

Translating Research into Improved Patient Care in Pulmonary Arterial Hypertension

Sébastien Bonnet^{1,2}, Steeve Provencher^{1,2}, Christophe Guignabert^{3,4}, Frédéric Perros^{3,4}, Olivier Boucherat¹, Ralph Theo Schermuly⁵, Paul M. Hassoun⁶, Marlene Rabinovitch⁷, Mark R. Nicolls^{8,9}, and Marc Humbert^{3,4,10}

¹Pulmonary Hypertension Research Group, Centre de Recherche de l'Institut de Cardiologie et de Pneumologie de Québec, Québec City, Québec, Canada; ²Department of Medicine, Université Laval, Québec City, Québec, Canada; ³INSERM UMR_S 999, Hôpital Marie Lannelongue, Le Plessis-Robinson, Paris, France; ⁴Université Paris-Sud and Université Paris-Saclay, Kremlin-Bicêtre, Paris, France; ⁵Universities of Giessen and Marburg Lung Center, Member of the German Center for Lung Research, Justus Liebig University Giessen, Giessen, Germany; ⁶Division of Pulmonary and Critical Care Medicine, Department of Medicine, Johns Hopkins University, Baltimore, Maryland; ⁷Division of Pediatric Cardiology, Department of Pediatrics, and ⁸Division of Pulmonary and Critical Care Medicine, Department of Medicine, Stanford University School of Medicine, Stanford, California; ⁹VA Palo Alto Health Care System, Palo Alto, California; and ¹⁰Assistance Publique-Hôpitaux de Paris, Service de Pneumologie, Centre de Référence de l'Hypertension Pulmonaire Sévère, Département Hospitalo-Universitaire Thorax Innovation, Hôpital de Bicêtre, Paris, France

We have witnessed significant advances in the management of pulmonary arterial hypertension (PAH) (1), a rare and severe condition characterized by intense pulmonary vascular remodeling, vasoconstriction, endothelial dysfunction, inflammation, and *in situ* thrombosis (2). More than 10 drugs targeting the endothelin, nitric oxide, and prostacyclin pathways (3) have been developed and commercialized. At present, available therapies have been shown to decrease patients' risk of short-term mortality (4) and clinical worsening (5). However, despite these advances with modern treatment modalities, most patients continue to experience poor quality of life (6, 7), and long-term prognosis remains poor (8–10). Intriguingly, despite the publication of numerous preclinical studies proposing new therapeutic targets for PAH (11), few have reached clinical trial phases (12) and none of these innovative therapies are currently approved for clinical use.

The journey from the discovery of a potential therapeutic target to its commercialization is long, complex, and costly. The series of clinical trials can cost hundreds of millions of dollars. Moreover, patient time commitment for current and upcoming PAH clinical trials is in the range

of months to years, thus limiting the number of patients with PAH to be enrolled in trials testing for other therapies. In addition, even when animal studies show promising results, more than 80% of these therapies ultimately fail when tested in humans (13). Potential reasons for unpredicted clinical response in humans and delayed drug development may include the inherent limitations of the currently available animal and *in vitro* models that mimic (part of) the spectrum of the human disease, as well as bias in the analysis or reporting of findings and limited data reproducibility (14). Importantly, this is not unique to PAH, because translating research into improved patient care exists across diseases (13, 15). Nonetheless, given the limited financial resources, the persistent medical need for improved therapy in PAH, and the restricted study population available for clinical trials, there is a need to reduce the number of false positive signals in preclinical studies and to optimize the development of innovative therapeutic targets through performance of clinical trials based on more robust experimental data. The current review discusses the challenges, pitfalls, and opportunities in preclinical research to foster drug development in PAH.

Exploratory versus Confirmatory Preclinical Research: Two Different and Complementary Modes of Investigation

Exploratory and confirmatory PAH preclinical studies are two important components of translational research. In exploratory research, investigators seek to provide a better understanding of the pathophysiological processes and to identify key cellular and molecular signaling pathways/targets involved in PAH development and/or progression. In confirmatory investigation, researchers seek to provide strong, reproducible, and detailed information on the dosage and toxicity of potential drug candidates to decide whether the drugs can be tested in PAH clinical trials. In this case, more stringent study design is recommended (Table 1). Taken together, it is clear that the prerequisites for these two modes of investigations differ and that the two different, albeit complementary, experimental strategies must be used.

Human Samples in PAH Preclinical Studies

Despite the importance of scientific results obtained from animal models, most of these

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Correspondence and requests for reprints should be addressed to Sébastien Bonnet, Ph.D., 2725, chemin Sainte-Foy, office A2134, Québec, PQ, G1V 4G5 Canada. E-mail: sebastien.bonnet@criucpq.ulaval.ca

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Table 1. Key Recommendations for Optimal Methods and More Stringent Study Design for Confirmatory Preclinical Studies in Pulmonary Arterial Hypertension

General methodological considerations

- Predetermined power calculation to define adequate sample size yielding to relevant results
- Unbiased approaches using randomization and blinded analyses
- Registration of preclinical studies to be considered to minimize publication/reporting bias
- Confirmation of results by independent investigators

In vitro studies

- Validation of the *in vitro* findings *in situ* and within various animal models
- Limitation of the number of passages and characterization and purity assessment of the primary cell cultures
- Careful selection of controls that most likely represent healthy cells isolated and cultured according to the same protocols
- Replication of the results in multiple cell lines
- Better reproduction of the cellular natural microenvironment (e.g., three-dimensional cell culture models in which cells are grown within extracellular matrix gels, pathological hypoxia, shear stress)

