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Authors

George, M Patricia
Gladwin, Mark T
Graham, Brian B

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TRANSLATIONAL REVIEW

Exploring New Therapeutic Pathways in Pulmonary Hypertension
Metabolism, Proliferation, and Personalized Medicine

M. Patricia George[1], Mark T. Gladwin[2,3], and Brian B. Graham[4,5]

[1]Division of Pulmonary, Critical Care, and Sleep Medicine, Department of Medicine, National Jewish Health, Denver, Colorado; [2]Pittsburgh Heart, Lung, Blood and Vascular Medicine Institute, Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania; [3]Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Pittsburgh and UPMC, Pittsburgh, Pennsylvania; [4]Division of Pulmonary, Critical Care, Allergy, and Sleep Medicine, Department of Medicine, Zuckerberg San Francisco General Hospital, San Francisco, California; and [5]Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of California San Francisco, San Francisco, California

Abstract

In this review, we explore the main themes from the 62nd Annual Aspen Lung Conference (hypoxia, cellular metabolism, inflammatory pathways, aberrant proliferation, and personalized medicine) and highlight challenges and opportunities in the coming decade of pulmonary vascular disease.

Keywords: pulmonary hypertension; cellular proliferation; personalized medicine; hypoxia signaling

At the 62nd Thomas L. Petty Annual Aspen Lung Conference, pulmonary experts gathered at the sixth conference in this series devoted to the topic of pulmonary vascular disease. The meeting brought together experts from around the world to discuss and debate challenges and opportunities in the field of pulmonary vascular disease. This inaugural State-of-the-Field article summarizes the key themes in pulmonary hypertension (PH) and lays out opportunities in which we can make progress in this disease.

Since the first Aspen Lung Conference devoted to the pulmonary circulation in 1962, we have experienced incredible progress in the field of PH. The pulmonary arterial hypertension (PAH) epidemics resulting from the ubiquitous use of anorexigens accelerated a desperate call for treatments in this disease. The first treatment for PAH in 1981 was also the first heart–lung transplant, and the first medical therapy to improve survival was described in 1990 (1, 2). Over the next 30 years, scientific discovery has led to the expansion of available medications to treat this disease, with 14 U.S. Food and Drug Administration (FDA)-approved medications for PAH. More recently, strategies have evolved to treat patients with milder disease with multiple agents in combination, as the use of upfront combination therapies has been shown in multiple trials to delay disease progression (3, 4).

Treatments thus far exploit three mechanistic pathways of pulmonary vasodilation: the nitric oxide (NO) pathway, endothelin pathway, and prostacyclin pathway. In an early concept of this disease, it was postulated that, similar to Knudsen's two-hit hypothesis in cancer pathogenesis, PAH pathogenesis involved a compilation of risk factors, from underlying disease risk factors and genetic mutations to infections and toxicogenic insults that lead down a pathway of endothelial dysfunction and development of PAH (5).

Since the publication of this model, researchers are working to better

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Conference Summary from the 62nd Annual Thomas L. Petty Aspen Lung Conference.

Correspondence and requests for reprints should be addressed to M. Patricia George, M.D., National Jewish Health, 1400 Jackson Street, Denver, CO 80206. E-mail: georgem@njhealth.org.

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understand the mechanistic underpinnings of PAH, both within and beyond endothelial dysfunction, and in so doing explore novel therapeutic approaches to this disease. On a certain level, many have noticed parallels between the progress in PAH and that in the field of oncology. We now use upfront combination therapy, similar to upfront induction chemotherapy in various cancers, and the field is moving from nonspecific treatments toward more molecularly driven, mechanistic approaches. Importantly, new therapies have emerged that move us beyond vasodilation, vasoconstriction, and endothelial dysfunction, toward more complex signaling pathways that regulate hypoxic and metabolic signaling, proliferation, apoptosis, senescence, and inflammation. With the development of multiple, expensive, and potentially more toxic therapeutics, the necessity of developing a precision approach to disease phenotyping and rationale targeting of highly effective interventions becomes paramount.

With remarkable success, the field of pulmonary vascular biology has identified a cornucopia of important signaling pathways that seem to contribute to, and, if targeted, modify, PAH pathogenesis. The discussant, Dr. Mark Gladwin, highlighted these many pathways that increase reactive oxygen species (ROS) production and stability: loss of superoxide dismutase, increased expression and activity of Nox2 or 4, xanthine oxidase (XO); reduce NO (endothelial nitric oxide synthase uncoupling and arginase expression); inflammation (NFAT, IL-2, TNFα, IL1β, and activated macrophages and complement); prolyl hydroxylase 2-HIF2α (hypoxia-inducible factor 2α) activation; endothelial apoptosis with vascular drop-out and pruning; metabolic changes involving glycolytic shifts, fatty acid oxidation, and anaplerosis involving right heart cardiomyocytes and vascular smooth muscle; effects of age, dysregulated proteostasis, autophagy and mitophagy; shear stress and vascular stiffness with matrix deposition, and activation of MMP-8, TSC2-Yap-TAZ, calcification-RUNX2; reduced levels of BMP9 (bone morphogenic protein 9) and BMPR2 (bone morphogenic protein receptor 2) with enhanced TGFβ (transforming growth factor β) signaling; insulin resistance and lipotoxicity; and major changes in transcriptional networks and epigenetic reprogramming. The discussant highlighted the array of clinical trials that seek to target these pathways (Table 1) and asked the audience to conduct a thought experiment, imagining they were on an investment advisory board, on which interventions would be most likely to change clinical outcomes in a meaningful way for patients on the current background therapies.

Table 1. You Are Serving on an Advisory Board in Pulmonary Hypertension Future Therapeutics: Which Pathway Would You Invest In?

A. Inhibit PHD2
B. Inhibit mTORC
C. Inhibit HIF-1α
D. Activate FoxO1
E. Activate AMPK
F. Inhibit arginase, glutaminase (anaplerosis)
G. Inhibit PDK (glycolysis)
H. Inhibit CPT1A (Fatty acid oxidation)
I. Inhibit PlGF (angiogenesis tip and breach cells)
J. Improve insulin sensitivity or activate ketogenesis (e.g., metformin, pioglitazone, and SGLT-2)
K. Inhibit TGFβ, activin, GDF signaling (sotatercept)
L. Increase BMPR2 function (e.g., FK506, Elafin, and Ataluren [PTC124])
M. Inhibit interstitial inflammatory macrophages (e.g., IL-6, IL-1b, and TNF)
N. Inhibit BRD (epigenetic reader) with RVX-208
O. Infuse extracellular vesicles from mesenchymal stem cells (MSC-EV)

Definition of abbreviations: AMPK = AMP-activated protein kinase; BMPR2 = bone morphogenetic protein receptor type 2; BRD = bromodomain-containing proteins; CPT1A = carnitine palmitoyltransferase 1A; FK506 = Tacrolimus; FoxO1 = forkhead box O1; GDF = growth differentiation factor; HIF = hypoxia inducible factor; mTORC = mammalian target of rapamycin complex; PDK = pyruvate dehydrogenase kinase; PHD2 = prolyl hydroxylase domain; PlGF = placental growth factor; PTC124 = Ataluren; SGLT-2 = sodium glucose co-transporter 2; TGFβ = transforming growth factor β.

The Aspen Lung Conference intentionally brought together experts in the fields of oncology and pulmonary vascular disease to explore common perspectives and mechanisms in these diseases. In this State-of-the-Field review, we explore the main themes from the conference—hypoxia, cellular metabolism, inflammatory pathways, aberrant proliferation, and personalized medicine—and highlight challenges and opportunities in the coming decade of pulmonary vascular disease (Figure 1).

Central Theme: Hypoxia—Larissa Shimoda, Ph.D., and Celeste Simon, Ph.D.

State-of-the-Art Speakers Dr. Larissa Shimoda and Dr. Celeste Simon discussed the roles of hypoxia signaling in pulmonary hypertension and malignancy, respectively. Hypoxia signaling is a foundational mechanism present in many etiologies of human PH—predominantly in World Health Organization (WHO) Group 3 disease but also present in WHO Group 1 disease—and underlies several experimental models of pulmonary hypertension such as hypoxia-treated rodents. The quintessential prolyl hydroxylase domain (PHD)–von Hippel-Lindau (VHL)–HIF1 pathway was discussed in the context of both PH and cancer. Dr. Shimoda discussed the evidence that hypoxia signaling is relevant in the etiology of PH and proposed a unifying theory for how hypoxia signaling may contribute to PH pathogenesis, whereas Dr. Simon discussed the role of hypoxia signaling in cancer and how it relates to aberrant metabolism.

