosidase treatment, indicating that it is unable to leave the ER and pass through the Golgi. Taken together, these studies indicate that the BMPR2<sup>Δex2</sup> mutant product is expressed at high levels but that it is incorrectly processed and retained in the ER. These findings suggest that dominant inhibitory effects of this mutant allele on BMP signaling result from its sequestration in the ER. Further studies are ongoing to establish the mechanism by which BMPR2<sup>Δex2</sup> is retained in the ER and what impact this has on the processing of other BMP signaling pathway components in the ER.

### 1.33

#### mTOR Complex 2 regulates energy levels, proliferation and survival of vascular smooth muscle cells in pulmonary arterial hypertension

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Increased proliferation and survival of pulmonary arterial vascular smooth muscle cells (PAVSMC) are key pathophysiological components of vascular remodeling in pulmonary arterial hypertension (PAH). PAVSMC in PAH have metabolic shift to glycolysis, but the molecular link between disturbed metabolism and increased cell proliferation and survival remains elusive. mTOR, a key regulator of cell growth and proliferation, acts through two distinct complexes, rapamycin-sensitive mTORC1 that modulates cell growth via S6K1 and 4E-BP1 and rapamycin-resistant mTORC2 that activates Akt. We previously demonstrated that chronic hypoxia-induced PAVSMC proliferation requires activation of both mTORC1-S6K1 and mTORC2-Akt. Here, we show that PAVSMC from subjects with idiopathic PAH (human PAH PAVSMC) and from rats with chronic hypoxia-induced pulmonary hypertension (PH) have altered cellular ATP levels, elevated proliferation, increased protein levels of anti-apoptotic Bcl2 and decreased levels of pro-apoptotic Bim compared to control PAVSMC. Glycolytic inhibitor 2DG, but not mitochondrial inhibitor rotenone, decreased ATP levels, inhibited proliferation and promoted apoptosis in human PAH and rat PH PAVSMC suggesting that PAVSMC proliferation and survival in PAH depend on glycolytic metabolism. Rapamycin-inhibited human PAH and rat PH PAVSMC proliferation, but had little effect on cellular ATP levels and cell survival. Inhibition of mTORC2-Akt by siRNA rictor or specific Akt1/2 inhibitor decreased cellular ATP levels, inhibited proliferation and induced apoptosis in human PAH and rat PH PAVSMC while having little effect on mTORC1. Collectively, these data demonstrate that mTORC2-Akt regulates energy levels, proliferation and survival of PAVSMC in PAH and suggest that targeting mTORC2-Akt in addition to mTORC1 may be beneficial to inhibit PAVSMC proliferation, induce apoptosis and attenuate pulmonary vascular remodeling in PAH.

## 1.34

**Dual blockade of IL4 and IL13 in schistosomiasis-associated pulmonary hypertension**

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Schistosomiasis-associated pulmonary arterial hypertension (Sc-PAH) is one of the leading causes of PAH worldwide. We have previously found that IL13 signaling alone is sufficient but not necessary to cause experimental schistosomiasis-associated pulmonary hypertension (Sc-PH) in a mouse model. IL4 is potentially redundant with IL13, and thus we hypothesized that the genetic dual blockade of both IL4 and IL13 would prevent the vascular remodeling in this disease. IL4<sup>-/-</sup>IL13<sup>-/-</sup> (4/13KO) and wild-type (WT) mice (both with C57BL6/J background; 4-5 per group) were sensitized to *Schistosoma mansoni* eggs, and then intravenously challenged with 5,000 *S. mansoni* eggs. One week after injection, right ventricular catheterization was performed, followed by analysis of the lung tissue. Both uninfected WT and uninfected 4/13KO mice had normal right ventricular systolic pressures (RVSP) at baseline, but both infected WT and infected 4/13KO groups had an elevated RVSP. However, by quantitative histological analysis, only infected WT mice had pulmonary vascular remodeling. The 4/13KO mice had smaller peri-egg granulomas and were less effective at clearing the *S. mansoni* eggs compared to the WT mice. Separately, intravenous egg challenge alone resulted in an elevated RVSP in 4/13KO mice; we have previously shown this is inadequate to cause an elevated RVSP in WT mice. Although mice lacking both IL4 and IL13 develop PH after infection with *S. mansoni*, they do not have pulmonary vascular remodeling, and the PH appears to be primarily due to vascular obstruction by undegraded eggs.