In vivo experiments

- Comprehensive phenotypical characterization of animal models
- Standardization of experimental conditions (e.g., route of administration and dose of Sugen, duration of hypoxia, timing of the intervention)
- Confirmation of *in vivo* findings in >1 animal model, taking into account genetic diversity, age, and comorbidities
- Appropriate splitting of age-/sex-matched littermates between control and treatment groups
- Realization of pharmacodynamic studies (e.g., tolerability, duration of the effect)
- Use of meaningful end points (e.g., survival, comprehensive hemodynamics)
- Comparison of the efficacy of the tested molecule alone or in combination with current clinical therapies
- Confirmation of drug efficacy in larger animal models to be considered

studies have been hampered by the fact that the models do not entirely encompass the typical features of human PAH. Human samples of high quality from patients with PAH and appropriate control subjects are thus invaluable resources for improving our understanding of PAH and validating emerging hypotheses. Human tissues may serve for exploratory studies aimed at demonstrating candidate cellular and molecular processes, genetic or proteomic markers (16) associated with PAH, critical histological and phenotypical characterization, as well as isolation and culture of pulmonary vascular cells (Figure 1) (17). In addition to characterizing human PAH, these samples are useful to rigorously support our assumption that animal models of PAH that share a similar end-stage pathology with the human disease are actually based on similar pathogenic mechanisms and further validate the translation potential of animal models before undertaking clinical studies.

Human PAH lungs (tissues and cells) have been most commonly obtained at the time of lung transplantation. Preestablished

protocols are mandatory to ensure the use of optimal techniques for acquiring, preserving, and processing lung tissue samples, such as those described by the Pulmonary Hypertension Breakthrough Initiative (www.ipahresearch.org/PHBI-Research-Project.html). More rarely, human PAH tissues have been obtained at the time of “warm”/early autopsy. Because PAH is associated with systemic metabolic, mitochondrial, inflammatory, and angiogenic disturbances (18–20), early autopsy has the advantage of allowing access to a wide variety of organs of interest. Nonetheless, extreme care is required to ensure that tissues are obtained before cellular and protein degradation occurs. In both cases, however, tissue samples are obtained from patients with end-stage PAH, when proteins and signaling pathways may be influenced by the patients’ treatment or merely represent a consequence of long-standing disease rather than mechanisms accounting for PAH development or progression. However, it is virtually impossible to obtain human PAH lungs from untreated patients with early disease or during longitudinal

assessments of disease evolution. The development of specialized endoarterial biopsy catheters may soon overcome this limitation, and future clinical trials must demonstrate the safety of this approach in patients (21).

Although we believe that the confirmation of new pathogenic features in human PAH significantly strengthens the conclusions of preclinical experiments, the use of human tissue samples is also associated with important responsibilities and challenges. Ethical approval, patient/family informed consent, and preservation of participants’ confidentiality are crucial prerequisites for such procedures. Although increasing the external validity of new findings, interindividual variability among patients with PAH is expected to be more common, when compared with experimental animal models, potentially requiring larger sample size. Given the effort required to harvest and prepare human tissues, the diagnosis of PAH should be fully validated according to current guidelines (1). The availability of clinical data is thus mandatory to confirm the suitability of the samples used for experiments.

The choice of cells and tissues with which PAH samples are compared is also crucially important. Control samples should be age and sex matched. Ideally, control subjects should also be matched for their underlying disease (e.g., scleroderma patients without PAH vs. scleroderma-associated PAH). Although this is desirable for samples obtained from living patients, it is virtually impossible to obtain true control samples for lung tissues. In many cases, researchers have relied on resected lung tissue for cancer. However, special attention is required to obtain tissues sufficiently distal from the tumor, which may significantly influence the phenotype and genotype of neighboring cells (22). More rarely, lungs from unused organ donors or at the time of early autopsy after the sudden death of patients with no lung disease have been used. In all cases, equivalent tissue specimens should be collected from the same organ areas. This is particularly important within the lungs, as distal versus more proximal pulmonary arteries (PAs) may significantly differ phenotypically. In addition, the same handling and processing must be used. Taken together, careful selection of control tissues that most likely represent healthy lungs/tissues is crucial.

