Full conference report: The first Keystone Symposia Conference on pulmonary vascular disease and right ventricular dysfunction – Current concepts and future therapies

Scientific Organizers: Georg Hansmann, Stephen L. Archer, and Margaret R. MacLean
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TUESDAY SEPTEMBER 11

Welcome and keynote address

Speaker: Dr. Dimmeler

The scientific organizers, Drs. Hansmann, Archer, and MacLean welcomed everybody to Monterey and opened the conference. Dr. Dimmeler was the first speaker of the day to give the keynote lecture. She summarized cellular based therapies for ischemic heart diseases and presented encouraging results using bone marrow-derived cells (BMC). Interestingly, only very few of the transplanted cells engrafted in the host organ differentiated and were detectable over a long period of time. The mechanisms preventing differentiation and retention of the transplanted cells and how this transient cell pool elicits its positive effects, remain elusive. In their recent studies, her group showed that epigenetical silencing of eNOS prevented the endothelial fate of early EPCs, CD34(+) cells, and mesoangioblasts. Jumonji D3, a histone demethylase, is induced by hypoxia and injury, removing repressive marks on endothelial promoters. Pharmacological intervention with inhibitors of methyltransferase or HDAC-induced EC fate in BMC underlines the drugs' therapeutic potential. Dr. Dimmeler gave further examples on how disease states affect epigenetic factors. She showed that ischemia and myocardial infarction increased miR-92a and miR-34a and that their inhibition enhanced recovery after ischemia. Dr. Dimmeler further exemplified how epigenetic factors participate in cell to cell communication. She provided evidence that Krüppel-like factor (KLF) was induced in EC by flow and controlled the expression of miR-126, 143, and 145; the latter miRs were released from EC and affected neighboring SMC. As a proof of principle, a c-elegans miRNA was overexpressed in EC and shuttled to SMC through a transwell membrane in vitro.

The role of stem cells, progenitor, and differentiated blood cells in pulmonary vascular disease and repair

Speakers: Dr. Kourembanas, Dr. MacLean, Dr. Hassoun, Dr. Ormiston, Dr. Chrobak, Dr. Stewart

Dr. Kourembanas used a hyperoxia-induced neonatal lung injury mouse model and a hypoxic PAH mouse model to study the effect of transplantation of mesenchymal stromal cells (MSC) or administration of MSC conditioned medium (CM) on disease prevention or regression. Her group showed that a single infusion of MSC-CM could reverse fibrosis and alveolar injury, PA remodeling, and PH in the hyperoxic BPD model. Furthermore, her lab could reverse established hypoxia-induced PH by transplantation of MSC. Dr. Kourembanas discussed potential mechanisms of MSC action. Her results indicated a paracrine effect, especially since CM was efficient in reversing the phenotype. Fractionation of MSC-CM identified exosomes as the active cell non-autonomous component, capable of preventing or reversing lung disease-associated PAH.

Dr. MacLean illustrated potential mechanisms in PH, which might account for the gender specific differences of the disease and higher incidence in women. She provided evidence for an interaction of the serotonin and estrogen-signaling pathway. She explained that serotonin antagonistic drugs, 5HT1b receptor, serotonin transporter (SERT) and TPH1 were shown to be involved in PH. In transgenic mice lacking TPH1, hypoxia-induced PH was ablated, while SERT gene overexpression induced PAH in aged female mice. Interestingly, ovariectomy in these mice abolished PH, and substitution of 17ß estradiol restored it. Dr. MacLean's group showed that 17ß estradiol induced Tph1, 5HT1B, and SERT in PASMC. Inhibitors of Tph1 and 5HT1B inhibited estrogen-induced proliferation

of the cells. The estrogen metabolizing enzyme CYP1B1 was increased in SERT+ mice, Sugen/hypoxic mice, and hypoxic mice. The metabolite 16-OHE1 induced PASMC proliferation and PH in rodents. Inhibitors of the metabolite rescued RV hypertrophy in the hypoxia and Sugen model of PAH. Her results indicated a signaling circuit of the serotonin and estrogen pathways, which might account for gender differences in the prevalence and severity of PH.

Dr. Hassoun presented evidence from the literature that several inflammatory diseases cause PAH and that the phenotype is characterized by inflammatory infiltrations and activation of immune cells. Studies from his lab demonstrated an association of PAH with the macrophage migration inhibitory factor (MIF). MIF is a lymphokine involved in cell-mediated immunity. Dr. Hassoun showed that MIF was induced in the right ventricle upon hypoxia or stress, which promoted angiogenesis and could be abrogated by genetic deletion.

Dr. Ormiston explored the function of NK cells in PAH. He detected an increase of functional defective CD56 dim/CD16+ cells and a change in the KIR receptor profile of NK cells of PAH patients. The NK cells were also impaired in their lytic activity and cytokine induction. He explained that there was no change in KIR gene polymorphism, but that the cells exhibited a pathological response to TGFβ signaling. Dr. Ormiston was able to recapitulate the human phenotype in the rat MCT and mouse hypoxia model, which would allow further investigation of the NK function in PH.

Dr. Chrobak investigated the transcription factor GATA6 in human PAH. She analyzed lung samples of SSc-PAH and IPAH patients and detected a decreased GATA6 expression in PAEC and PASMC. In the mouse hypoxia and rat MCT model of PH, GATA6 was rapidly downregulated in the EC. Accordingly, mice with conditional endothelial loss of GATA6 spontaneously developed PAH shown by increased RVP, RV hypertrophy, inflammation, and vascular remodeling. ChIP analysis identified genes controlling vascular tone, EC activation, and vascular remodeling as direct targets of GATA6.