## 1.35

**Evidence of BMPR2 alternative splicing as a novel genetic modifier of HPAH penetrance**

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The molecular reasons of why only 20% of BMPR2 mutation carriers develop PAH remain unknown. Here we present data that show that this reduced penetrance is likely due to BMPR2 alternative splicing. BMPR2 gene product is alternatively spliced to produce two splice variants: isoform-A (full-length); and isoform-B (missing exon 12). Several lines of evidence point to the importance of exon 12 in HPAH pathogenesis: (1) deletion of exon 12 is a common HPAH-inducing-BMPR2-mutation; and (2) mice overexpressing exon 12-deleted BMPR2 transcripts develop PAH phenotypically similar to human HPAH. Here we analyzed the relative amounts of these isoforms mRNA by real-time PCR analysis in PBMCs of 46 BMPR2 mutation-positive HPAH-patients and 31 BMPR2 mutation-positive unaffected-carriers. Our data show that affected individuals had relatively higher isoform-B expression compared to isoform-A expression (B/A ratio) than unaffected individuals (P<0.002). We then proceeded to determine the molecular mechanism behind this differential isoform expression. Analysis of exon 12 sequence identified a putative exonic splice enhancer whose mutations result in exon 12 skipping leading to increased isoform-B mRNA. Using siRNA and Western blot analysis we identified three splicing factors that bind to this splice enhancer and regulate BMPR2 exon 12 skipping. We then conducted a focused drug screen and identified FDA-approved pharmacological agents, which alter the activity of this enhancer resulting in alteration of the splice isoform ratio in either direction. In conclusions, penetrance of BMPR2 related HPAH can be predicted by BMPR2 isoform ratio; this isoform ratio is controlled by an exonic splice enhancer and its

associated splicing factors; this isoform ratio can be manipulated in either direction by FDA-approved pharmacological agents.

## 1.36

**Urokinase plasminogen activator receptor is a marker of pulmonary venous smooth muscle cells**

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The study of vascular pathophysiology in pulmonary arterial hypertension has primarily focused on the precapillary circulation; comparatively little attention has been afforded to the venous system. Recent evidence, however, suggests that pulmonary veins (PVs) play an important pathophysiological role in severe pulmonary hypertension. One challenge to studying the PVs is an inability to reliably identify them, by anatomical means, in the lung parenchyma. Furthermore, no specific molecular markers to PVs have been identified. We hypothesized that a systematic molecular characterization of normal PVs would identify distinct molecular markers—an important initial step to further research of PVs in pulmonary vascular disease. A mixture of agarose and fluorescent beads was retrogradely injected into the pulmonary veins through the left ventricle of mice while the aorta was clamped. Veins (20-200 μm) were identified under tissue section using fluorescent microscopy. Arteries, veins, and parenchyma were dissected using laser capture microdissection and RNA purified. qRT PCR arrays tailored to vasculogenic transcripts were used to characterize expression profiles of veins, arteries, and parenchyma for comparison. A list of candidate transcripts upregulated in veins was generated. Commercial antibodies were purchased and protein expression in paraffin embedded lung tissue was assessed. Pulmonary veins could be successfully and reliably identified by retrograde filling with fluorescent beads. Importantly, fluorescent beads were absent in arteries and airways. Comparison of RNA expression profiles between arteries and veins identified several candidates which were differentially expressed across vessel types. Of these, urokinase plasminogen activator receptor (UPAR) was expressed in pulmonary venous smooth muscle cells, distinguishing veins from arteries or lymphatics. Mounting evidence suggests the PVs play an important pathophysiological role in severe PAH; yet they remain poorly understood and understudied. Using a back-filling technique with fluorescent tags, the PVs were reliably identified for use in laser-capture microdissection and RNA purification. Comparison of RNA expression profiles between veins, arteries, and parenchyma successfully identified candidate markers of the pulmonary veins. Characterization of protein expression using immunohistochemistry-confirmed UPAR as a distinguishing marker of pulmonary venous smooth muscle cells. This research represents an important initial discovery in our understanding of the pulmonary veins in human disease.