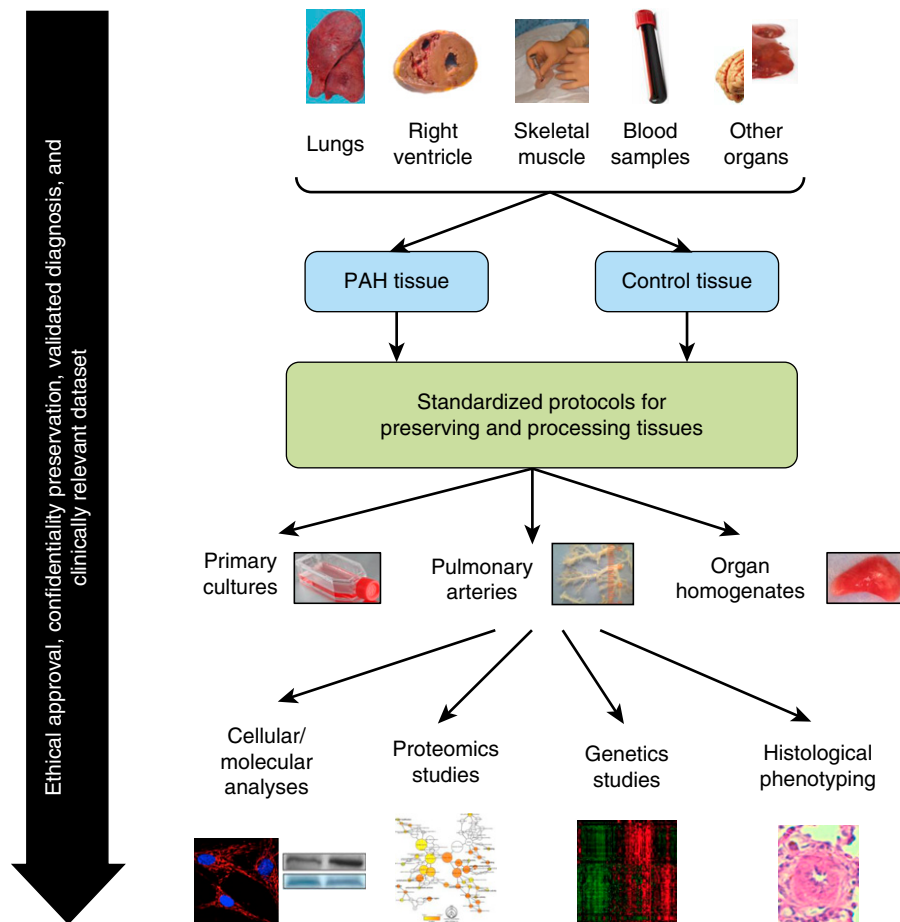


Figure 1. Pulmonary arterial hypertension (PAH) preclinical studies using human specimens. The access to human samples represents an invaluable resource for improving our understanding of PAH. Throughout this process, ethical approval, informed consent, and preservation of participants' confidentiality are crucial prerequisites. The availability of a clinically relevant data set to confirm the suitability of the samples is also essential. Control samples should be matched for age, sex, and localization. In addition, careful selection of control tissues that most likely represent healthy tissues is crucial. The elaboration of preestablished and standardized operating procedures to ensure optimal techniques for acquiring, preserving, processing, and eventually destroying tissue samples is required. Finally, the creation of a structured PAH biobank and network facilitating access to human tissue samples is essential.

Finally, few lung transplantations are performed for PAH worldwide yearly (23). Therefore, every effort should be made not to miss opportunities to collect specimens. The creation of structured PAH network and biobank facilities dedicated to harvesting and preserving explanted lung tissues, facilitating access to human tissue and ensuring homogeneity in tissue processing, is thus warranted because human tissues are currently underexploited in PAH experimental research. Despite the present ethical concerns and complex national and local regulations, such samples should ideally be placed in a public/network

repository with governance rules allowing others to have access, based on scientific rationale, to such invaluable material to promote pathophysiological and therapeutic target discoveries.

Lessons from Conventional Two-Dimensional Culture Systems to Organs-on-Chip Technologies

The extensive structural and functional remodeling of the vasculature in PAH lungs involves changes in all three layers of the vessel wall (intima, media, and adventitia)

and are the consequence of abnormal cellular hypertrophy, hyperplasia, inflammation, apoptosis, migration, and accumulation of extracellular matrix (ECM) (24–26). Many different cell types contribute to the dynamic nature of this process, including—among others—pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs), fibroblasts/myofibroblasts, and pericytes, as well as various inflammatory and immune cells (Figure 2).

Conventional two- and three-dimensional culture systems have made substantial contributions to PAH translational research and biomedical discovery and are being widely used (Table 2). Indeed, the conservation of an abnormal cellular phenotype outside their natural habitats has been demonstrated for primary cultures at early passages. In severe PAH, PAECs and PASMCs exhibit hyperproliferative and apoptosis-resistant phenotypes contributing to the extensive pulmonary vascular remodeling observed in PAH (27–32), as well as proinflammatory phenotypes (33, 34).

These cells are useful to unravel the pathophysiological mechanisms of this disease, to identify intrinsic abnormalities and to explore the panel of secreted factors contributing to aberrant cell–cell and cell–matrix communication. The cellular response can be tested in the presence of small-molecule agonists or antagonists under high-throughput conditions. Furthermore, they can also help in characterizing cell phenotypes from genetic studies and determining the activities of a promising therapeutic agent or even serve in cell-based assays for high-throughput screening. Although abnormal cell phenotypes are conserved over several passages *in vitro*, that does not necessarily imply that molecular expression patterns or distinct cellular responses will be identical to the *in vivo* situation. Moreover, culture conditions such as media or substrate stiffness may further add to phenotypic changes. It is thus crucial to study how these various pulmonary vascular cell types derived from human PAH lungs behave outside their pathological microenvironment.