VHL syndrome is a dominantly inherited familial cancer syndrome predisposing to a variety of malignant and benign tumors, including renal clear cell carcinoma, caused by a germline mutation in the VHL gene (6). The VHL protein is a component of a complex that includes elongin B, elongin C, and cullin-2, which is involved in the ubiquitination and degradation of the transcription factor family of HIF proteins (6). When sufficient oxygen is present, the PHD family of enzymes uses oxygen and α-ketoglutarate to hydroxylate HIF protein proline residues. The VHL protein complex detects these hydroxylated prolines and ubiquitinates the HIF protein: the ubiquitination marks result in proteosome-mediated protein degradation (7). In summary, HIF proteins are constitutively synthesized, and the PHD–VHL–HIF system regulates the rate of degradation. This system maintains a low level of HIF protein in the cell in settings of normoxia, but in hypoxia the

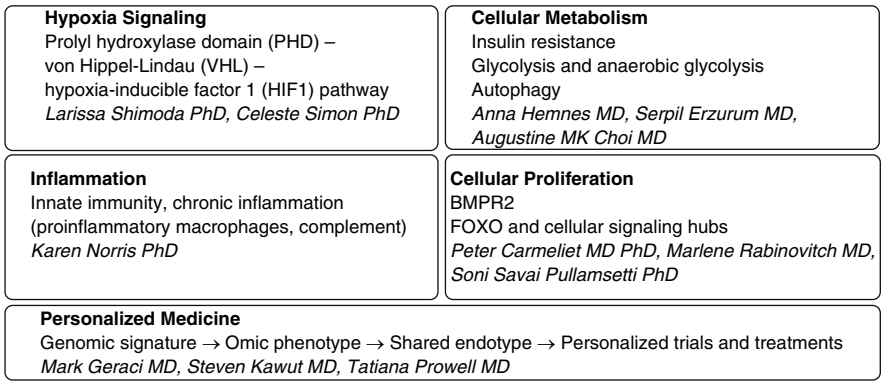

Figure 1. These were the main themes in the field of pulmonary hypertension discussed in the 62nd Thomas L. Petty Aspen Lung Conference, "Exploring New Therapeutic Pathways in Pulmonary Hypertension." BMPR2 = bone morphogenetic protein receptor type 2; FOXO = forkhead box O.

PHD–VHL system activity decreases, resulting in HIF protein accumulation on the timeframe of hours (Figure 2A). HIF proteins function as dimers, with an α and a β chain. There are three α-isoforms (HIF1α, HIF2α and HIF3α) and one shared β subtype (HIFβ, also called ARNT). In the cell nucleus, HIFα-HIFβ dimers bind hypoxia-responsive element (HRE) sequences in gene promoters and positively regulate gene transcription (8).

In humans, there is evidence of increased HIF1α expression in PH lesions and cells from patients with PH, although other authors have reported decreased expression (9, 10). Exposing pulmonary artery endothelial cells to hypoxia causes an increase in HIF1α expression (9, 11). Whole-body HIF1α heterozygous expression in mice resulted in protection from hypoxia-induced pulmonary hypertension, and as did HIF1α deletion in smooth muscle cells (9, 11). HIF2α heterozygous mice are also protected from pulmonary hypertension, and several groups have more recently shown that HIF2α deletion in endothelial cells is protective against hypoxia-induced PH (12–14).

Linking the HIF pathway with WHO Group 3 PH more clearly, there are adaptive genetic mutations that have occurred in three disparate populations living at high elevation: Tibet, Ethiopia, and the Andes. All three populations have an increased rate of mutations in the PHD–VHL–HIF pathway, including in the genes HIF2α, HIFβ, prolyl-4-hydroxylase 2 (PHD2), and PHD3 and several key targets of HIF including VEGFβ (vascular endothelial growth factor β) and the 2 endothelin receptors (15). As a maladaptive mutation, in the Chuvash region, there is a VHL mutation that limits binding and ubiquitination of HIF2α, resulting in HIF2α gain of function, causing pathologic polycythemia and increased pulmonary hypertension incidence. This human disease is phenocopied in VHL-deficient mice and mice lacking PHD2 in endothelial cells (15).

There are several potential downstream mechanisms by which HIF signaling may trigger PH vascular pathology. The HIF proteins have hundreds of targets, and a large number of them have been implicated in PH, including Kv channels; Na–H exchange transporters; the TRPC1 (transient receptor potential cation channel subfamily C member 1) calcium channel; aquaporin-1; DRP-1 (dynamin-related protein 1), which causes mitochondrial fission; multiple proteins in the glycolytic pathway; multiple growth factors and other cytokines including SDF1α and endothelin-1; and arginase 2, which decreases NO availability (7). Together, these targets can result in diverse pathologic phenotypes including cellular contraction, proliferation, migration, changes in phenotype (such as endothelial–mesenchymal transformation), and immune cell recruitment and activation. Dr. Shimoda proposed a general, unifying theory on the role of HIF in PH, which incorporates many of these pathologic signaling pathways and phenotypes (Figure 2B). In this model, endothelial cells, smooth muscle cells, and fibroblasts all have increased HIF signaling resulting directly from hypoxia, and indirect HIF-triggered paracrine signaling, resulting in pathologic pro-PH cellular phenotypes (8).

HIF signaling in PH is an attractive potential therapeutic target, and there are several compounds available or in different stages of development. Digoxin and acraflavine cause HIF inhibition and partially prevent hypoxic PH (16). Two compounds that may be specific for HIF2α, C76 and PT2567, prevent PH in animal models (13, 17). Critically, however, PT2567 is not effective when the treatment is delayed. The aggregate of these data raises questions regarding which HIF isoform to target, at which time point, and in which cell types, if it should be done directly or indirectly, and if there may be sex-dependent phenotypes.

The discussant considered two areas of relevance to current HIF-oriented research. One area that has not been significantly explored is the chemical biology that regulates the inhibition of the prolyl hydroxylase that is vital to oxygen sensing and hydroxylation of the HIFs. This enzyme—for example, the HIF PHD2—is a non-heme ferrous iron–containing, 2-oxoglutarate–dependent dioxygenase that regulates HIF by hydroxylating two conserved prolyl residues in N-terminal oxygen degradation domain and C-terminal oxygen degradation domains of HIF-1α. Although it is inhibited when there is insufficient oxygen bound to the enzyme, insufficient iron, or insufficient α-ketoglutarate, it is also likely that the iron center can be oxidized by ROS and that the enzyme can be further modulated by posttranslational signaling or changes in expression driven by transcriptional and epigenetic events.

The second area involves the debate around the most dominant prolyl hydroxylase and HIF (1 or 2) and the cell type (endothelium or smooth muscle or bone marrow–derived cells) that modulate pulmonary vascular remodeling in PAH. Recent data using endothelial and smooth muscle–specific knockout of both HIF1 and HIF2α suggest that endothelial PHD2 and HIF2α have a major effect on the development of PH in mouse models, with significant additional contribution from bone marrow–derived cells (18). A clearer understanding of the relative contributions of these isoforms and cell types is likely necessary to move to new specific inhibitors of PHD2 that are in development.