## 1.37

**Cytochrome P450 1B1 influences the development of pulmonary arterial hypertension**

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The incidence of idiopathic and heritable PAH is up to three-fold higher in women than men. A mutation in the gene encoding the estrogen-metabolizing enzyme cytochrome P450 1B1 (CYP1B1) is associated with increased incidence of PAH in patients harboring BMPR-II mutations. CYP1B1 expression is increased in pulmonary arterial smooth muscle cells (PASMCs) in experimental and human PAH. Here we investigate the hypothesis that CYP1B1 promotes the development of PAH by assessment of hypoxia-induced PAH in mice deficient of CYP1B1

(CYP1B1<sup>-/-</sup> mice). Right ventricular systolic pressure (RVSP), right ventricular hypertrophy (RVH) and pulmonary vascular remodeling (PVR) were assessed. WT littermate mice were studied as controls. Following exposure to 2 weeks chronic hypoxia, male CYP1B1<sup>-/-</sup> mice showed a reduction in RVSP and PVR while both male and female CYP1B1<sup>-/-</sup> mice exhibited reductions in RVH. Pulmonary arteries from CYP1B1<sup>-/-</sup> mice had reduced vasoconstriction to serotonin in males and females. Immunohistochemistry staining confirmed increased expression of CYP1B1 in remodeled pulmonary arteries of patients with idiopathic and heritable PAH. 17 $\beta$  estradiol-induced proliferation of human PSMCs (hPASCs) was inhibited with the selective CYP1B1 inhibitor, 2,3',4,5'-tetramethoxystilbene. Furthermore, the CYP1B1 metabolite 16 $\alpha$ -hydroxyestrone stimulated proliferation in hPASCs. In conclusion, our findings suggest that increased 17 $\beta$  estradiol metabolism via CYP1B1 may influence PAH pathogenesis.

### 1.38

#### 17 $\beta$ -Estradiol (E2) attenuates hypoxia-induced pulmonary hypertension through an estrogen receptor-dependent mechanism that involves decreased ERK1/2 activation

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We investigated whether the protective effects of E2 in hypoxia-induced pulmonary hypertension (HPH) are mediated by estrogen receptor (ER)-activation, or by CYP450- and catechol-O-methyltransferase (COMT)-dependent conversion to E2 metabolites. Furthermore, we investigated whether E2 protection is associated with decreased proliferative and/or increased antiproliferative signaling. Adult male Sprague-Dawley HPH rats (PO<sub>2</sub>=380 mmHg; 2 weeks) were treated with E2 alone (75 mcg/kg/d), or cotreated with E2 and (a) ER-antagonist ICI182,780, (b) CYP450-inhibitor ABT, or (c) COMT-inhibitor OR-486. Standard hemodynamic and remodeling endpoints including cardiac output (CO) were assessed. Additional studies were performed in E2-treated hypoxic male wild-type, ER- $\alpha$ <sup>-/-</sup>, and ER- $\beta$ <sup>-/-</sup> mice; with complementary experiments in primary rat pulmonary artery (PA) endothelial cells (PAECs) and smooth muscle cells (PASCs) in 1% or 21% O<sub>2</sub> for 48 hrs. P<0.05 was considered significant. E2-treatment attenuated hypoxia-induced increase in RVSP, RVSP/CO, RV/(LV+septum) and hematocrit, and attenuated the hypoxia-induced decrease in CO. E2 decreased hypoxia-induced PA and RV remodeling, inhibited proliferative ERK1/2-activation in lung and RV, and increased expression of cell cycle-inhibitor p27<sup>Kip1</sup> and autophagy marker LC3-II. ER-blockade, but not E2 conversion-inhibition, attenuated E2's effects. E2 protection was attenuated in ER- $\beta$ <sup>-/-</sup> HPH mice. In hypoxic (but not in normoxic) PAECs, E2 (1 nM-1  $\mu$ M) dose-dependently attenuated proliferation (BrdU), ERK1/2-activation, and VEGF-secretion, while enhancing p27<sup>Kip1</sup>-expression and autophagy. ER-blockade attenuated E2 effects on proliferation and ERK1/2-activation. Antiproliferative E2 effects were also observed in hypoxic PASCs. E2 protection in HPH is mediated by ER-activation without a requirement for metabolite conversion. This is associated with decreased proliferative signaling, and increased cell cycle-inhibition and autophagy. ER- $\beta$  appears necessary for E2 protection. Elucidating E2 signaling mechanisms may reveal potential therapeutic targets in HPH.