Although there are clear limits to translate *in vitro* findings to the human disease, several measures should be adopted to increase the translational efficiency of the

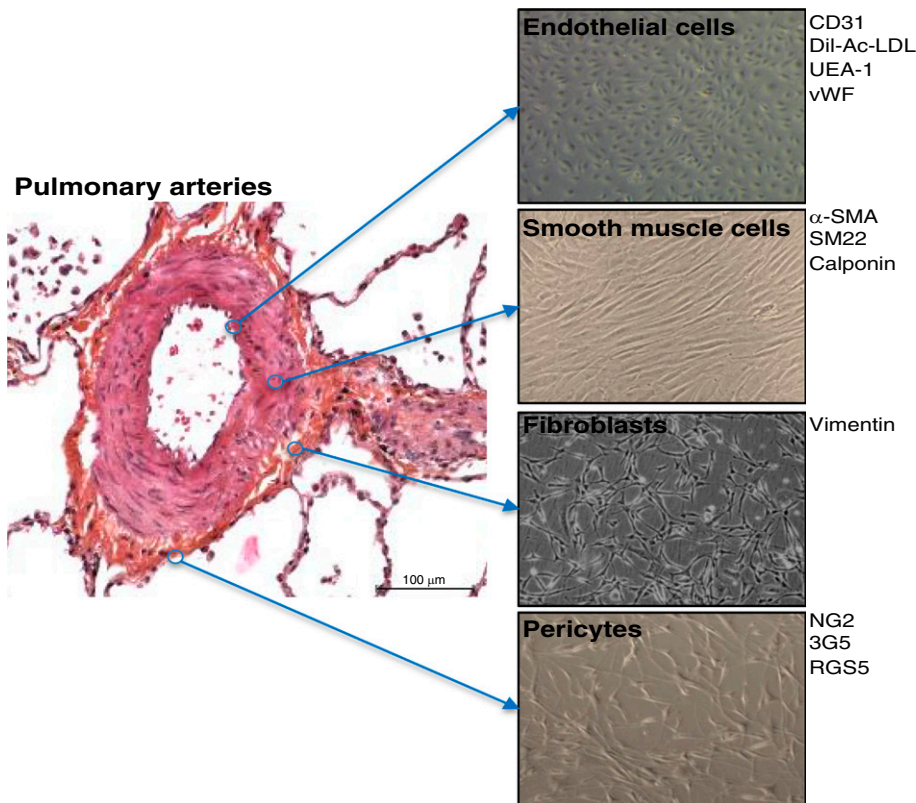


Figure 2. Key cell types constituting the vascular wall of small pulmonary arteries and contributing to pulmonary vascular remodeling. 3G5 = 3G5-reactive ganglioside antigen; α -SMA = α -smooth muscle actin; CD = clusters of differentiation; Dil-Ac-LDL = 3,3'-diiodoacetyl-LDL = 3,3'-diiodoacetyl-LDL; UEA-1 = *Ulex europaeus* agglutinin 1; vWF = von Willebrand factor.

in vitro studies. Among the most important of these are to validate *in situ* the relevance of the *in vitro* findings. The research problem should also be defined as precisely as possible to choose the most relevant cell type and technique to use. Characterization and purity assessments of the primary cell cultures should also be assessed to ensure that they maintain phenotypic properties with passaging. As stated previously, the availability of a clinically relevant data set, appropriate selection of control cells, and the fact that the same protocols for the isolation and culture of “control cells” and “PAH cells” must be employed are essential.

Better *in vitro* models are also needed to more accurately mimic human disease. Indeed, the pulmonary vascular remodeling associated with PAH implies intense production of matrix structure. Indeed, cells interact physically and functionally with components of the ECM that actively contribute to the creation of a permissive

pericellular/extracellular microenvironment for cell proliferation, differentiation, survival, and migration in PAH. In this context, several three-dimensional cell culture models in which cells are grown within ECM gels could be used to facilitate expression of differentiated characteristics that allow, for example, the study of tube formation and cell–matrix interactions. Pathological tissue hypoxia and hemodynamic forces (shear stress) are also two other key components of the cellular microenvironment in PAH that influence signaling pathways and cell function. To investigate the *in vitro* effects of chronic exposure to hypoxia, specially dedicated settings could be considered to lower oxygen to 0.1–1% as well as pharmacological inhibitors of prolyl hydroxylation such as cobalt chloride (CoCl_2 , a hypoxia mimetic), desferrioxamine, and dimethylxalylglycine (a nonspecific inhibitor of prolyl hydroxylase domain-containing proteins)

(35, 36). Similarly, *in vitro* steady shear stress flow patterns could be generated by various types of dedicated apparatus to deliver a specific, unidirectional, and constant flow rate across a cultured endothelial monolayer in a geometrically uniform flow chamber. Various levels of shear stress could be applied to pulmonary microvascular endothelial cell monolayers, ranging from physiological (2.5 dyn/cm^2) to high shear (15 or ultimately 21 dyn/cm^2), and maintained up to 72 hours or increased gradually with various flow profiles at intervals of 24 hours (37, 38). Finally, the availability of coculture technology could also be useful when answering specific biological questions and for studying both physiological and pathophysiological interactions between different cell populations (e.g., immune cells with PSMCs) (39). Similarly, a range of different coculture systems, two- and three-dimensional culture systems, as well as organs-on-chip technologies have also been developed for specific experimentation (40). Nevertheless, even with the best *in vitro* strategies, primary cell cultures may not always be appropriate or practical for some specific experimentation and do not fully reconstitute features of clinical PAH. Therefore, their strengths and weaknesses should be taken into account and most commonly be combined with *in situ* observations and animal models to validate *in vitro* observations and optimize adequate translational research.