Dr. Simon discussed how hypoxia signaling plays a key role in cancer. Many solid tumors are limited in their growth extent by their blood supply, and hypoxia commonly occurs in the center of tumors, resulting in pO2 levels $<$10 mm Hg, at which HIF activation likely occurs. The

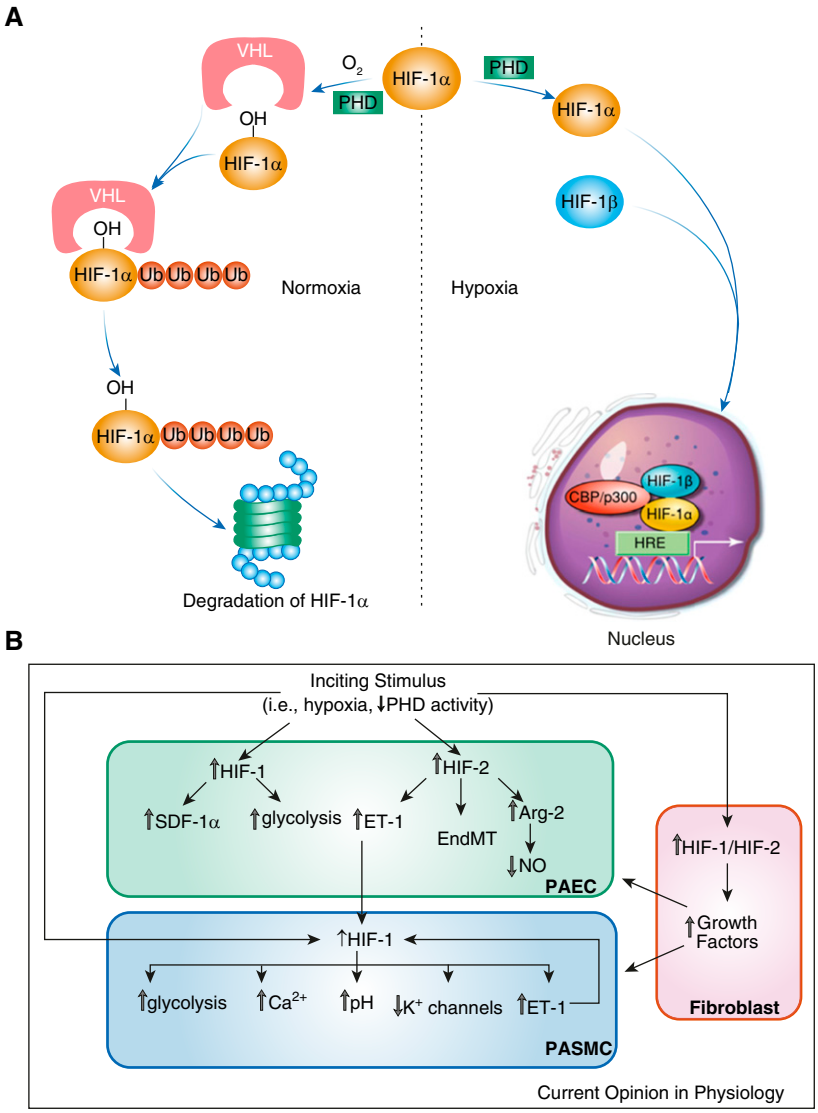

Figure 2. (*A*) In a normoxic environment, HIF-1α interacts with PHD and is ubiquitinated and targeted for degradation. In a hypoxic environment, HIF-1α and HIF-1β target to the nucleus, recruit coactivators CBP and p300, and lead to transcription of an HRE domain. CBP = CREB-binding protein; HIF = hypoxia inducible factor; HRE = hypoxia response element; p300 = EP300 or E1A binding protein; PHD = prolyl hydroxylase domain; Ub = ubiquitous; VHL = Von Hippel Lindau protein. Reprinted from Reference 7. (*B*) Increased HIF-1 levels in PAEC leads to increased glycolysis and SDF-1a, which recruits immune cells. Increased HIF-2 levels increase ET-1 production, promote endothelial-to-mesenchymal transition (EndMT), and lead to increased Arg 2, leading to reduced NO production. ET-1 then stimulates PASMC in a paracrine manner to produce HIF-1 and leads to mitochondrial metabolic changes, vasoconstriction, and proproliferative effects. Increased HIF-1 and HIF-2 in fibroblasts can lead to growth factors that act on both PAECs and PASMCs. Arg 2 = arginase 2; ET-1 = endothelin-1; NO = nitric oxide; PAEC = pulmonary artery endothelial cell; PASMC = pulmonary artery smooth muscle cell; SDF-1α = stromal-derived factor 1α. Reprinted by permission from Reference 8.

diffusion distance of oxygen in tissue is thought to be ~150 μm (19, 20). Furthermore, lower oxygen levels are associated with worse outcomes in patients, although by unclear mechanisms (19). Oxygen is critical for many fundamental biologic processes, such as the unsaturation of fatty acids required in fatty acid anabolism. However, HIF signaling itself is critically important.

Renal clear cell carcinoma is characterized by increased HIF activity (often by VHL loss-of-function mutations as noted above) and significantly increased fatty acid production, packing the cytosol and giving rise to the "clear cell" feature. Transcriptional profiling of the cancer cells versus normal kidney cells identified significant increases in the expression of fatty acid metabolism genes, whereas there were significant decreases in the genes that promote gluconeogenesis. One of the enzymes in particular is fructose bisphosphate (FBP1), which catalyzes the metabolism of fructose-1, 6-bisphosphate to fructose-6-phosphate, a necessary step in generating glucose from noncarbohydrate sources. Interestingly, a nonmetabolic function of FBP1 was identified: it can migrate to the nucleus where it serves as a binding partner and negative regulator of HIF1α (21). In these cells in which FBP1 expression was decreased, there was thus an increase in HIF1α activity and an increase in glycolysis, that is, glucose metabolism in the opposite direction from gluconeogenesis.

Central Theme: Cellular Metabolism and Inflammation—Anna Hemnes, M.D., Serpil Erzurum, M.D., Augustine M. K. Choi, M.D., and Karen Norris, Ph.D.

Cellular Metabolism

According to the WHO, the worldwide prevalence of overweight and obesity is projected to be greater than 3.3 billion by the year 2035, with the prevalence of diabetes reaching over 500 million (WHO website). These data remind us that a modern epidemic of noncommunicable cardiovascular disease threatens the developing world. The impact of insulin resistance in obesity and metabolic syndrome on the lung have been the topic of much research in lung diseases, and there certainly seems to be a pathogenetic role of this in pulmonary vascular disease as well (22, 23).

Insulin resistance, characterized by elevated triglyceride to calculated high-density lipoprotein ratio, elevated hemoglobin A1c, and impaired oral glucose tolerance testing, is common in PAH (24–26). Because of the association of insulin resistance with PAH, researchers have looked at the potential impact of this state on the PAH vasculature and right ventricle and have suggested that this may be an adjunctive therapeutic target in patients with PAH.

To understand the impact of metabolism, it is important to revisit

metabolic pathways in healthy individuals. Energy production and metabolism are centered around four basic pathways: aerobic glycolysis and oxidative phosphorylation, anaerobic glycolysis, fatty acid oxidation, and glutaminolysis (driving anapleurosis). There is another proproliferative pathway that has been described in both cancer biology and pulmonary hypertension, namely, aerobic glycolysis, otherwise known as the "Warburg effect" (27) (Figure 3A). It is hypothesized that although this pathway may be inefficient in terms of ATP production, 4 mol ATP per 1 mol glucose compared with 36 mol ATP in oxidative phosphorylation, this pathway and glutaminolysis are useful in producing nicotinamide adenine dinucleotide phosphate and acetyl-coA for fatty acid and amino acid synthesis to produce cellular biomass in proliferating cells (27–29).

Dr. Anna Hemnes discussed ways in which patients with insulin resistance demonstrate changes in the pulmonary vasculature as well as right ventricle (RV). In both compartments, there are altered cellular energetics with increased glycolysis and decreased fatty acid oxidation and glucose oxidation. In the RV, interestingly, there was a sevenfold higher lipid content in patients with PAH versus controls, measured by human proton magnetic resonance spectroscopy (30). Supporting this, Dr. Karen Norris shared data from her nonhuman primary model of human immunodeficiency virus (HIV)-PAH; macaques with PAH have increased glucose uptake in their right ventricle compared with non-PAH controls (31). Dr. Hemnes discussed ways in which treating metabolic syndrome may positively affect pulmonary vascular disease. Reversal of metabolic syndrome in a patient with PAH through gastric bypass surgery was associated with significantly improved hemodynamics and cardiac function (32). Metformin inhibits fatty acid oxidation, has been shown to reduce RV myocyte lipid deposition, and is currently being further studied in a randomized controlled trial (ClinicalTrials.gov NCT03617458) (33). Additional inhibitors of fatty acid oxidation, ranolazine, and trimetazidine are also under study in patients with PAH (e.g., ClinicalTrails.gov NCT01174173 and NCT03273387). Dr. Gladwin shared data from a two-hit animal model using vascular endothelial growth factor receptor blocker SU5416 in a rat with a double-leptin receptor defect (ZSF1) to model obesity-related PH associated with heart failure with preserved ejection fraction, highlighting a potential role for metformin plus oral nitrite in the treatment of this disease (34). Metformin and nitrite reversed pulmonary pressures and vascular remodeling when dosed at the time of SU5416, and also improved insulin resistance mediated through the activation of SIRT3 and AMP-activated protein kinase (34). This same effect was not seen in the standard SU5416-hypoxia rat model of PAH in Sprague-Dawley rats, suggesting that this could be a potential treatment for those with metabolic syndrome (35).