### 1.39

#### Induced pluripotent stem cells as a model for heritable pulmonary hypertension

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Heritable pulmonary hypertension (HPAH) is characterized by profound vascular endothelial (EC) dysfunction and remodeling, accompanied by mutations in the gene encoding the BMP Type 2 receptor (BMPR2). Rodent models to date do not adequately recapitulate the disease pathology. A novel option for understanding the pulmonary vascular dysfunction manifested in HPAH is the use of patient-derived induced pluripotent stem cells (iPSCs). We hypothesized that HPAH patient derived iPSC cells and directed EC differentiation would prove to be an effective model in order to understand the consequences of BMPR2 mutation. To demonstrate that HPAH patient derived iPSC would be a valid model to study cell-based mechanisms of disease, in collaboration with Drs. Kotton and Austin, we generated transgene free, karyotypically normal iPSC lines from skin fibroblasts obtained from an HPAH patient. iPSC exhibited an ES cell-like morphology. Sequencing of genomic DNA confirmed the retention of mutation in the BMP3ACr1 iPSC cell line. These Hu iPSC were subsequently differentiated into multipotent mesenchymal cells. Differentiation of iPSCs into mesenchymal cells was demonstrated by surface marker expression of CD 29, 73, 105, Stro-1 and lack of the hematopoietic markers CD45, CD3 and CD14. Multipotent differentiation to mesenchymal lineages was also represented by bone (alkaline Phosphatase and Von Kossa stain), and (alcian blue and Aggrecan stain) and adipose/fat, oil red O stain. Endothelial differentiation was demonstrated by expression of VE-cadherin (CD144) and PECAM-1 (CD31) and lack of CD45, CD14, CD3. The use of iPSC cells derived from HPAH patients with known BMPR2 mutations provides an alternative source of patient-derived vascular cells to model disease.

### 1.40

#### A novel mechanism to explain enhanced pulmonary artery vasoconstriction in female rabbits

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In women the incidence of certain forms of pulmonary arterial hypertension is 4-fold greater than that observed in men. Mechanisms to explain the female predominance are scarce but likely relate to hormonal changes that contribute to the pathogenesis of the disease. We previously reported that arachidonic acid and the 15-lipoxygenase (LO)-derived arachidonic acid metabolite, 15-hydroxyeicosatrienoic acid (15-HETE) produced more vasoconstriction in pulmonary arteries of female compared to male rabbits. Expression of 15-LO protein was greater in females and 15-LO expression increased in males in response to estrogen treatment. The overall objective of the present study was to determine mechanisms that explain female sex/estrogen modulation of 15-LO expression in the pulmonary vasculature. Because it is known that cytokines increase 15-LO mRNA and protein expression, we incubated pulmonary arteries with 17 $\beta$ -estradiol (1  $\mu$ M, 18 hrs.) and found that the protein expression of interleukin 4 (IL-4) increased compared to controls. Blockade of IL-4 production with IBMX prevented estrogen-induced 15-LO expression. Additional studies explored the hypothesis that the ability of estrogen to increase cytokine expression results in the activation of the transcription factor, STAT3 that then signals to increase 15-LO gene transcription. To test this, activated STAT3 expression was compared in pulmonary arteries of females and males with and without estrogen treatment. Isolated male pulmonary arteries incubated with estrogen had increased activated STAT3 expression compared to controls. Interestingly, control pulmonary arteries from females also had greater expression of activated STAT3 compared to males. Further defining the mechanisms of estrogen and 15-HETE interactions in the pulmonary vasculature in models of PAH will likely provide new insights in understanding their role in the pathogenesis of PAH.