***In Vivo* Preclinical Research in PAH**

Animal models have played a crucial role in drug development in PAH. Multiple animal models have been developed to mimic as much as possible the abnormalities seen in the human PAH condition, to decipher the molecular mechanisms involved in the development and progression of the disease and to test the therapeutic potential of drugs. Although these models contribute to a better understanding of the pathology and therapeutic innovation, they display substantial limitations (Table 3). Among these, the monocrotaline exposure model (rats) and chronic exposure to hypoxia model (mice and rats) (the latter model now frequently combined with

Table 2. Phenotypic Signature of Primary Cultures of Pulmonary Vascular Cells Derived from Patients with Severe Pulmonary Arterial Hypertension

	Phenotypic Signature in Human Pulmonary Hypertension	References
Pulmonary endothelial cells	Proproliferative and apoptosis-resistant phenotype	27, 29, 70
	Proinflammatory phenotype	33
	Smooth muscle-like mesenchymal phenotype	44, 71, 72
	Decreased tube formation	73
	Increased migratory potential	28
	Changes in metabolic processes	74, 75
Pulmonary arterial smooth muscle cells	Proproliferative and apoptosis-resistant phenotype	28, 76–83
	Proinflammatory phenotype	34
	Increased migratory potential	28
	Senescent phenotype	84, 85
	Changes in metabolic processes	41, 80, 86–90
Pulmonary fibroblasts	Proproliferative and apoptosis-resistant phenotype	91–94
	Proinflammatory phenotype	94, 95
	Increased migratory potential	91, 94
	Changes in metabolic processes	87
Pulmonary pericytes	Proproliferative phenotype	96, 97
	Decreased migratory potential	97
	Decreased tube formation	97

administration of the vascular endothelial cell growth factor receptor inhibitor SU5416 [Sugen]) are the most commonly investigated models. Fawn-hooded rats, which spontaneously develop PH in a serotonin-dependent fashion that replicates some PAH features, have also been used (41). More recently, the first models of pulmonary veno-occlusive disease induced by alkylating agents have been described (42, 43). Using advances in transgenic technologies, mutant mice and rats have been generated to mimic features of the most common genetic cause of PAH, namely *BMPR2* loss of function (44). Because the generation and characterization of these new genetic models are expensive and time-consuming, they should ideally be shared through a public repository. It must also be kept in mind that although mice allow extensive genetic manipulations, they generally display modest vascular remodeling compared with rats. Importantly, few animal models have been developed and validated for other types of pulmonary hypertension, potentially explaining the limited therapeutic advances beyond group 1 disease. Developing such relevant animal models should thus be a priority for the scientific community.

As observed for virtually all multifactorial disorders, current animal models are useful but not perfect, and they

mimic only part of the PAH pathophysiological process. This may explain why animal models are frequently considered poor predictors of whether an experimental drug can become an effective treatment. For this reason, the rationale for choosing one model should be stated (45, 46), and performing studies using more than one rodent model is recommended, whenever possible. This approach is now often applied in PAH preclinical exploratory studies (47, 48). Sometimes, however, the real reason is that confirmatory preclinical studies were not rigorously designed (Table 1). The probability that a research finding is indeed true not only depends on the level of statistical significance, but also on the prior probability of it being true (before doing the experiments) and the statistical power of the study (14). For confirmatory studies, it is thus mandatory that hypotheses and analyses be planned *a priori* to minimize multiple testing. In addition, the research problem should be defined as precisely as possible with prespecified hypotheses and analyses to minimize multiple testing, and the rationale for choosing one model should be stated (45, 46). Predetermined power calculation to define adequate sample size yielding relevant results is also recommended. Although registration would pose a challenge for exploratory studies, some kind of registration for confirmatory

experiments should also be considered to minimize publication/reporting bias. Moreover, confirmatory studies by independent investigators would provide a promising approach to increase robustness of and confidence in novel therapeutic targets. Such an approach would require a shift in the present attitude toward the value of such studies for funding and publication.

Another major problem remains the heterogeneity in the experimental design and execution, even within a same model. In the Sugen-hypoxia model, for example, there is considerable inconsistency in terms of choice of route of Sugen administration (49, 50), duration of hypoxia, and initiation and termination of treatment. Therefore, better standardization of the experimental design will certainly contribute to better reproducibility.

When studies are designed to confirm the curative potential of a molecule, researchers should also investigate what dose animals can tolerate, whether the drug reaches the relevant tissue at the required dose, and how the drug is metabolized. Moreover, treatment of the animals should be initiated after noninvasive confirmation (e.g., echocardiography) of the full establishment of PH, as it is critical to demonstrate whether a molecule improves PAH or only slows down its progression. In addition to short-term studies, the potential benefit of new drugs should be determined after longer periods of treatment through determination of key pulmonary and systemic hemodynamic parameters and standardized morphometric analyses of pulmonary vascular remodeling, such as PA wall thickness, the percentage of muscularized distal PA, and right ventricular hypertrophy. Cardiac output, systemic blood pressure, right ventricular function, exercise tolerance, long-term toxicity, and overall survival should also be monitored when possible (Figure 3). Moreover, confirmatory studies should ideally compare the efficacy of the tested molecule with current clinical therapies (47), because new drugs are likely to be given to patients with PAH who are already receiving standard PAH therapies. Finally, the use of preclinical larger-animal models more closely related to humans, such as pigs (51, 52) or nonhuman primates (53), could result in increased clinical success rates of drugs in development. However, working with larger animals is extremely expensive and fraught with ethical concerns.