Dr. Stewart reviewed the endothelial cell pathology in PAH and recent attempts to influence the disease by the transplantation of endothelial progenitor cells. A well-established source of cells for transplantation is late outgrowth EPC, also called endothelial colony forming cells (ECFC). Dr. Stewart's group derived ECFC from PAH patients, with or without BMPR2 mutations, and performed unbiased micro-array, proteome, and phospho-proteome screens to detect changes in disease. A proteomic approach revealed a downregulation of V type proton ATPase, cAMP protein kinase type1, alpha actinin, and laminin B as well as an upregulation of translationally controlled tumor protein (TCTP). TCTP is known to be growth promoting and anti-apoptotic. He demonstrated that TCTP is induced in the Sugen rat model (here: normoxia) and hypothesized that TCTP is released in apoptotic nanovesicles of EC and stimulated proliferation of SMC. His analysis of the phospho-proteome detected only one less phosphorylated protein, while phosphorylation was generally upregulated. This finding indicated a global upregulation of kinase pathways, even in the background of BMPR2 "loss of function."

Workshop 1: How to translate basic research findings into improvement of patient care—The role of industry

Speakers: Dr. Punnoose, Dr. Horn, Dr. Thomas

Dr. Punnoose applied a computerized model to simulate the outcome of the implantation of right ventricular assist devices in PAH. According to their analysis, partial RV support with RV-assist devices would reduce RA pressure, augment LV filling, and cardiac output, while PA pressure and wedge pressure would potentially increase. Both placement of the inflow cannula in RA or RV increased RVAD pressures significantly, but no significant difference between locations was predicted.

Dr. Horn presented different ways and devices for RV assist and the indications depending on INTERMACS stages. She pointed out that more data is needed for new continuous flow devices. Available results suggested that these devices are more efficient, and she proposed to initiate the assist at earlier INTERMACS stages.

Dr. Thomas discussed the approach and key factors of commercial research and drug development. He defined the major steps such as target identification and target validation, which is the prerequisite for subsequent clinical investigation. He emphasized the importance of patient samples as a central source for the target identification. Due to the lack of biopsies, transplants are often the only tissue source, but he questioned the usefulness since these samples are end-stage disease samples. Dr. Thomas also presented specific results of their study of Imatinib for the treatment of PAH. In the HxSu model, Imatinib decreased PA pruning, RV fibrosis, inhibited TPH1 expression, and reduced 5-HT levels. His study of TPH1 KO suggested that serotonin is involved in Imatinib action, but also revealed TPH1 independent effects. Dr. Thomas explained that the detailed confirmation of pathway activity might help to better predict the responder population.

Growth factors, TGF-beta/BMP signaling, and pulmonary vascular disease

Speakers: Dr. Humbert, Dr. Bloch, Dr. de Caestecker, Dr. Thistlethwaite

Dr. Humbert reviewed the current knowledge of the relevance of inflammation, associated cytokine actions, and

immune mechanisms described in PAH pathophysiology. At the end of his talk, he presented recent findings of his lab, which suggested that the inflammatory infiltrations around the PA were tertiary lymphoid organs with a centrifugal arrangement of T and dendritic cells.

Dr. Bloch elaborated the effect of different BMPR2 mutations on the development of PAH. He explained that the study of the BMP pathway in pulmonary EC and SMC with genetic mouse models was complicated by cardiac defects imposing upon gene deletion using classical Cre drivers, like SM22 or Tie2. His lab therefore isolated PAEC or PASMC from mice expressing a floxed allele of the GOI and added the Cre recombinase in vitro to induce deletion. They observed that loss of one Bmpr2 allele mainly affected BMP 4 and 7 mediated Id1 gene-expression in PASMC. Loss of both alleles selectively activated the response on Bmp 7 via an alternative ActRIIa pathway. PAH-associated BMPR2 mutations also govern mutations in the cytoplasmic tail domain (TD). Dr. Bloch showed that decay-resistant deletion of the TD was embryonically lethal in the homozygous. Heterozygous mice exposed an increased response to BMP7 because of a loss of the inhibitory effect of the TD on ALK2-mediated BMP7 signaling. BMP4 signaling was not affected since it rather depended on ALK3.

Dr. de Caestecker's work provided a putative disease mechanism of BMPR non-sense mutations with mRNA decay. Bmpr2deltaEx2/+ mice were susceptible to PH under certain stimuli. The mutant allele expressed a truncated product in pulmonary endothelial cells, which did not locate to the cell surface, but was retained in the ER. Treatment with chemical chaperones restored cell surface expression und re-established signaling through SMAD 1/5/8 and therefore provided a potential future therapy.

Dr. Thistlethwaite summarized the current understanding of the function of the notch pathway in PAH. Notch 3 expression was specific to smooth muscle cells, while Notch 1 was expressed in endothelial cells. PAH patients exposed increased Notch 3 ICD and Hes5 levels, which correlated with the severity of the disease. Animal models resembled these findings and would therefore allow studying the molecular mechanisms. Notch 3 was shown to induce proliferation of smooth muscle cells, and KO mice were resistant to hypoxia-induced PA hyperplasia and did not develop hemodynamic signs of PAH. Smooth muscle specific over-expression of Hes5, resembling Notch pathway activation, induced severe smooth muscle cell hypertrophy. Dr. Thistlethwaite pointed out the therapeutic potential of interference with the notch pathway. The finding would support that DAPT reversed RVSP without affecting the systemic BP in a mouse model of hypoxia-induced PAH.