Exploring metabolism beyond insulin resistance led into mitochondrial energetics. Dr. Serpil Erzurum discussed the seminal observations in the early 1990s, showing that NO levels were lower in cells from the explanted lungs of patients with PAH, and how this aberrancy was linked to metabolic pathways in the endothelial cell (36). To make NO, two domains on endothelial NO synthase must be phyosphorylated to produce NO; however, in PAH, this phosphorylation is deficient. In large part, this is associated with increased mitochondrial arginase 2 activity in patients with PAH, leading to ornithine, and downstream to α-ketoglutarate, ultimately feeding into the Krebs cycle, a process known as anaplerosis, which supports cellular proliferation during the aerobic glycolytic shift (37). Interestingly, dysfunction of the arginine–NO pathway in PAH through metabolism of arginine to ornithine by arginase can then feed into anaplerosis pathways and drive cellular proliferation, as ornithine is metabolized to glutamate. Further studies in pulmonary endothelial cells from patients with idiopathic PAH tied an increase in HIF-1α levels to decreased NO production and downregulation of manganese superoxide dismutase (9). Given the central role of mitochondrial metabolism and dysfunction to the development of PAH, Dr. Erzurum and colleagues explored whether there were certain mitochondrial DNA haplotypes that were associated with greater risk of PAH, and they found that mitochondrial haplogroup L was protective against PAH, whereas haplogroups N and M were associated with increased risk of PAH (38).

Cells, in addition to using metabolic signaling pathways to best support cellular proliferation and existence in a low-oxygen environment, also use cellular housekeeping processes to support optimal usage and recycling of damaged or unused cellular materials in a process called autophagy. Dr. Augustine Choi discussed that autophagy is being studied in many diseases, from neurodegenerative disease and aging to pulmonary disease and oncology (39) (Figure 3B). In cancer, autophagy can be both tumor suppressive and tumor promoting, depending on the context (39, 40). In healthy cells, autophagy helps remove damaged proteins and unneeded organelles, reducing oxidative stress in the cell, and thus may play a tumor-suppressive role. Monoallelic mutations in the gene encoding the autophagy protein, Beclin-1, have been found in breast, prostate, and ovarian cancers, suggesting loss of a tumor-suppressive effect of autophagy (39). However, autophagy is also found to be upregulated in tumor cells, as it suppresses the induction of p53 tumor suppressor protein and helps maintain functional mitochondria to survive the nutrient-depleted tumor microenvironment and cellular stress and support ongoing proliferation (41).

In pulmonary hypertension, it is not yet clear whether autophagy has an adaptive or maladaptive role, and perhaps, similar to the role of autophagy in cancer, it will also be context dependent (40, 42). PH pathogenesis is associated with increased cellular proliferation and apoptosis (43). Autophagy serves as a cellular survival mechanism and inhibits apoptosis and necrosis (43). This autophagy–apoptosis cross-talk in endothelial cells and smooth muscle cells may play an important role in pulmonary vascular remodeling in pulmonary hypertension (43). Chen and colleagues describe a model in which there may be different phases of autophagy, whereby early in response to an environmental stressor (e.g., inflammation, hypoxia, drug/toxin, shear stress, genetic mutation, ROS), autophagy may be controlled and protective in endothelial cells, whereas when it is excessive, it leads to apoptosis of these same cells. Later, it may become more of a proproliferative signal in endothelial cells. Similarly, it may also have a prosurvival/proproliferative effect on smooth muscle cells. One of the challenges is that there is no easy way to measure the level of autophagy *in vivo* in human patients, making it challenging to intervene at the right time in this process (42).

Inflammation

Because metabolism is so closely linked to inflammatory pathways, Dr. Marlene Rabinovitch has called for us "to consider immunity a unifying hypothesis explaining this disease" (44) (Figure 4). There is now a

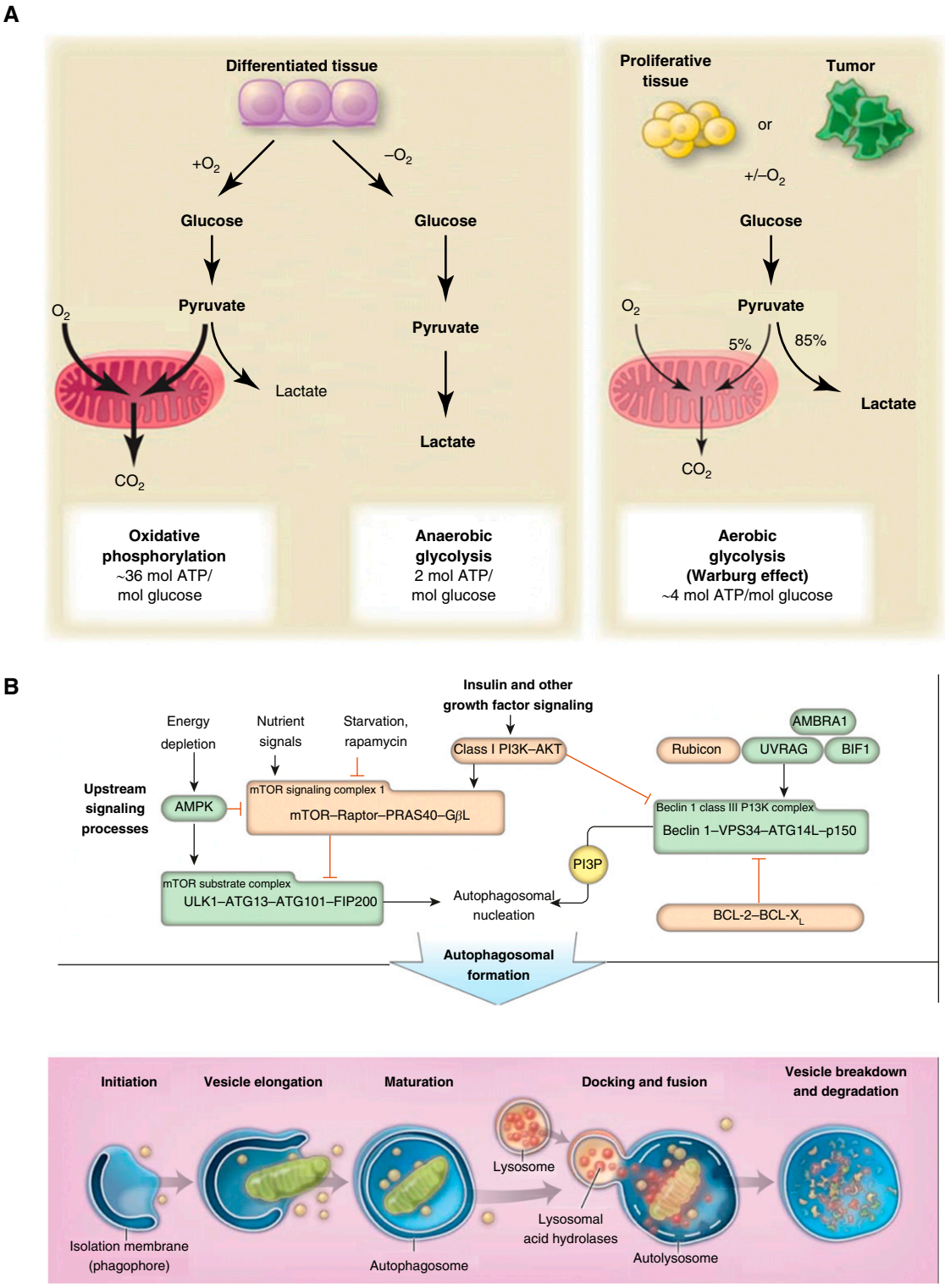

Figure 3. (*A*) In normal cells in the presence of oxygen, the predominant metabolism is that of oxidative phosphorylation, to yield 36 mol ATP per mol glucose. In a hypoxic environment, the cell shifts to anaerobic glycolysis, yielding 2 mol ATP per mol glucose. Proliferative cells (e.g., cancer cells) have an ability to undergo aerobic glycolysis, whereby they generate 4 mol ATP per mol glucose, but additional products (acetyl coenzyme A and nicotinamide adenine dinucleotide phosphate) are needed to generate biomass in proliferating cells. Reprinted by permission from Reference 27. (*B*) In the setting of energy, nutrient, and metabolic signals, the mTOR substrate complex and Beclin class III PI3K complex lead to formation of the autophagosome. Reprinted by permission from Reference 39.