Table 3. Preclinical Models of Pulmonary Hypertension

Species	Model (Ref)	Pros	Cons	Comments
Rat	Induced Monocrotaline (98)	Inexpensive Important vascular remodeling (intimal hyperplasia, medial hypertrophy, and adventitial thickening), and right ventricular failure Provides insights into inflammatory and matrix remodeling mechanisms of PAH Used for the development of most current PAH-targeted therapies	Associated with pulmonary interstitial edema, some features of myocarditis, and hepatic veno-occlusive disease that are not typically present in human PAH (99) Most experimental treatments appear to improve or fully reverse the pathology (100). For example, dexfenfluramine and dasatinib, two agents known to increase the risk of developing human PAH (101, 102), paradoxically protect or reverse PH in MCT rats Conversely, some currently available therapies that are effective in human PAH have limited efficacy in MCT rats (e.g., bosentan) Disease progression toward death might be too fast for compensatory mechanisms to develop	To overcome the easy reversal of PH, some authors have attempted to combine the administration of MCT with other interventions, such as pneumonectomy or chronic hypoxia
	Chronic hypoxia (103)	Inexpensive and widely available	No obstructive intimal lesions Pulmonary vascular remodeling induced by chronic hypoxia exposure alone is reversible after cessation of hypoxia, contrasting with human PAH, which occurs under normoxic conditions and is associated with irreversible vascular lesions Severity of the phenotype differs among rat strains (Sprague-Dawley rats are highly susceptible, whereas Fischer rats are relatively resistant to hypoxia)	Assumed to be representative of group 3 (PH secondary to lung disease and/or hypoxia) rather than PAH
	Sugen-hypoxia (50)	Administration of SU5416 in combination with exposure to hypoxia was shown to intensify the vascular remodeling process, leading to the appearance of plexiform lesions Virtually unresponsive to current PAH treatments, correlating well with the unresponsiveness of PAH to some treatments in humans	Sugen induces an emphysema-like phenotype SU5416 injection is accompanied by airspace enlargement (emphysema) (50) Intraspecies variation in susceptibility has been reported (104, 105)	Sugen has been administered to various strains, including athymic nude rats, to decipher the role of regulatory T cells in the pathogenesis of PAH (106)
	Mitomycin (42, 43)	May allow the understanding of pulmonary veno-occlusive disease, a largely ignored and misdiagnosed form of PAH		The first rodent model of pulmonary veno-occlusive disease
	Pulmonary banding (107–109)	Allows study of chronic pressure-overloaded right heart hypertrophy and failure in the absence of pulmonary vascular remodeling	Only a few studies have characterized the PAB model	
	Spontaneous Fawn-hooded rats (41)	Spontaneously develop PH in a serotonin-dependent fashion that replicates some PAH features	Associated with developmental lung anomalies, including reduced number of alveoli (110) More than 6 mo is required to observe moderate PH in this strain Available data are limited on therapeutic treatments that have been tested in this model	
	Genetically modified <i>BMPR2</i> ^{Δ140Exon1/+} (44)	Powerful tool to dissect the underlying pathophysiological mechanisms of PAH	Low penetrance and expressivity, precluding their use for drug testing The identification of patient-relevant second triggers that would lead to high and consistent occurrence of PH would certainly facilitate their use in large-scale preclinical setting	First rat model of <i>BMPR2</i> heterozygosity generated by zinc finger nuclease-based gene editing of the first exon in the gene

(Continued)

Table 3. (Continued)

Species	Model (Ref)	Pros	Cons	Comments
Mouse	Induced Chronic hypoxia	Inexpensive and widely available	Reversal Displays modest vascular remodeling after exposure to chronic hypoxia	
	Sugen-hypoxia (111)	Allows the development of severe PH with more typical angioproliferative features of PAH compared with chronic hypoxia alone	Displays moderate vascular remodeling and PH only	
	Schistosomiasis mice (112)	May contribute to further understanding of underlying mechanisms such as the role of inflammation in PH associated with schistosomiasis		
	Genetically modified <i>Bmpr2</i> ^{+/−} (113)	Mice allow extensive genetic manipulations	Absence of right ventricular hypertrophy Severity of vascular remodeling in these animals tends to vary and to remain mild. This shortcoming has been addressed by genetically modifying the mice to express a dominant negative allele for the <i>BMPR2</i> gene in PSMCs, leading to vascular changes resembling plexiform lesions and severe PH (114)	Exposure of these mice to hypoxia (11% F _{IO₂} for 3 wk) results in vasoconstriction, muscularization of the small PA, and increased PA pressure BMPR2 deletion can be done selectively in either endothelial or smooth muscle cells by crossing animals with carriers of cell-specific Cre recombinase genes (113)
	SM22 α -rtTA \times TetO (7)- <i>Bmpr2</i> ^{R99X} (115)	Pulmonary vascular remodeling similar to plexogenic arteriopathy	Only one-third will develop elevated pulmonary pressures, vascular pruning, and muscularization of microvessels	Expressing a dominant-negative <i>BMPR2</i> gene (with an arginine-to-termination mutation at amino acid 899)
	Tg(<i>Alk1</i> -Cre)-L1 \times <i>Bmpr2</i> ^{fllox/fllox} (116)		Knockout mice developed mild PH, with only a subset of mice exhibiting RV hypertrophy and distal PA remodeling	
	<i>Sirt3</i> ^{−/−} (117)	Confirmed the implication of mitochondrial dysfunction		
	<i>Ucp2</i> ^{−/−} (118)	Confirmed the implication of mitochondrial dysfunction		
	<i>ApoE</i> ^{−/−} (119)	The fact that <i>ApoE</i> ^{−/−} mice spontaneously develop PH and PA remodeling suggested that insulin resistance and obesity contribute to the development of PH		
	S100A4/ <i>Mts</i> -1 (overexpression) (120, 121)	Pulmonary vascular remodeling similar to plexogenic arteriopathy is seen with this model May provide a possible explanation for sex differences, as seen in human idiopathic PAH	Low penetrance (males remain essentially unaffected)	Overexpressing <i>s100A4/mts1</i> gene under the control of the HMG-CoA reductase gene promoter
	Tg(SM22 α -Cre) \times <i>Pparγ</i> ^{fllox/fllox} (122)	Confirmed the implication of PPAR γ in the development of PAH	Mice with targeted deletion of PPAR γ in PSMCs developed only mild PH and pulmonary vascular remodeling	
	SM22 α -5-HTT ⁺ (overexpression) (123)	Allowed the assessment of targeting the serotonin signaling pathway to reverse pulmonary vascular remodeling		5-HTT-overexpressing mice exposed to chronic hypoxia developed RV hypertrophy and significant pulmonary vascular remodeling
	CC10-IL6 (overexpression) (58)	IL-6-overexpressing mice exposed to chronic hypoxia developed elevated RV systolic pressure and hypertrophy, as well as significant obliterative pulmonary vasculopathic changes	May be specifically representative of PAH with marked inflammatory implication only	This model documented that IL-6 promotes the development and progression of PA remodeling
	Endothelin receptor-B knockout model (124)	Confirmed that the entire endothelin signaling system plays an important role in PH and contributed to the development of endothelin receptor antagonists as a treatment for PAH		
	<i>Vip</i> ^{−/−} (125)	<i>VIP</i> ^{−/−} mice spontaneously develop moderate to severe PH with pulmonary vascular remodeling and RV hypertrophy	The exact role of VIP in PH remains unknown This model differs from human PH regarding hemodynamic severity and histology	