WEDNESDAY SEPTEMBER 12

Metabolic regulators in pulmonary vascular disease

Speakers: Dr. Carmeliet, Dr. Hansmann, Dr. Michelakis, Dr. Yong-Hu Fang, Dr. Mair, Dr. Morrell

Dr. Carmeliet explained basic mechanisms of resistance to anti-angiogenesis treatment like VEGF signaling through alternative pathways. He suggested new therapeutic approaches that would interfere at downstream metabolic checkpoints and would potentially prevent such escape mechanisms.

In the first half of his talk, Dr. Hansmann summarized the vasoprotective actions of the transcription factor PPARγ that acts downstream of BMP-RII, representing a rescue therapy for BMPR2 mutations/dysfunction. BMP-2 induces nuclear shuttling and DNA-binding of PPARγ in human pulmonary artery SMCs (PASMCs). Others had shown that PAH patients have decreased pulmonary mRNA expression of BMP-2, PPARγ, and ApoE. Dr. Hansmann and colleagues from Stanford could link all three factors and demonstrated (1) a novel antiproliferative BMP-2/PPARγ/apoE axis in PASMCs using small hairpin RNA interference and transgenic mouse techniques; (2) pharmacological PPARγ activation inhibited PDGF-BB-induced ERK phosphorylation and blocked PDGF-BB-induced proliferation of PASMCs isolated from a PAH patient with a frame-shift mutation in the BMP-RII gene; and (3) loss of PPARγ in vascular SMCs and ECs caused PAH in vivo. These findings indicate that BMP-RII dysfunction decreases endogenous PPARγ activity and enhances PDGF-BB/MAPK pathways and associated vascular remodeling. However, the observation that the PPARγ agonist rosiglitazone reversed PAH, RVH, and remodeling in the APOE -/- mouse in association with an 8-fold induction of adiponectin (APN) suggested that PPARγ target genes other than APOE may also be mediators of PVD reversal. Meanwhile, the vasoprotective effects of PPARγ-dependent signaling in the lung have been confirmed by others in additional transgenic PH models such as the APN -/- mouse. Dr. Hansmann proposed that a strategy aimed at activating PPARγ pathways could reverse the PAH phenotype. Of note, pioglitazone and rosiglitazone have been found to have quite different safety profiles in humans. Since recent clinical observations indicate that insulin resistance and dyslipidemia (low HDL cholesterol) are risk factors or disease modifiers ("2nd hits"), PPARγ activating agents might be beneficial in the future treatment of both insulin-resistant and insulin-sensitive PAH patients with or without BMPR2 mutations. Moreover, Dr. Hansmann presented preliminary data on plasma biomarkers in infants with congenital heart disease and increased, normal or decreased pulmonary blood flow. Finally, Dr. Hansmann presented work on a novel microfluidic capture device that can measure CD34+/KDR+ EPCs in low blood

volumes without preprocessing and showed these EPCs are decreased by 50% in the blood of PAH patients. The group proposed that number and function of circulating EPCs measured by the capture chip or other methods, therefore represent promising PAH biomarkers for disease progression and response to therapy.

Dr. Michelakis made the audience aware of the crucial function of mitochondria in PAH. Mitochondria appear to regulate apoptosis and inflammation, Ca++ and mROS levels, and act systemically via mitokines. Mitochondria in SMC of PAH patients were hyperpolarized, which inhibited apoptosis. Polarization of the mitochondria was regulated by pyruvate dehydrogenase (PDH), which shuttled pyruvate to the mitochondria. Inhibition of its kinase by dichloracetate (DCA) was shown to induce apoptosis and reduced the elevated HIF levels in PAH models. Dr. Michelakis reported an improvement of PA perfusion in several PAH patients included in the ongoing DCA study. He explained close interaction of mitochondria and the endoplasmatic reticulum and showed that, loss of Nogo, which regulated the ER mitochondria interaction, was preventative for hypoxia-induced PAH. PBA, a chemical chaperone, reversed hypoxia induced decrease in mitochondrial Ca++ and was preventative and reversed PAH in animal models. Dr. Michelakis reported that SIRT3 and UCP KO mice presented already at normoxia with a decrease in Krebs cycle intermediates, similar to PAH patients, and pointed out that individuals with mutations in these genes may be more susceptible to severe PAH.

Dr. Yong-Hu Fang reported that glutaminolysis and its associated genes (e.g., SLC7A5, SLC1A5) were induced in PAH. He studied the effect of inhibition of glutaminolysis by 6-Diazo-5-Oxo-l-Norleucine (DON) in the isolated working heart and observed an increase in cardiac output. In vivo studies unfortunately showed adverse effects, e.g., significant weight loss.

Dr. Mair discussed why women are more susceptible to PAH than men. She reported that estrogen induced hPASMC proliferation. Aromatase converts androgen to estrogen and two SNP in the promoter region of the aromatase gene were found to be associated with higher risk to develop portopulmonary PAH. Dr. Mair showed that anastrazol inhibition of aromatase reduced estrogen in normoxic and hypoxic rodents and improved pulmonary vascular remodeling and hemodynamic parameters in PAH. She provided evidence that hypoxia decreased BMPR2 expression in PH mouse lungs. This effect was reversed by in vivo inhibition of aromatase.

Dr. Morrell explained that BMPR2 mutations specify a quantifiable phenotype in human PASMC and EC. He stated that blood outgrowth EC (BOEC) would provide a useful platform to study the impact of BMPR2 mutation and EC function. He further showed that BOEC possessed stem-cell like properties and were a novel and efficient substrate for the generation of patient specific iPS cells. Dr. Morrell gave evidence that BOEC-iPS could be differentiated toward vascular cell lineages and therefore potentially provides a patient specific disease model for genetic forms of PAH.