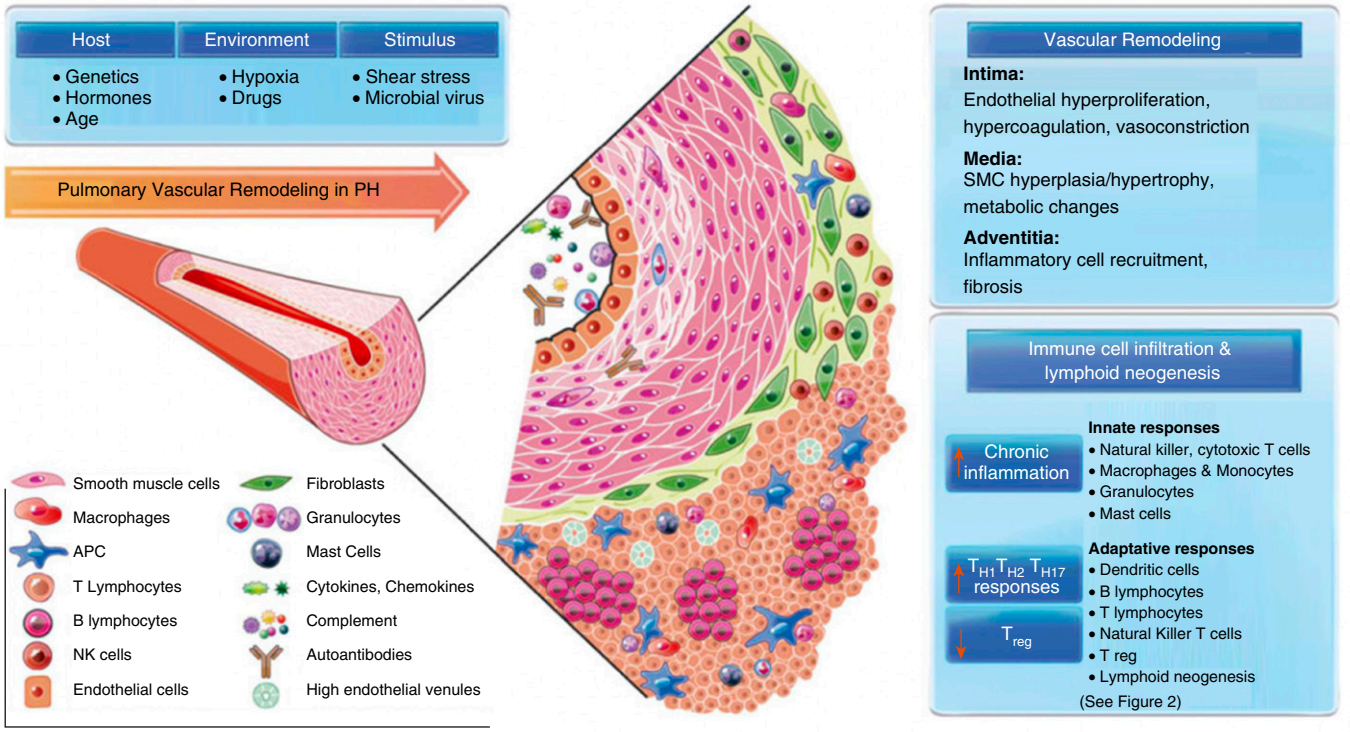

Figure 4. In the appropriate host, with appropriate stimuli (e.g., hypoxia, drugs, shear stress, or virus), pulmonary vascular remodeling occurs in the setting of immune cell infiltration and tertiary lymphoid follicle formation. Immunologic effects include cell-mediated and innate immune responses, contributing to pulmonary vascular remodeling. NK = natural killer; PH = pulmonary hypertension; SMC = smooth muscle cell. Reprinted by permission from Reference 44.

growing body of literature describing roles of innate and adaptive immunity in PAH pathogenesis. Tertiary lymphoid follicle formation reported in explanted lungs from patients with idiopathic PAH supports a role for adaptive immunity in PAH (45). At Aspen, the speakers delved into the role of the innate immunity via monocyte and macrophage-mediated inflammation in pulmonary vascular disease.

BMPR2 signaling prevents recruitment of inflammatory cells by suppressing GM-CSF (granulocyte macrophage colony-stimulating factor) expression, whereas human lung tissue from patients with PAH shows increased GM-CSF staining around pulmonary arteries and in plexiform lesions (46). Interestingly, GM-CSF increases macrophage recruitment and enhances pulmonary hypertension in hypoxic mice, whereas blockade of GM-CSF prevents macrophage recruitment and pulmonary hypertension in this model (46). Upstream from GM-CSF, dysregulated complement signaling may play an important role in PAH: in humans and in the hypoxia mouse model of PAH, hypoxia activates the alternative complement pathway, and increased complement staining is seen around arteries of those with pulmonary vascular disease. Dr. Kurt Stenmark has proposed that hypoxia or other injury to the blood vessel may expose neo-epitopes that are recognized by antibodies that in turn trigger the alternative complement pathway. This in turn leads to increased GM-CSF expression and the recruitment of monocytes and macrophages, leading to pulmonary vascular remodeling (47).

Dr. Norris described a nonhuman primary model of HIV-PAH using simian immunodeficiency virus (SIV), in which 50% of SIV-infected macaques will develop PAH, and the PAH animals have increased frequencies of proinflammatory, nonclassical monocytes as well as increased frequencies of proinflammatory, nonclassical macrophages in the BAL fluid (48). Increased frequencies of these monocyte and macrophage phenotypes were associated with worse PH severity, and the PH animals had greater frequencies of tissue-resident proinflammatory M1-like and M2a-like macrophages and lower frequencies of antiinflammatory M2c-like macrophages (48). Treatments with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), starting either at the time of SIV infection or at 4 months after infection, reduced the proinflammatory M1-like and profibrotic M2a-like macrophages, without altering the antiinflammatory M2c macrophage frequencies, and decreased the development of PH in this model (49).

Central Theme: Aberrant Proliferation—Peter Carmeliet, M.D., Ph.D., Marlene Rabinovitch, M.D., Soni Savai Pullamsetti, Ph.D.

Excessive cellular proliferation is one of the hallmarks of both cancer and pulmonary hypertension. Dr. Sèbastien Dumas from the laboratory of Dr. Peter Carmeliet in Leuven, Belgium, started by looking on the role of fatty acid metabolism in endothelial cell proliferation. One of the regulators of fatty acid oxidation is carnitine

phosphyltransferase 1α, which is required for long-chain fatty acid transport across the mitochondrial membrane—a rate-limiting step in fatty acid metabolism. β-oxidation of long-chain fatty acids generates acetyl coenzyme A, which enters the trichloroacetic acid cycle: this is necessary for both angiogenesis and lymphangiogenesis (50, 51). The Carmeliet laboratory has discovered that knocking out carnitine phosphyltransferase 1α in endothelial cells is therapeutic against the excessive angiogenesis that occurs in macular degeneration, whereas angiogenesis is rescued by replacement of trichloroacetic acid cycle intermediates, such as by acetate supplementation, which leads to acetyl coenzyme A (50). He discussed how metabolomics can be studied in a forward direction starting with genetic abnormalities and moving forward to phenotype, or a reverse direction by starting with the phenotype and then identifying the underlying metabolic abnormalities. His group is actively investigating how the endothelial cell metabolic phenotype can change during angiogenesis, and the heterogeneity in different endothelial cell subpopulations.

Dr. Rabinovitch discussed the many roles of BMPR2 signaling in maintenance of vascular homeostasis relevant to PH. These include the targets PPARγ/Wnt and apelin, which promotes endothelial regeneration (52). Of note, Dr. Majka also described a mechanism of Wnt signaling in the vascular homeostasis (53). Dr. Rabinovitch described the role of BMPR2 in endothelial–mesenchymal transition via another BMPR2 target, Slug, which functions as a transforming factor (54). Functionally, BMPR2 knockout in smooth muscle cells caused persistent PH when the hypoxic stimulus was removed. BMPR2 also blocked the recruitment of inflammatory cells, with BMPR2 deficiency causing an upregulation of GM-CSF and monocyte recruitment (46). Consistent with this concept, GM-CSF worsened hypoxic PH, whereas anti-GM-CSF abrogated hypoxic PH associated with similar changes in macrophage density. Lastly, Dr. Rabinovitch described proteins from old viral fragments embedded into the human genome, termed an endogenous retrovirus, that induces inflammation, which can promote PH (55).