## 1.41

### QTc prolongation round in animal models of pulmonary hypertension is a marker of survival and is reversible by the fatty acid oxidation inhibitor, trimetazidine

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We hypothesized that prolongation of the QTc interval observed in rodent models of pulmonary hypertension is (a) modifiable with metabolic therapy and (b) also occurs in human PH. In rodent models of pulmonary arterial hypertension (PAH) and pure RVH induced by pulmonary artery banding (PAB), the QTc interval is prolonged, reflecting downregulation of repolarizing Kv channels in RV myocytes. We measured fatty acid oxidation (using a dual isotope technique) and monophasic action potential duration (MAPD) in the RV Langendorff model. In vivo we recorded QTc in PAB versus sham rats. PAB rats were treated with partial inhibitors of fatty acid oxidation (pFOXi-ranolazine and trimetazidine) versus placebo. We measured the expression of the repolarizing cardiac K<sup>+</sup> channel, Kv1.5, Hexokinase 2, a mediator of glycolysis, and the fatty acid transporter, FATP/CD36 in the RV by qRT-PCR. We measured the QTc interval and prospectively examined the impact of QTc interval on survival in 202 patients on PAH-specific therapy. Both trimetazidine and ranolazine decreased fatty acid oxidation in experimental RVH. In rats having undergone sham surgery the mean QTc was 0.122 s. Four and 8 weeks after PAB, the QTc increased to 0.205 s and 0.213 s, respectively (versus 0.122 s in Sham rats). Treatment with trimetazidine for 8 weeks decreased the QTc to 0.19 s. Ranolazine had no effect on QTc. MAPD was increased in PAB versus sham rats and returned to baseline after treatment with trimetazidine (MAPD50 18 ms in Sham compared with 27 ms in PAB models and 20 ms in PAB + trimetazidine [ $P < 0.01$  between PAB MAPD50 and PAB + trimetazidine MAPD50]). RV Kv1.5 mRNA expression was decreased in PAB rats and was partially restored by trimetazidine. Trimetazidine also decreased the expression of HKII and FATP/CD36. Ranolazine did not significantly affect HKII but did decrease FATP/CD36 expression. QTc intervals were longer in PH patient versus controls ( $454.8 \pm 29$  ms versus  $429.8 \pm 18$  ms,  $P < 0.001$ ). QTc interval did not differ based on PH etiology or therapy. On multivariate analysis, a QTc  $\geq 480$  ms was an independent predictor of mortality (HR 4.21, 95% CI 1.31–13.49). QTc prolongation is a feature of experimental RVH and human PH. QTc prolongation has prognostic significance. In the PAB model, QTc prolongation is amenable to metabolic therapies with pFOXi that are approved for human use for other cardiovascular conditions.

## 1.42

### Calpain activates intracellular TGF- $\beta$ 1 in pulmonary vascular remodeling of pulmonary hypertension

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In the present study we examined the role of calpain in collagen synthesis and pulmonary vascular remodeling in two models of pulmonary hypertension. We found that calpain inhibition using conditional knockout of calpain-4 prevented calpain activation and increases in right ventricular systolic pressure (RVSP), right ventricular hypertrophy, as well as collagen deposition and thickening of pulmonary arterioles of mice with hypoxic pulmonary hypertension. Moreover, the specific calpain inhibitor MDL28170 prevented the progression of RVSP, right ventricular hypertrophy as well as collagen deposition and thickening of pulmonary arterioles of rats

with established pulmonary hypertension induced by monocrotaline. Calpain inhibition did not affect increases in PDGF-BB and EGF but ameliorated the increase in active TGF- $\beta$ 1 in hypertensive lungs. Furthermore, inhibition of calpain using MDL28170 or calpain siRNAs attenuated EGF- and PDGF-BB-induced increases in calpain activity, proliferation, and collagen-I synthesis in pulmonary artery smooth muscle cells. EGF and PDGF-BB increased intracellular active TGF- $\beta$ 1 and p-Smad2/3 but not intracellular total TGF- $\beta$ 1 or active TGF- $\beta$ 1 and total TGF- $\beta$ 1 in the culture medium. Cell-impermeable TGF- $\beta$  neutralizing antibody did not block EGF- and PDGF-BB-induced increases in collagen-I and p-Smad2/3. However, the cell-permeable-specific Akt5 inhibitor SB431542 or Smad2/3 inhibitor SIS3 prevented EGF- and PDGF-BB-induced increases in collagen-I and p-Smad2/3 and hypoxic pulmonary hypertension. Additionally, calpain inhibition attenuated EGF- and PDGF-BB-induced increases in intracellular active TGF- $\beta$ 1 and p-Smad2/3. Finally, lung tissues from patients with pulmonary arterial hypertension demonstrated concordant higher levels of calpain activation and intracellular active TGF- $\beta$  in smooth muscle cells of pulmonary arterioles. Our data provide the first evidence that calpain mediates EGF- and PDGF-BB-induced collagen synthesis and proliferation of pulmonary artery smooth muscle cells via an intracrine TGF- $\beta$ 1 pathway in pulmonary hypertension.