Workshop 2: MicroRNAs and iPS cells-novel tools and targets in cardiovascular biology and pulmonary vascular research

Speakers: Dr. Grieve, Dr. Drake, Dr. Margaillan, Dr. Prewitt, Dr. Spiekerkoetter

The afternoon workshop was opened by Dr. Grieve, who reported that several miRNAs were dysregulated during the development of PAH. She studied the effect of interference with miR451, which was upregulated in PAH. Prophylactic application of antimir451 in the rat hypoxia model reduced RV pressure as compared to nonsense control. Interestingly, neither RV pressure nor other PAH parameters were changed in the miR451 KO, suggesting perhaps the existence of effective compensatory mechanisms for genetic miR451 loss in the pulmonary system.

Dr. Drake reviewed the literature about interaction of TGFbeta-smad signaling and microRNAs. Some miRNAs were responsive to BMP9 treatment in PAEC, unless a BMPR2 mutation was evident. The lysosomal pathway degraded BMPR2. Chloroquin reduced lysosomal degradation of BMPR2 and rescued smad-mediated miRNA processing in HPAH cells, like miR21 and miR27.

Dr. Margaillan explained that RUNX2 regulated HIF1 activation in HEK. RUNX2 was target of miR204, which was shown to be downregulated in PAH patients, conversely HIF1- and RUNX2 were upregulated in PAH. Expression of miR204 in vitro in PASMC reversed proliferation and re-established apoptosis. Furthermore, he reported that in vivo application, systemic or by nebulization, in a sugen rodent model ameliorated vessel remodeling and reversed PAH.

Dr. Prewitt reported that BMPR2+/- PAEC have a decreased barrier function, while displaying increased caveolae-like structures. She showed that Caveolin1 was atypically located intracellular, which could be reversed by a dynamine inhibitor (dynasore), suggesting increased caveolar endocytosis. She described that Src kinase was constitutively activated and that inhibition of the kinase with PP2 conversely improved PAEC barrier function.

Dr. Spiekerkoetter hypothesized that increase of BMPR2 signaling in HPAH might prevent or reverse already established PAH. The group established and performed a high-throughput screen with 3600 FDA approved drugs. Tacrolimus (FK506), Ascomycin, and Rapamycin were

the top 3 hits. FK506 rescued BMP signaling in PAEC from IPAH patients and promoted EC proliferation and tube formation. She further reported that low-dose FK506 was able to prevent and reverse hypoxia-induced PAH in a BMPR2 endothelial KO and the sugen hypoxia rat model. They currently conduct a phase II clinical trial to test the safety and efficacy of low-dose FK506 treatment in human.

The right ventricle in pulmonary hypertension: Cardiomyocyte function and hemodynamic performance

Speakers: Dr. Archer, Dr. Kühne, Dr. Redington, S. Rowan

Dr. Archer made the audience aware of metabolic changes in PAH and RVH patients that should be addressed in future trials, especially since they might be reversible. He reported that HIF1 regulated Pyruvate Dehydrogenase Kinase 1 (PDK1) in vessels, while Foxo1 controlled PDK4 in the right ventricle. PDK inhibited pyruvate dehydrogenase (PDH), the enzyme connecting the glycolysis pathway with the citrate cycle by transforming pyruvate to acetyl-CoA. Aerobic glycolysis protected from apoptosis, while a counteracting glycolytic shift and reduction of PDH in the affected RV in PAH was observed. He showed that the metabolic changes could also be found in the MCT rat model of PAH. They applied a dual isotope technique to the RV working model, which allowed monitoring of oxidative lipid metabolism and glucose oxidation. Dr. Archer provided evidence that DCA treatment improved the metabolism, hemodynamics, and right ventricular output. He further reported that Ranolazine and Trimetazidine, inhibitors of fatty acid beta-oxidation, improved the outcome in PAH and RVH models.

Dr. Kühne stated that cardiac MR would provide a multitude of information, which could be used for an integrated and multi-level functional analysis of the highly complex right ventricular pathophysiology. He exemplified the ability to define pressure-volume loaded RV sub-compartments by MRI, e.g., inflow, infundibulum, and trabecular compartments. Furthermore, intra-cardiac flow kinetics could be analyzed for measuring energy loss of the in- and outflowing blood and ultimately pump efficiency of the RV. He showed that diffusion tensor MRI detected the mayor direction of myofibers and elucidated that there is no adaptive response of the RV upon pressure overload, as it would be known for the LV. Dr. Kühne further illustrated the potential of MRI to predict the outcome of a treatment by in silico simulation of biofluid dynamics.

Dr. Redington gave a detailed overview of the right and left ventricular interaction in PAH. The RV would be adapted to low afterload and would have a low contractile reserve. It reacted on increased afterload with prolongation of the systole. The LV contributed significantly to the RV pressure and flow, but this influence was diminished once the RV increased in size. Moreover, the consecutive compression of the LV reduced LV function, and the increased duration of RV systole lead to reduced LV preload. He discussed potential therapeutic strategies like RV inotropic agents (e.g., phosphodiesterase 5 inhibitor), use of the Novalung for acute RV unloading and the exposure of the RV or AP to the LV pressure or the binding of the aorta.

S. Rowan reported that gremlin, a BMP antagonist, was increased in human PAH lung samples as well as in hypoxic rodent models of PAH. He explained that gremlin was recently also described to act as a VEGFR2 agonist, thereby activating eNOS, which would be in contrast to its effects mediated by interference with BMP signaling. They therefore examined haplo insufficiency of gremlin in a mouse hypoxia model. Reduced gremlin lead to increased eNOS expression and activity in early hypoxia, reduced vascular remodeling, and attenuated the hypoxic increase in PVR when compared to wild-type mice.