Related to the central role of BMP9-BMPR2 signaling in PAH pathogenesis, the discussant Dr. Gladwin highlighted recent related work suggesting a hypothesis that a balance between reduced BMP9-BMPR2 signaling in PAH results in unopposed upregulation of TGFβ and activin signaling that drive myogenic and fibrogenic differentiation. Loss-of-function BMP signaling pathway mutations have been documented in heritable PAH and its vascular overlap syndromes that affect genes integral to BMP signaling in the endothelium, including the BMP type 2 receptor BMPR2, the type I receptor ALK1, Endoglin, co-SMAD4, SMAD9, and the cognate ligand for this receptor complex itself, BMP9 (56). The involvement of loss-of-function mutations impacting BMP ligands and effectors as opposed to activin, GDF, and TGFβ has led to a hypothesis that unopposed TGFβ, SMAD2/3 signaling, in the face of insufficient BMP signaling, may drive PAH (57). This hypothesis has now been tested in preclinical models and in a new large phase 2 study of ACTRIIA-Fc (Sotatercept), which is a soluble activin type II receptor extracellular domain expressed as an immunoglobulin-Fc fusion protein, serving as a selective activin/GDF ligand trap (58). Preliminary data have been released to the news media suggesting positive effects of this antibody on clinical outcomes in patients with PAH.

Dr. Gladwin also highlighted fascinating new developments in the field suggesting that altered BMP9 and BMP10 signaling through both BMPR2 and Alk1 can drive the development of PAH, portopulmonary hypertension, hepatopulmonary syndrome, and hereditary hemorrhagic telangiectasia (HHT), seemingly divergent syndromes that can present with either pulmonary hypertension or pulmonary arteriovenous malformations (AVMs). In zebrafish models of HHT, either low levels of BMP10 derived from cardiomyocytes and carried to the vasculature in flowing blood or low levels of its receptor Alk1 (also reduced in human HHT) lead to the formation of AVMs. In mouse models and patients with PAH, low levels of BMP9 can phenocopy loss of its cognate receptor BMPR2 (57, 59). Loss-of-function mutations in GDF2 in heritable PAH cause low BMP9 production and signaling (56). Remarkably, reduced production of BMP9 from the liver in cirrhosis is associated with both portopulmonary hypertension and the hepatopulmonary syndrome, suggesting that alterations in ligand (BMP9, BMP10, or heterodimers) or receptors (BMPR2 or Alk1) can drive the phenotypes of pulmonary hypertension or pulmonary AVMs across divergent diseases (60, 61).

Dr. Soni Savai Pullamsetti discussed the role of transcription factors in PH and cancer. Indeed, there are a large number of signaling hubs, including transcription factors that are shared between PH and cancer pathology, including FOXO, platelet-derived growth factor (PDGF), and several others (62) (Figure 5). These shared signaling abnormalities likely contribute fundamentally to the shared pathologic phenotypes, including proliferation, migration, altered metabolism, inflammation, and apoptosis resistance. One example is the loss of PPARγ, which is observed in human PAH, contributes to vascular remodeling experimentally, and can be corrected by treatment with the PPARγ agonist pioglitazone to treat experimental pulmonary hypertension (63). Another example is the loss of FoxO1, which is also observed in human PAH. Its blockade contributes to experimental PH, and the correction of FoxO1 with paclitaxel or the FoxO1 target FoxM1 with thiostrepton can treat experimental disease (64, 65). Other transcription factors associated with both cancer and PH include p53 and NF-κB. The Pullamsetti group recently reported a more direct association between PH and cancer, observing an increased rate of PH among patients with lung cancer (by pulmonary artery diameter) and in animal models of lung cancer (66). Pathologically, there were shared features, including inflammation by cellular infiltrates and cytokines associated with both diseases, and blocking NFκB signaling suppressed both inflammation and the experimental PH.

Dr. Pullamsetti then discussed the possibility of therapeutically targeting transcription factors, an approach actively being studied in cancer. As noted, several of the compounds used in experimental studies such as pioglitazone are approved for other indications and could be repurposed. Investigations in cancer have already led to the approval of several compounds directly targeting transcription factors including ruxolitinib (targeting STAT proteins) and vorinostat (targeting histone deacetylases). Altered epigenetic histone acetylation is a widespread signature in PAH, and targeting

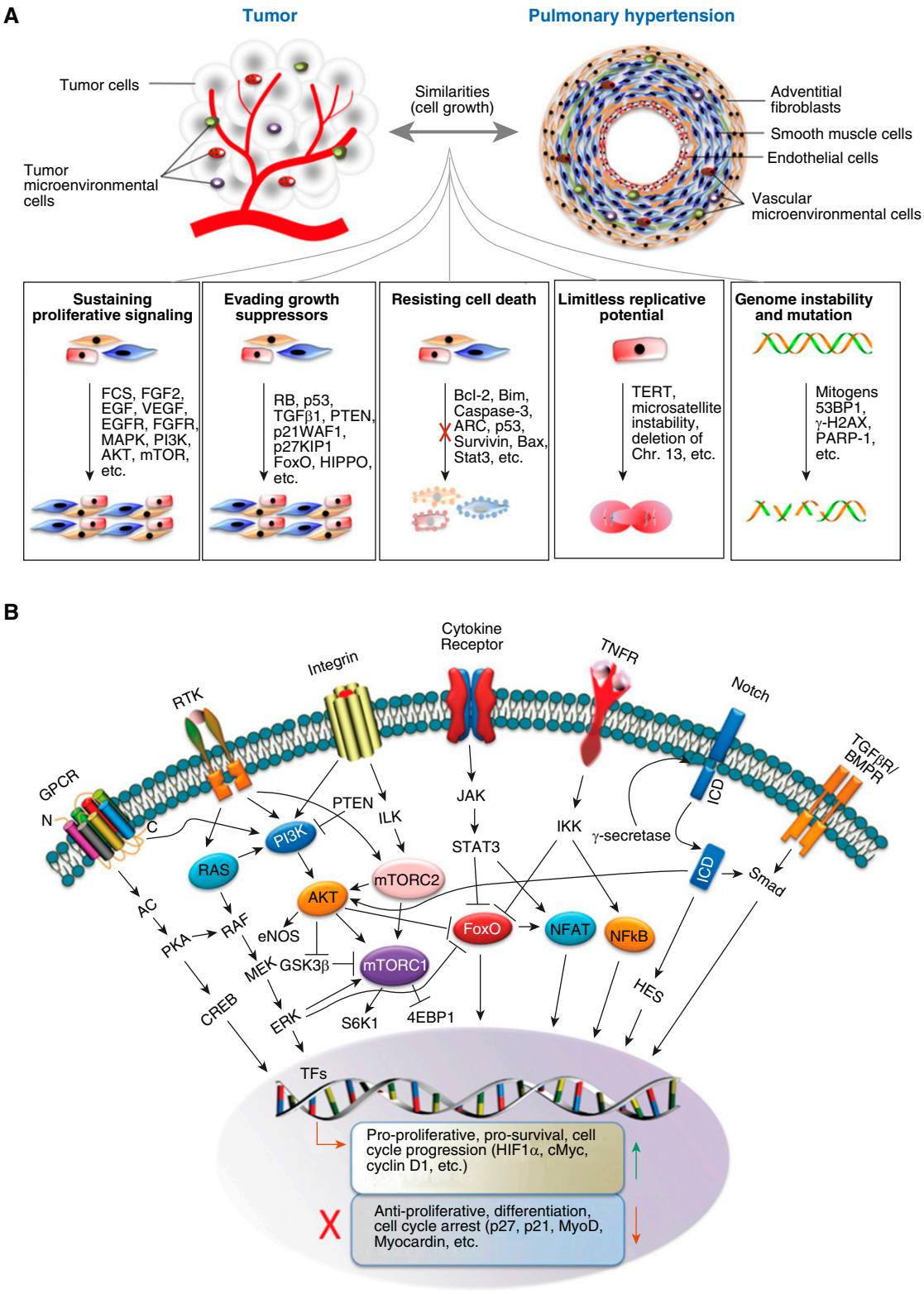

Figure 5. (*A*) There are shared pathways of cellular proliferation in tumorigenesis and pulmonary vascular disease, leading to sustained proliferative signaling, evasion of growth suppressors, apoptosis resistance, limitless replicative potential, and genome instability and mutation. 53BP1 = p53-binding protein 1; AKT = v-akt murine thymoma viral oncogene homolog; ARC = apoptosis repressor with caspase recruitment domain; Bax = Bcl-2–associated X protein; Bim = Bcl-2–interacting mediator of cell death; Chr. = chromosome; EGF = epidermal growth factor; EGFR = EGF receptor; FGF-2 = fibroblast growth factor 2; FGFR = FGF receptor; γ-H2AX = histone H2A variant H2AX phosphorylated at Ser-139; HIPPO = MAPK = mitogen-activated protein

the epigenetic reader BRD4, which binds acetylated lysine residues, has been effective in experimental PH models, using the compounds JQ1 and RVX208 (apabetalone) (67, 68). In the future, bioinformatic methods will be useful to identify which transcription factors are most likely to be successful, by defining interaction networks and hierarchies (69).