## 1.43

### Downregulation of SERCA2a in maladaptive but not adaptive RVH

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Patients with pulmonary hypertension can develop either an adaptive right ventricular hypertrophy (RVH) and retain their right ventricular (RV) function or a maladaptive RVH leading to rapid RV failure. The mechanism underlying the transition from adaptive to maladaptive state is unclear. Sarco (endo) plasmic reticulum Ca<sup>2+</sup> ATPase (SERCA2a) plays a key role in cardiac contractility by regulating cardiomyocyte calcium. SERCA2a expression and activity is depressed in experimental and clinical left heart failure. However, very little is known about changes in SERCA2a expression and activity in RV failure. We sought to study the expression of SERCA2a in rats with adaptive (pulmonary artery banding (PAB)) or maladaptive (monocrotaline-induced pulmonary hypertension (MCT)) RVH. In the PAB model, PAB versus sham surgery was performed in male Sprague Dawley rats (n=6 in each group). In the MCT group, male Sprague Dawley rats were randomized to either a single subcutaneous injection of monocrotaline (60 mg/kg) or sterile water (control, n=5 each group). PAB animals were sacrificed at 8 weeks whereas MCT-treated rats were sacrificed at 4 weeks (at which time they manifested RV failure). The mRNA expression of SERCA2a, quantified using qRT-PCR, was significantly decreased in MCT versus control RV. However, in PAB-induced RVH there was no statistically significant decrease in SERCA2a expression as compared to RVs from the sham animals. SERCA2a expression is significantly down-regulated in maladaptive RVH but not in adaptive RVH. The upstream signaling that mediates this down-regulation in maladaptive RVH needs further study. This finding suggests SERCA2a gene therapy using adeno associated viral vectors merits investigation.

## 1.44

### The cell-penetrating homing peptide CAR selectively enhances pulmonary effects of systemically coadministered vasodilators in a preclinical model of severe pulmonary arterial hypertension

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A recent study has identified a cell-penetrating homing peptide, CARSKNKDC (CAR), which specifically recognizes the neovasculature of wound tissue and also homes to hypertensive pulmonary arteries. We hypothesized that CAR would selectively augment the pulmonary vasodilatory effect of a coadministered substance by facilitating its transportation into pulmonary vascular tissue. Severe pulmonary arterial hypertension (PAH) was induced in rats by SU5416 injection followed by 3 weeks of exposure to hypoxia and 2 weeks of return to normoxia. Intravenously administered fluorescein-labeled CAR accumulated in the remodeled pulmonary arteries, while no or low signal was detected in other organs, except for the kidney where CAR is excreted. A simple coadministration of CAR (without conjugation with the vasodilators) enhanced pulmonary but not systemic vasodilatory effects of the Rho kinase inhibitor fasudil and the tyrosine kinase inhibitor imatinib in catheterized PAH rats. These results suggest that CAR selectively enhanced the pulmonary activity of the coadministered drugs by facilitating their transportation into the hypertensive arteries where CAR homes. The exact mechanism by which the co-administered CAR enhances the pulmonary effects of drugs remains to be determined. Our results suggest a new paradigm in the treatment of pulmonary diseases, using a cell-penetrating homing peptide to selectively augment pulmonary vasodilation by non-selective vascular drugs without potentially problematic conjugation process.

#### 1.45

### Possible involvement of endothelial mesenchymal transition in the formation of plexiform lesions in a preclinical model of severe pulmonary arterial hypertension