THURSDAY SEPTEMBER 13

MicroRNAs in proliferative vascular disease

Speakers: Dr. Baker, Dr. Voelkel, Dr. Paulin, Dr. Bonnet, Dr. Stankiewicz

Dr. Baker reported that miR145 was elevated in the vascular wall/SMC and complex lesions, but not the serum of iPAH and hPAH patients. He showed that genetic ablation of miR145 and antimiR145 protected mice against hypoxia-induced PH (reduction in SRVP and vascular remodeling). GSEA of a micro-array on PA samples from miR145 KO was enriched for coagulation, thrombin, and wnt signaling. Dr. Baker provided experimental evidence that miR145 antagonism repressed wnt signaling. They further studied intranasal and s.c. ways of administration antimiR145 and showed that intranasal application was effective for pulmonary knock down, but had less systemic effects. His lab just started experiments on large animal models, like the pig stent and vein graft model and observed an immediate loss of miR145, followed by reinduction after 7 days. His studies on miR21 suggested that the miR was upregulated in the vascular wall in hypoxia. Interestingly, ablation worsened pulmonary hemodynamics in hypoxia and miR21 KO presented with basal lesions and age-related pulmonary changes in normoxia. However, this result could not be reproduced with antimiR. In the miR21 KO, BMPR2 was downregulated in normoxia, Stat3, Timp3, and Pdcd4 up in hypoxia, suggesting them as targets of miR21 signaling.

Dr. Voelkel reviewed the literature and presented own results on histone deacetylation in RV disease and PAH, and discussed the potential use of HDAC inhibitors for treatment. He reported that HDAC inhibition reduced inflammation, vascular remodeling, and myocyte hypertrophy in LV disease, while decreased HDAC activity

in the lung is associated with COPD and HDAC inhibition induced emphysema. Dr. Voelkel described that Pan-HDAC inhibition with trichostatin worsened RV dysfunction in the binding model and induced loss of capillaries, fibrosis, and cardiomyocyte hypertrophy in the SuHx model. Dr. Voelkel exemplified differences of the SuHx and banding model. HDAC 1–3 were induced and PGC1 affected in the hypoxia, but unchanged in the PAB model. He provided experimental evidence of ER stress and mitochondrial dysfunction in the SuHx model, as CHOP, Gadd34 were upregulated, and sphingosine 1 phosphate (S1P) to Ceramide imbalanced. He hypothesized that ER stress and mitochondrial dysfunction were upstream regulators of HDAC, PGC1, and VEGF, among others, in PAH and RV disease.

Dr. Paulin hypothesized that specific miRNA signatures would characterize the transition from fetal to adult and from a compensated to decompensated RV in disease. In a TaqMan Array, they elucidated 7 differentially regulated miRNAs in the developing and decompensating RV (miR208, 132, 338, 200b, 155, 92a, 328). She further provided evidence for a Mef2-miR208 signaling loop, which was driving the transition from a compensated to a decompensated RV state. Pressure overload induced a switch from aMHC to bMHC. aMHC is the host gene of miR208, which would target Med13-NCoR1 and consecutively influence the expression of metabolic genes. She showed that RV decompensation correlated with loss of miR208, activation of Med13/NCoR1, and loss of Mef2.

Dr. Bonnet summarized previous results, suggesting that STAT3 regulates Pim1/Nfat, Bmpr2, and Hif1, which accounted for the inflammation, mitochondrial, metabolic, and Ca disorders observed in PAH. He hypothesized that miRNAs might play a role in the regulation of STAT3. To answer this question, the group performed a TaqMan Low Density Array (TLDA) analyzing 377 miRNAs of two human PAH samples. miRNA 204 was revealed to be the only significantly downregulated miRNA. Further studies of his lab showed that miR204 restoration reduced STAT3 and vascular remodeling in experimental PAH, although STAT3 is not a direct target of miR204. He suggested an involvement of the Src pathway, since SHP2 is upregulated in PAH, but repressed by miR204. Dr. Bonnet went on describing how DNA repair mechanisms may contribute to the PAH phenotype. He reported that DNA damage was observed in PAH and essential components of the DNA repair mechanisms were activated in PAH, e.g., yH2AX, 53BP1, and PARP1. Interference with DNA repair by ABT888 further enhanced the PAH phenotype. He provided evidence that PARP1 activation in PAH accounted for the induction of miR204/Nfat/Hif. Inhibition of PARP1 in the Sugen rat hypoxia and MCT model improved PH. Dr. Bonnet thereby exemplified how DNA damage resulted in epigenetic changes during the course of the disease. In the last part of his talk, he described how miRNAs influenced the exercise intolerance observed in PAH. Exercise intolerance was supposed to be related to a change in micro-vascular density of the skeletal muscle. The group observed a correlation between downregulation of miR126 and upregulation of SPRED1 and loss of micro-vessels in PAH patients. Expression of miR126 in muscular EC restored angiogenesis, while in vivo antagomir induced exercise intolerance and reduced vascular density. Taken together, he provided evidence for an implication of miRNAs in the etiology of PAH and skeletal muscle dysfunction and the implication of DNA repair machinery in miRNA dysfunction.

Dr. Stankiewicz described a rare developmental disorder, i.e., alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV), which is associated with FOXF1 deletion or mutation. FOXF1 is specifically expressed in the fetal lung and placenta. His studies identified de novo non-coding deletion copy number variations, 250 kb upstream of FOXF1, and defined a 75 kb regulatory region. He showed that this region was only deleted on the maternal chromosome 16. The regulatory region contained a differentially methylated subunit harboring GLI2 binding sites and a fetal lung specific EST, likely representing a large intergenic non-coding RNA (lincRNA). Dr. Stankiewicz proposed a complex tissue specific long-range regulation model of FOXF1, with interplay between the fetal lincRNA and the methylation-controlled GLI2 binding site, which showed an incomplete paternal imprinting.