Central Theme: Personalized Medicine: Who Progresses and Why?—Mark Geraci, M.D., Steven Kawut, M.D., Tatiana Prowell, M.D.

The presentation of PAH in patients is heterogeneous, with some people responding to medication and others progressing despite aggressive therapy. In the sessions on personalized medicine, a framework for deep phenotyping as well as how to build clinical trials and personalized treatments was presented (Figure 6) (70).

Dr. Mark Geraci presented the 2010 National Heart, Lung, and Blood Institute Task Force proposed recommendations for directions needed in pulmonary vascular research, and among the list were the following recommendations: *1*) Encourage systems biology analysis to understand and define interaction between lung vascular genetics, epigenetics, metabolic pathways, and molecular signaling; *2*) Develop strategies using appropriate animal models to improve the understanding of the lung vasculature in health and in conditions that reflect human disease; and *3*) Improve lung vascular disease molecular and clinical phenotype coupling (71).

Since that call to action, researchers have been working to bridge from genes to phenotypes in PAH. One such bridge was the discovery that prostacyclin synthase promotor polymorphisms that are associated with increased transcriptional activity are overrepresented in BMPR2 mutant carriers that do not develop PAH (72). This protective association may help explain the incomplete penetrance of this autosomal dominant genetic mutation. A systems analysis of the PAH lung transcriptome, the largest to date, presents strong evidence for the estrogen receptor pathway, modulators of inflammation, innate immunity, and Wnt pathway processes. Many of the pathways identified were consistent with those that are currently known to play a role in PAH pathogenesis, but novel upstream regulators were also identified, allowing for new research investigations (72). Ongoing deep phenotyping research is made possible through the Pulmonary Hypertension Breakthrough Initiative and the Pulmonary Vascular Disease Phenomics Program (PVDOMICS), an eight-center clinical study creating a registry of 1,500 patients with PH (Group 1–5 disease), patients at risk for PH, and healthy controls. Blood samples are being run to look at genome, transcriptome, proteome, metabolome, coagulome, and cell biome. Combined with imaging, clinical chemistry, and hemodynamics, the goal is to describe different endophenotypes of PH (73).

When we discuss novel treatment targets in PAH and exploring them in clinical trials, it is important to consider trial design. Although we have made remarkable advances in treating PAH in the last 30 years, the approach to studying PAH in randomized controlled trials is largely unchanged. At Aspen Lung Conference, Dr. Steven Kawut proposed that research into new treatments for PAH should dovetail with research into new ways for studying therapeutics in PAH.

One area the pulmonary vascular disease field can learn from is the field of oncology and trials in breast cancer. Dr. Tatiana Prowell shared lessons from oncology drug development on the following topics: modernizing eligibility criteria, use of prognostic and predictive biomarkers, and development of novel (surrogate) endpoints. Many potential trial recipients have been traditionally excluded from oncology clinical trials (e.g., central nervous system involvement, HIV positivity, prior malignancy, and marginal performance status), and the result is greater health disparity and slow accrual to trials in patients who poorly represent those who will receive the drug in the postmarketing setting (74). Patients are excluded for many reasons, including potential to confound treatment effect, fear of drug–drug interaction (e.g., antiseizure or antiretroviral medications), concerns for patient safety and risk of jeopardizing a drug development program, outdated concerns (e.g., limited life expectancy in patients with treated HIV or brain metastases), and convention (e.g., exclusion of men from breast cancer trials). However, use of exclusion criteria limits the generalizability of results and may potentially exclude patients who benefit from therapy, leading to two joint statements from the American Society of Clinical Oncology and Friends of Cancer

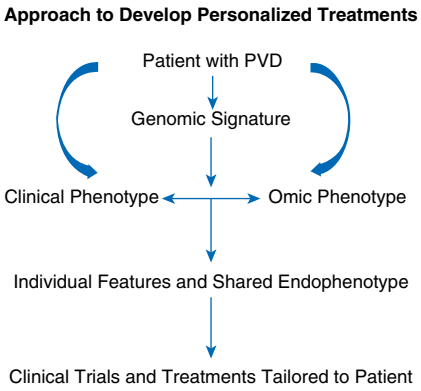

Figure 6. Approach to develop personalized treatments for patients with pulmonary vascular disease (PVD). Reprinted from Reference 70.

Figure 5. (*Continued*). kinase; p21WAF1 = cyclin-dependent kinase inhibitor 1A; p27KIP1 = cyclin-dependent kinase inhibitor 1B; p53 = tumor protein 53; PARP-1 = poly(ADP-ribose) polymerase 1; PTEN = phosphatase and tensin homolog; RB = retinoblastoma; Stat3 = signal transducer and activator of transcription 3; TERT = telomerase reverse transcriptase; TGF-β1 = transforming growth factor-β1; VEGF = vascular endothelial growth factor. (*B*) Molecular pathway cross-talk illustrates the challenge of interfering with signaling pathways at the receptor level and perhaps highlights the concept of targeting downstream cell signaling hubs and transcription factors that lead to proproliferative, prosurvival signaling. Reprinted from Reference 62. 4EBP1 = 4R-binding protein 1; AC = adenylyl cyclase; BMPR = bone morphogenetic protein receptor; CREB = cyclic AMP–responsive element binding protein; eNOS = endothelial nitric oxide synthase; ERK = extracellular signal–regulated kinase; GPCR = G protein–coupled receptor; GSK3β = GSK 3β; HES = hairy/enhancer of split; ICD = intracellular domain; ILK = integrin-linked kinase; JAK = Janus kinase; MEK = mitogen-activated protein kinase kinase; mTORC1 = mTOR complex 1; mTORC2 = mTOR complex 2; MyoD = myogenic differentiation 1; PKA = protein kinase A; RAF = rapidly accelerated fibrosarcoma; RAS = rat sarcoma; RTK = receptor tyrosine kinase; S6K1 = p70 S6 kinase 1; STAT3 = signal transducer and activator of transcription 3; TF = transcription factor; TGF-βR = TGF-β receptor; TNFR = TNF receptor.

Research calling for more inclusiveness in oncology clinical trials (75, 76). As we develop genetic and other biomarkers of disease in PAH, it will be important to study biomarker-negative patients, as the chosen biomarker may not always predict response and biomarker-negative patients may benefit from the trial drug. Choosing the appropriate surrogate endpoints also poses a challenge, as they may not end up being associated with the true endpoint of interest. In oncologic trials, a meta-analysis of clinical trials using pathological complete response showed that it was a poor surrogate for overall survival or event-free survival (77).