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We have reported that the severe, sustained pulmonary hypertension in a very late stage of the SU5416 plus hypoxia/normoxia-exposed rat is accompanied by the formation of plexiform lesions that are indistinguishable from those observed in human pulmonary arterial hypertension. The major cellular components that form the plexiform lesion are the hyperchromatic and oval-shaped core cells and endothelial cell marker positive lining cells. The origins and characteristics of these types of cells, however, remain unknown. We have performed immunohistochemical analyses of these lesions to further characterize the cellular composition. Adult male Sprague Dawley rats were injected subcutaneously with SU5416 (20 mg/kg; a VEGF receptor blocker), and exposed to hypoxia (10% O<sub>2</sub>) for 3 weeks followed by a return to normoxia for an additional 10 weeks. At 13 weeks after the SU5416 injection, hemodynamic parameters were measured and lungs were fixed for immunohistochemical staining. All rats developed severe pulmonary hypertension. Immunohistochemical analyses showed the lining cells were positive for mesenchymal as well as endothelial cell markers, while they were negative for the stem cell markers CD133 and c-Kit. Results for the core cells were not uniform—i.e., some but not all core cells were positive for the fibroblast marker S100A4 and  $\alpha$ -smooth muscle cell actin and were negative for endothelial and stem cell markers. These results raise the possibility that an endothelial mesenchymal transition may be involved in the formation of the plexiform lesion in this model.

#### 1.46

### Reversion of vascular remodeling in pulmonary hypertension—Impact of AMD-1

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Pulmonary hypertension (PH) can be induced by chronic alveolar hypoxia which results in a vascular remodeling process. In this study, we investigated whether pulmonary vascular remodeling observed in the mouse model of hypoxia-induced PH could be reversed by reoxygenation (reverse remodeling). Furthermore we sought to identify new genes that may trigger this process. Mice exposed to chronic hypoxia (21 days, 10% O<sub>2</sub>) were re-exposed to normoxia (for up to 42 days). Reversal of PH during reoxygenation was evident by decreased right heart hypertrophy, right ventricular pressure, and muscularization of small pulmonary vessels compared to hypoxic controls. Microarray analysis from these mice revealed S-Adenosylmethionine decarboxylase 1 (AMD-1) as one of the most downregulated candidates. As AMD-1 was already shown to be important for polyamine synthesis and cell proliferation, we focused on the impact of AMD-1 in the development and the reversal of pulmonary hypertension. In situ hybridization revealed AMD-1 localization in pulmonary vessels. AMD-1 silencing by siRNA decreased proliferation of pulmonary arterial smooth muscle cells and diminished phosphorylation of PLC- $\gamma$ 1. Furthermore, AMD-1<sup>-/-</sup> mice exhibited attenuated PH when exposed to chronic hypoxia compared to wild-type controls. Promoter analysis revealed that AMD-1 could be regulated by Egr1 as a consequence of EGF stimulation. These findings indicate that in the animal model of hypoxia-induced PH, vascular remodeling can be reversed by reoxygenation. AMD-1 may be involved in both the remodeling of pulmonary arteries during chronic hypoxia and the process of reverse remodeling.

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### A critical role for calpain in the development of pulmonary hypertension

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We have recently identified a critical role for a novel gene family, calpain, in the chronic hypoxia and monocrotaline animal models of PH. The calpains are a family of cytosolic, Ca<sup>2+</sup> activated, neutral cysteine proteases, many of which are ubiquitously expressed. When activated, these enzymes cleave a broad spectrum of functionally important targets that regulate cytoskeletal organization, cell proliferation, migration, apoptosis, and platelet aggregation—all key processes in the development of PH. Inhibiting calpain activity attenuated the development of PH. Mice overexpressing calpastatin (CAST), the highly specific endogenous and inhibitor of calpain developed less RV hypertrophy, a lower right ventricular pressure, and decreased pulmonary vascular remodeling compared to control animals after exposure to hypoxia for 3 weeks. Inhibition of calpain activity with calpeptin (250  $\mu$ g/kg, SQ twice daily), a highly permeable small molecule inhibitor of calpain, attenuated the development of monocrotaline-induced PH. Twenty-eight days after MCT injection, the animals developed significantly less right ventricular hypertrophy and pulmonary vascular remodeling. Biochemical analysis of lung lysates revealed increased calpain 1 and 2 expression with MCT and a return toward baseline in those animals treated with calpeptin. On a cellular level, the pulmonary artery smooth muscle cells (PASMC) isolated from patients with PH or animal models of PH possess increased migratory and proliferative phenotype. PASMC isolated from CAST overexpressors demonstrated decrease migration compared to PASMC obtained from wild-type C57BL6 when exposed to hypoxia. We are currently evaluating the role of calpain in smooth muscle cell proliferation.