Innovative clinical PAH trials

Speakers: Dr. Michelakis, Dr. Rich, Dr. Nicolls

Dr. Michelakis gave a historical overview over DCA treatment, a drug that was first applied in patients with the malignant brain tumor, glioblastoma. DCA induced a rapid decrease in the mitochondrial potential, induced PDH and consecutively reduced proliferation, and initiated apoptosis in glioblastoma. Regular brain tissue appeared to be unaffected by treatment. He also summarized the animal data that suggested that DCA could be a potential therapy of human PAH. Dr. Wilkins took over the second part of the talk to present the rationale and design for the first trial of DCA in patients with PAH. This trial has been conducted at the University of Alberta and the Imperial College of Medicine. The primary outcome of the current trial is safety and tolerance of the drug (phase I). Secondary outcomes are changes in PVR, RV mass, and volume by MRI, lung glucose uptake measured by 18-FDG-PET, pulmonary blood flow, 6-minute walk distance, and quality of life. Included are PAH patients WHO class II–IV on standard background and PAH therapy. Three treatment groups were defined 3 mg/kg, 6.25 mg/kg, and 12.5 mg/kg BID. Twelve patients have been recruited so far, while the trial is ongoing.

Dr. Rich discussed caveats in clinical trial design for PAH. He summarized the status quo of the current clinical trials in PAH. There were 23 RTC involving 3780 patients. Meta analysis showed an improvement of the 6-minute walk and 16-week survival, but no effect on the hemodynamics under the medications studied. He pointed out that, data acquired in several accomplished studies are not publicly available and therefore lost for the research community. According to Dr. Rich, there is also a lack of knowledge on how and where the drugs work and which subpopulations of patients might benefit or not. He determined the variable phenotypes of the patients and the definition of adequate endpoints as common issues of clinical trials on PAH. Considering his first point, he mentioned that the WHO classification is quite old (1998) and would not reflect morphological variations of PAH, but rather be clinical symptom oriented. According to him, this involved the danger that a study might target a single clinical parameter rather than taking the entire phenotype into account. Dr. Rich stated that FDA requirements did not meet parameters good to evaluate the success of a new treatment. He said it would not be obvious as of when to expect a therapeutic effect of a new treatment. A study might be terminated even before an improvement is detectable, e.g., RVEF. Dr. Rich elaborated on two further issues when designing PAH trials. First, the call for multi-center clinical trials would make the organization of studies complicated and expensive. As a result, pharmaceutical companies might refrain from joining these endeavors, which would have disastrous consequences for the development of new therapies. Second, randomized trials might not be able to identify subgroups, which profit from a certain treatment. PAH is a heterogeneous disease, and improvement in a subgroup might not be reflected in the average of the whole population. Dr. Rich therefore voted to establish a new study design, which should be prospective, but biomarker stratified. He went on presenting the Pulmonary Hypertension Academic Research Consortium. Members included academics, regulatory authorities, industry and physician medical societies. Its organization structure includes a steering committee, 5 working groups, and a pediatric advisory committee. He praised it as a new approach for the organization of clinical trials, since all members agree on such important terms as to share the acquired data, definition on endpoints, inclusion, diagnostic and success parameters, and the collection and banking of patient-derived specimen.

Dr. Nicolls presented the rationale for a treatment of SSc-PAH with rituximab. Cytotoxic T cells, CD20+ B cells, IgG, and complement depositions were found in plexiform lesions and PA walls of SSc-PAH patients. While regulatory T cells are compromised, the disease is characterized by an activation of B cells and circulating auto-antibodies, targeting the endothelium. They hypothesized that SSc-PAH patients would demonstrate an improvement or stabilization in pulmonary vascular resistance and right-ventricular end-diastolic volume index within 24-weeks past-treatment with rituximab. Inclusion criteria for the study were diagnosis of systemic sclerosis, SSc-PAH diagnosis within 5 years, and initiated PAH treatment for 12 weeks. The change in pulmonary vascular resistance was defined as the primary endpoint. He reported that, so far, 14 patients were enrolled. Preliminary results were still pending. Dr. Nicolls concluded that immune mediators contribute to PAH development and that different PAH conditions may be susceptible to different adjuvant immunotherapies.

FRIDAY SEPTEMBER 14

Pulmonary hypertension in parenchymal lung and thromboembolic diseases

Speakers: Dr. Krasnow, Dr. Thebaud, Dr. Mitchell, Dr. Zhou, Dr. Jamieson, Dr. Ogawa

Dr. Krasnow provided insights into the development of the pulmonary artery. His lab used the Cre-lox technology to label single cells in a tissue-specific manner and followed their fate with multi-color reporter systems. They utilized a Tbx4 enhancer Cre, which labeled lung mesenchymal cells, a ubiquitously labeling Cre-Hprt-Cre, a mesothelial Wt1 driven Cre, and VE-cadherin Cre for endothelial labeling. Dr. Krasnow provided evidence that the pulmonary vessels did not arise by vasculogenesis from the lung mesenchyme or by angiogenesis from vessels outside the lung. His group's studies with the endothelial specific Cre revealed that the vessels developed by reorganization of the immature endothelial plexus inherent to the lung. He showed that the plexus was actually perfused and connected to the blood circulation, while it would undergo what he described as a leak-proof remodeling and plexus coalescence, to form a mature, organized, branched vascular bed. Dr. Krasnow then described how mesenchymal cells reorganize along the endothelial tube to build the adventitial layers. The first layer of cells aligned with the endothelium and expanded by longitudinal division, followed by a radial division budding off cells to form the second layer, which in turn expanded by longitudinal division, and so on.