In pulmonary hypertension, Dr. Kawut suggested that novel treatment targets could focus more on clinical endpoints of symptoms, health-related quality of life, and physical activity, surrogate endpoints, understanding of treatment heterogeneity, and review of responder analyses. Dyspnea is almost universal in PAH and can be assessed with the Borg dyspnea scale (with a 0.9-unit change being significant), and PAH Symptoms and Impact questionnaire (PAH-SYMPACT) (78, 79). Dyspnea predicts quality of life (80). There are several quality-of-life tools that are used in PAH, including Cambridge Pulmonary Hypertension Outcome Review (CAMPHOR) and emPHasis-10 (81). Measuring activity with accelerometry is reliable, and average daily activity levels are negatively associated with energy (CAMPHOR) and both mental and physical fatigue (83). Surrogate endpoints are a lab measurement or physical sign that is a substitute for a clinically meaningful endpoint directly measuring how a patient feels, functions, or survives and is expected to predict the effect of therapy. Six-minute walk test and hemodynamics are actually poor surrogate endpoints (84, 85). There may be a role for using the multiparameter risk assessment calculators as surrogates, but more data are needed to know if they would be a true surrogate. Where the multiparameter risk assessment may help is in isolating those patients at highest risk who may be most likely to have a treatment effect in a study. Finally, some of our most recent and powerful data have come from composite endpoint studies (3, 4). Although these provide increased precision and power, and also allow for incorporating several endpoints that are meaningful to patients, caution must be exercised as weighting hard endpoints like death and hospitalization with soft endpoints like change in 6-minute walk distance or increase in medication may weaken inferences. There may be a dilution in effect (mortality in one arm might be diluted by increased hospitalization in another arm, which could result in a "null" finding).

Concluding Remarks and Thoughts from Our Conference Summarizer—Mark Gladwin, M.D.

To understand the complexity of this field, the organizers set up five areas of discussion: hypoxia signaling, cellular metabolism, inflammation, cell proliferation, and personalized medicine. However, after experiencing presentations from the State-of-the-Art speakers, we find that themes of altered cellular signaling (genetic/epigenetic pathways), metabolism, and inflammation were woven throughout all of the talks, suggesting multiple new opportunities for therapeutic interventions (Figure 7).

At the conclusion of the conference, Dr. Gladwin highlighted the challenges and opportunities in the field of pulmonary vascular biology. In an orphan disease with more than 14 FDA-approved medications in the past 30 years, in many ways we are victims of our own success, with many pathways and approved drugs but so few patients with this rare disease. We face challenges of slow enrollment into clinical trials as well as competing clinical trials, and as we learn more and get more specialized in a more nuanced understanding of endophenotypes, we generate even smaller groups of potentially eligible patients for trials. Within this context, new agents must work in the setting of background pharmacotherapy, and new drugs must be significant disease modifiers and reverse rather than just mitigate established disease. This is increasingly important as we face higher costs of specialty pharmaceuticals and a requirement for a value proposition that new drugs must drive substantive effects on patient-centered outcomes and survival. Furthermore, the pathways before us are complex, involving multiple cell types and spanning organ systems, with cross-talk between bone marrow, skeletal muscle, adipocytes, right ventricle, and pulmonary arterial cells. The current state of knowledge in autophagy exemplifies a context-specific and time-specific nature to whether it is adaptive or maladaptive in cancer and potentially PH pathogenesis. And even beyond the vascular focus of disease mitigation, we also continue to be challenged in understanding the effects on the right ventricle in the context of coupling to the pulmonary vasculature, and how drugs, hormones, metabolism, and environment modify the pulmonary vasculature versus the heart.

The many pathways highlighted in the conference emphasize ways in which we can look to advance our field beyond endothelial dysfunction and treatment with vasodilator therapy, toward using targeted therapies to address the underlying cellular and metabolic dysfunction and pulmonary vascular remodeling. One of the challenges as we discover more pathways involved in experimental and in human PAH is the overlap in these signaling pathways and other pathways, such that treating one cellular signaling pathway may not abrogate parallel proproliferative pathways. One way to address this is through understanding of major disease-modifying signaling hubs and potentially looking toward epigenetic signaling, as in cancer research, although this method is in its infancy. Indeed, targeting pathways that are important for maintaining normal system homeostasis

Figure 7. Rather than distinct categories, the themes of the 62nd Aspen Lung Conference of genetic/epigenetic signaling, cellular metabolism, and inflammation are intertwined and drive cellular proliferation.

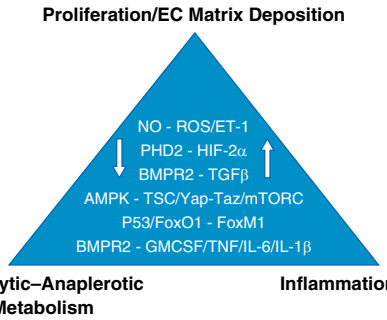

Figure 8. Three major pathways appear to dominate current thinking: cellular proliferation and extracellular matrix deposition, glycolytic–anaplerotic metabolism, and inflammation. Within this context, a number of pathways are dysregulated based on downregulation to upregulation: NO to ROS and ET1, PHD2 to HIF-2α; BMPR2 to TGFβ; AMPK to TSC,Yap-Taz,mTORC; P53 to FoxO1, FoxM1 and BMPR2 to GMCSF/TNF α/IL-6/IL-1β. EC = extracellular.

(e.g., immunity, transcription, epigenetics, and metabolism) may have negative effects outside of the pulmonary vasculature.

To this end, Dr. Gladwin attempted to organize the major signaling hubs that are up- and downregulated into a hierarchy of importance based on the weight of evidence that these pathways are disease modifying. This is presented as a thought exercise and does not at all exclude other important areas of research. Three major pathways appear to dominate current thinking and are shown in Figure 8: cellular proliferation and extracellular matrix deposition, glycolytic-anapleurotic metabolism, and inflammation. Within this context, a number of pathways are dysregulated based on downregulation to upregulation: NO to ROS and ET1, PHD2 to HIF-2α; BMPR2 to TGFβ; AMPK to TSC,Yap-Taz,mTORC; P53 to FoxO1, FoxM1 and BMPR2 to GMCSF/TNFα/IL-6/IL-1β.

Additional challenges in our field fall into the endophenotyping of patients, with differences in sex, age, ethnicity, and precise phenotypes when we categorize patients into WHO Groups of PH. In searching for a "pure endophenotype" to study, we may not have the numbers of subjects in this orphan disease space unless we better design our clinical trials. However, in narrowing our subjects to define the "pure endophenotype" for our studies, defined by genetic or other biomarkers that will determine the right person for a specific therapy, for example, we should be careful not to exclude patients that may not have that biomarker that very well may benefit from the treatment. There is certainly a need for investigating PH therapies especially in patients with a combined WHO Group phenotype (e.g., the patient with combined post- and precapillary PH characterized by a mean pulmonary arterial pressure >25 mm Hg and pulmonary artery wedge pressure (PAWP) ⩾15 mm Hg but also with high transpulmonary pressure gradient or high pulmonary vascular resistance).

Moving forward, Dr. Gladwin suggested several ways to overcome challenges by considering the following strategies: looking at single-cell RNA sequencing in cells and localization in tissues; pursuing deep phenotyping of blood and tissue for responder enrichment; moving beyond single-cell culture studies to tissue multicell interactome studies analogous to work in the tumor microenvironment field; targeting important network "hubs" that modulate proliferation, hypoxic response, and inflammation; and considering combination chemotherapeutic agents with induction and maintenance phases. He emphasized the importance of using large and small animal models for specific aspects of human disease and proposed using multilaboratory randomized, double-blind, placebo-controlled trials of new agents in animal models before moving to human studies (86). Dr. Gladwin also encouraged the field to repurpose and test available drugs used in other diseases with established safety profiles from other patient populations to PAH. He also suggested advancing studies in larger cohorts with combined post- and precapillary pulmonary hypertension (e.g., PH associated with heart failure with preserved ejection fraction) and borderline PH (mean pulmonary arterial pressure 21–24). Finally, he challenged the field to create larger, publicly funded cooperative groups, leveraging PVDOMICS, pulmonary hypertension biological initiative, and others, similar to the Eastern Cooperative Oncology Group (ECOG) or the Autoimmunity Centers of Excellence, entities that are supported by the National Institutes of Health and work closely with the pharmaceutical industry to test chemotherapeutic agents.

Over the past 30 years, we have made incredible progress in the field of pulmonary hypertension, discovering 14 FDA-approved treatments and extending life expectancy in patients with this chronic and progressive disease. Based on the work presented and discussed at the 62nd Thomas L. Petty Aspen Lung Conference, we predict that in the next 30 years, as we seize on scientific opportunities before us, we will move toward truly effective and targeted therapies with the potential to reverse underlying pathology in patients with pulmonary hypertension. At the end of the discussion, the audience was asked again to "place bets" as expert consultants on the likely promise of interventions highlighted in Figure 1, recognizing the field's rapid success in identifying new therapeutic targets but also humility in our limited ability to make such predictions and continued reliance on safe, innovative, and properly resourced clinical trials. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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