Definition of abbreviations: Alk1 = activin receptor-like kinase 1; ApoE = apolipoprotein E; BMPR2 = bone morphogenetic protein receptor 2; CC10 = club cell 10 protein; F_{IO₂} = fraction of inspired oxygen; HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A reductase; 5-HTT = serotonin transporter; MCT = monocrotaline; PA = pulmonary artery; PAB = pulmonary artery banding; PAH = pulmonary arterial hypertension; PSMCs = pulmonary artery smooth muscle cells; PH = pulmonary hypertension; PPAR γ = peroxisome proliferator activated receptor γ ; rtTA = reverse tetracycline-controlled transactivator; RV = right ventricle; S100A4 (*Mts*1) = S100 calcium-binding protein A4; Sirt3 = sirtuin 3; SM22 α = smooth muscle protein 22- α ; TetO (7) = seven tetracycline operator (tetO) repeats; Tg = transgenic mice; Ucp2 = uncoupling protein 2; VIP = vasoactive intestinal peptide.

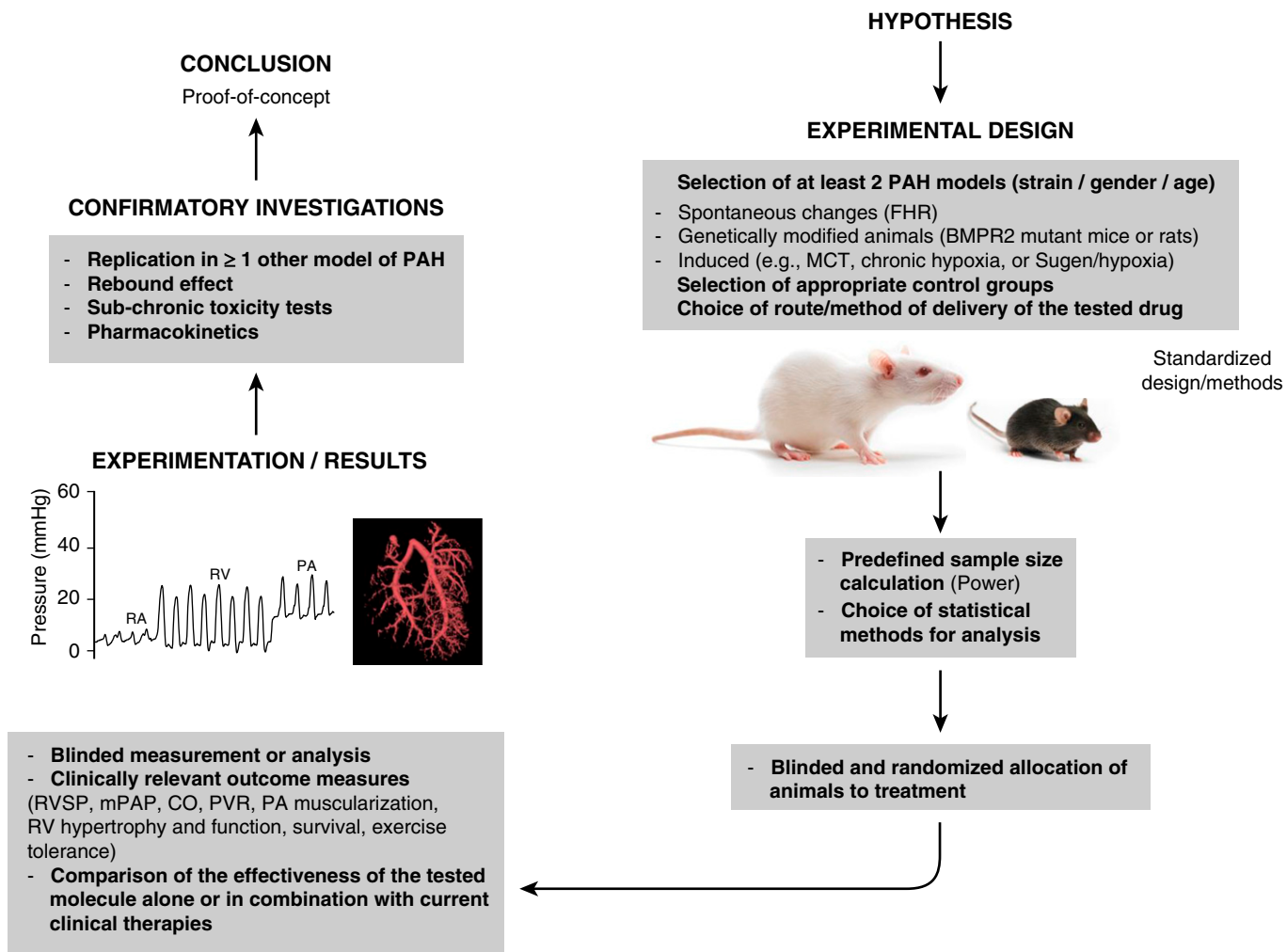


Figure 3. Example of a confirmatory study design using animal specimens. BMPR2 = bone morphogenetic protein receptor, type II; CO = cardiac output; FHR = fawn-hooded rat; MCT = monocrotaline; mPAP = mean pulmonary artery pressure; PA = pulmonary artery; PAH = pulmonary arterial hypertension; PVR = pulmonary vascular resistance; RA = right atrium; RVSP = right ventricular systolic pressure; RV = right ventricle.

Facing Challenges in Translating Confirmatory Preclinical Investigations into Clinical Trials

Although current treatments may delay disease progression, these therapies have not shown potential in reversing the vasculopathy that underlies PAH.

Novel potential targets of PAH drug development include vascular inflammation, metabolic derangements, and aberrant BMPR2 (bone morphogenetic protein receptor, type 2) signaling.

Among the first approaches to encounter these challenges are immunotherapy trials. The first multicenter blinded, randomized, placebo-controlled clinical trial (RCT) to evaluate an

immunotherapy in PAH began enrolling patients in 2010 and is testing the efficacy of B-cell depletion with rituximab in systemic sclerosis-associated PAH. ASC01 is a National Institutes of Health-sponsored trial scheduled to complete enrolment this year (clinicaltrials.gov, NCT01086540). This trial includes a number of mechanistic studies including evaluating B-cell phenotype and clonality, multiplex cytokine assays, and regulatory T-cell properties. For this study, as for other emerging PAH therapies, there is anticipation that this careful phenotyping may provide biomarkers that may help predict which patients will benefit most from a given adjuvant therapy.

Although ASC01 has targeted one aspect of the adaptive immune response,