Dr. Thebaud's presentation covered experimental treatment options for lung injury in CDH and BPD. He pointed out that newborns with CDH poorly responded to current treatment options and that new strategies would be needed. He hypothesized that endothelial colony forming cells (ECFC) might be one such. Angiogenesis and alveolar development are tightly connected. Dr. Thebaud provided some evidence for the existence of ECFC in the fetal lung, which had clonogenic potential and did induce de novo blood vessels in nude mice upon transplantation. Interestingly, the function of these ECFC was impaired in the nitrofen-induced

rat CDH model since they produced less CFU or vascular tubes. He showed that transplantation of ECFC in the MCT model (administration at P6) rescued the alveolarization defect and reduced PAH. Similarly, ECFC or umbilical cord blood (UCB) derived MSC/pericytes restored the alveolar structure and improved pulmonary hemodynamics and exercise performance in the O2 BPD model. He further demonstrated that conditioned medium of bone marrow derived MSC (BM-MSC) prevented inflammation and the course of ARDS in the LPS induced rodent model. UCB cells or conditioned medium reduced fibrosis in the bleomycin, and BM-MSC reduced airway remodeling in the ovalbumin asthma model. He summarized that human UCB are a rich viable source of potential repair cells, promising treatment of lung disease characterized by arrested alveolar development and PAH.

Dr. Mitchell explained that prostacyclins and their analogues would not only act by vasodilatation on classical cell surface IP receptors, linked to cAMP but also via interaction with cytosolic PPARß/δ. She provided evidence that PPARß/δ agonists are potential therapies for PH. Prostacyclins were able to bind and repress PKCα, enhance the AMPK pathway and prevented RV remodeling and improved hemodynamics in models of pulmonary hypertension. In a micro array they identified known PPARß/δ target genes were induced in the RV of treated mice.

Dr. Zhou presented his study of a cell-based approach to rescue monocrotaline-induced PAH. He explained that prostacyclin therapy of PAH is hampered by the very short half-life of the molecule. The enzymes COX (cyclooxygenase) and PGIS (prostacylcin synthase) are needed for prostacyclin biosynthesis from arachidonic acid. Dr. Zhou transplanted modified bone marrow-derived endothelial-like progenitor cells (ELPCs), stably expressing a COX isoform 1-PGIS fusion enzyme, in the MCT model. He hypothesized that these cells would be able to supply prostacyclin in vivo and ameliorate PAH. He provided evidence that a single time delivery of the engineered cells rescued MCT-induced PAH and offered a survival benefit for at least 4 weeks. Jugular vein delivery localized the transplanted cells mostly to the lungs and may minimize systemic effects.

Dr. Jamieson made the audience aware that PAH, as a result of chronic pulmonary embolism (CTEPH), was a potentially underestimated entity of PAH. The incidence was reported as 45,000 cases per year. He considered a right heart catheter as the diagnostic gold standard. A discordance of PVR and angiogram and a PVR >1000 would predict a worse outcome. He stated that a bilateral complete endarterectomy in circulatory arrest would be the mandatory treatment and a complete resolution in 90% of the patients could be expected.

Dr. Ogawa presented her findings regarding proliferation of cells isolated from patients with chronic thromboembolic pulmonary hypertension.

Workshop 3: Vascular metabolomics and proteomics—Where are the novel biomarkers for pulmonary vascular diseases?

Speakers: Dr. Rothman, Dr. Lavoie, Dr. Ahmad, Dr. Frazziano, Dr. Goncharova, Dr. Farrow

Dr. Rothman hypothesized that miRNAs may affect signaling pathways in PAH, better knowledge of miRNAs may therefore provide insights into disease pathology and the incomplete penetrance of BMPR2 mutations. They might further serve as biomarkers. Dr. Rothman et al. performed a preliminary study to identify differentially regulated miRNAs in blood samples taken during pulmonary catheterization. miR140-5p stratified PAH from other lung diseases without PAH and was downregulated in patients with iPAH and ScPAH. Dr. Rothman suggested SMURF1, SP1, and PDGFRα as potential targets and showed that transfection of pre miR140-5p prevented SMURF1 expression in hPASMC. Further investigation will include a longitudinal study of miRNA expression from diagnosis and disease treatment.

Dr. Lavoie hypothesized that BMPR2 mutations produce an imbalance in EC protein expression and/or activity that is integrally related to development of abnormalities in lung vascular function and structure in hereditary PAH. His lab performed a study aiming to identify dysregulated proteins in blood outgrowth EC of PAH patients with BMPR2 mutations as compared to healthy controls. They identified 22 dysregulated proteins out of a total of 416 proteins. He described that upregulated proteins, e.g., MCM7 or TCTP, activated cell proliferation, while downregulated proteins were involved in vasoconstriction, e.g., AMPK. Dr. Lavoie explained that TCTP is a tumor-associated protein inducing apoptosis-resistance and was markedly upregulated in lung biopsies of HPAH patients and in the Sugen normoxia rat model. They intend to use the Sugen model to further investigate the function of TCTP in PAH.

Dr. Ahmad pointed out that most animal studies of PAH were performed on male animals, despite a female prevalence of PAH in humans. His lab reached out to establish a rodent model of female PAH to determine gender differences in disease progression. Male and female rats underwent a left pneumonectomy, followed by MCT administration 7 days post-OP. Specimen from left and right ventricles were collected 10 days post-MCT and analyzed for gene expression by microarray. Dr. Ahmad's results suggest the successful establishment of a female PAH animal model, since female were more susceptible to PAH and developed more severe hemodynamic changes at an earlier time point. He showed that RV gene expression changed before

RV dysfunction occurred. The group detected a significant overlap of genes changed in RV and LV failure, and female animals exposed a more dramatic change in RV gene expression than male.

Dr. Frazziano talked about her studies on reactive oxygen species in the pressure overloaded right ventricle. She explained that NADPH oxidase (Nox) derived ROS. Nox2 was reported to be activated by Rac1 and cytosolic components, while Nox4 was constitutive active. Dr. Frazziano demonstrated that ROS (H_2O_2) increased in acute PAB. This was paralleled by an early upregulation of Nox4, while Nox2 was not changed, suggesting a function of Nox4 in acute PAB. Inhibition or absence of Nox2 prevented PAB-induced RV hypertrophy, indicating an involvement in the chronic phase of PAB.

Dr. Goncharova studied the function of mTORC2 in vascular SMC (PAVSMC) of IPAH. Her results indicated that mTORC2 is upregulated in IPAH PAVSMC and enhanced a glycolytic metabolism, resulting in higher ATP, increased proliferation, and survival. She further demonstrated that mTORC2 regulated mTORC1 signaling and Bim expression. Dual inhibition of mTORC1 and mTORC2 prevented proliferation and induced apoptosis in IPAH PAVSMC.

Dr. Farrow introduced the audience to the bronchopulmonary disease of preterm infants and emphasized that development of PAH would indicate a worse outcome. sGC and PDE5 regulated vasoconstriction by cGMP and were previously described to be involved in PPHN of term infants. Dr. Farrow explained that sGC and PDE5 were known to be redox and developmentally regulated. She hypothesized that these two proteins would be negatively impacted by preterm birth and exposure to hypoxia. Dr. Farrow provided evidence that sGC and cGMP are decreased, while PDE5 is increased in small PA using a hyperoxia-induced mouse model of BPD. The effect could be reversed by Sildenafil treatment.

Pulmonary arterial hypertension—Current concepts and future therapies

Speakers: Dr. El Kasmi, Dr. Wilkins, Dr. Archer

Dr. El Kasmi filled in for Dr. Stenmark. In his talk, he outlined the interdependence of inflammation, autoimmunity, and epigenetic changes in the progression of PAH. He hypothesized that adventitial fibroblasts would orchestrate initiation and perpetuation of vascular inflammation in PAH. He explained that these fibroblasts were known to expose a pro-inflammatory expression signature, which is retained even after isolation and in vitro culture. As further evidence, he provided data that conditioned medium from PAH fibroblast, induced markers of M2 activation in macrophages. Dr. El Kasmi showed that human and hypoxic calf PAH fibroblasts are enriched in CTBP-1, a redox sensor implicated in cell proliferation, migration, fibrosis, and inflammation. It is supposed to be part of a large chromatin-remodeling complex. Dr. Stenmark's lab performed RNA sequencing to elucidate targets of CTBP-1, which included genes associated with the "imprinted," activated phenotype of the PAH fibroblasts, e.g., HMOX1, PTGIS (down) or TGFβ1, and IGFBP4 (up). Dr. El Kasmi then went on to talk about the contribution of autoimmunity on the progression of PAH. He emphasized two proteins, LTBR and CCR7, interference with which influenced BALT development in experimental settings. Dr. El Kasmi further postulated that the pro-inflammatory phenotype of PAH fibroblasts would be the result of epigenetic changes. He showed that HDACs are constitutively activated in fibroblasts derived from PAH patients and that inhibition of these normalized CTBP-1 expression and attenuated the pro-inflammatory profile. Dr. El Kasmi described that a change in miRNAs equally contributed to the epigenetical changes. They identified a decrease in miR124, a miRNA involved in inflammation and cancer. Interestingly, HDAC inhibition upregulated miR124 and decreased CCL2/MCP-1 expression in PAH fibroblasts, again illustrating the interdependence of inflammation and epigenetic changes in PAH. Dr. El Kasmi summarized and pointed out that epigenetic as compared to genetic changes would be potentially drugable and need to be better understood.

Dr. Wilkins' lab used patient specimen of different origins (lung tissue, blood) to analyze, in an unbiased approach, differentially regulated, potential biomarkers. Potential hits were then analyzed in animal models or patient samples and validated on a patient collective for their predictive value. He described a reduction of miR150 in 145 PAH patients, a miRNA involved in the development of T, NK, and iNKT cells. Dr. Wilkins provided further data from mass spectrometry of PAH vasculature, elucidating an upregulation of chloride intracellular channel 1/4 (CLIC1/4), and periostin. He showed that CLIC4 was found in EC and plexiform lesions of PAH patients and in the MCT rat PH model. CLIC4-induced EC permeability and proliferation and stabilized Hif1α. According to Dr. Wilkins, monitoring of the metabolic activity might be another useful marker to evaluate the severity and course of PAH. He mentioned that FDG-PET would already be an established marker in oncology.

Dr. Archer summarized the meeting, focusing on the translational aspects of this first Keystone meeting of RVD and PVD. He closed the session by introducing the audience to defects in mitochondrial fusion and fission and the implications in PAH. He reported that mitochondrial fusion and fission was recently reported to be perturbed in small lung cancer. Mitochondrial fission was considered to be a checkpoint of mitosis, since mitochondria needed

to undergo fission to allow cytokinesis. DRP-1-induced mitochondrial fission and was itself inhibited by Mdivi-1. Dr. Archer showed that fissogenic DRP-1 and Fis-1 were upregulated, while fusogenic Mfn-2 was downregulated. Inhibition of DRP-1 by either Mdivi-1 expression or interference with siDRP-1 led to a G2M phase arrest and abolished SMC proliferation.

Dr. Hansmann then thanked the participants, speakers, organizers, and Keystone staff, emphasized the need for comprehensive evaluation and closed the meeting.

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