two innate immune mediators are the focus of forthcoming clinical trials: leukotriene B₄ (LTB₄) and neutrophil elastase. Targeting these mediators has posed unique challenges to development. Macrophage-derived LTB₄ was identified as a potentially important cause of endothelial injury that may also be involved in the genesis and maintenance of the occlusive arteriopathy (54). Ubenimex (Eiger Biosciences, Palo Alto, CA) is a leukotriene A₄ hydrolase inhibitor, which inhibits LTB₄ biosynthesis; ubenimex has been used as a chemotherapy adjuvant in Japan and required orphan drug designation for use in the forthcoming U.S. PAH trial (clinicaltrials.gov, NCT02664558). There is evidence to suggest that different forms of PAH may result from different types of immune

injury (e.g., helper T-cell type 1 [Th1]/Th17 vs. Th2 immunity) (55), and it is not currently clear how various patients with PAH will respond; it may depend on whether their immunity is skewed toward one phenotype or serum levels of LTB₄ may predict drug responsiveness. Another approach targeting innate immunity will use the drug elafin (Proteo Biotech Inc, Kiel, Germany) to inhibit neutrophil elastase activity, which is also strongly implicated in PAH pathogenesis and BMPR2 activation (56, 57). Because elafin has only previously been given to humans as a single intravenous injection in Europe, chronic subcutaneous therapy will require Good Laboratory Practices studies in at least one large-animal species as well as a phase 1 clinical trial before a multicenter, phase 2 RCT in patients with PAH takes place. IL-6, a molecule that bridges both innate and adaptive immunity, and that appears to have a causal role in PAH pathogenesis (58–60), is being tested in an open-label phase 2 safety trial of tocilizumab. As with the rituximab trial, a principal issue is whether the immune suppression caused by IL-6 modulation is safe in an already tenuous population.

Apoptosis signal-regulating kinase 1 (ASK1) is a ubiquitously expressed redox-sensitive serine/threonine protein kinase that activates the p38 mitogen-activated protein kinase and Jnk pathway. A phase 2, dose-ranging, randomized and placebo-controlled study of GS-4997 in patients with PAH has been initiated (clinicaltrials.gov, NCT02234141), which addresses safety, tolerability, and efficacy.

Dysfunctional BMPR2 signaling is implicated in the pathogenesis of PAH (61). A study screening 3,756 U.S. Food and Drug Administration–approved drugs identified FK506 (tacrolimus) as an effective up-regulator of BMP signaling, and low-dose FK506 was an effective treatment for experimental PAH (62). Preliminary data also suggested that compassionate use of low-dose FK506 in three patients with end-stage PAH might have been associated with clinical benefit (63). On the basis of these findings, a phase 2a RCT in PAH was initiated to evaluate the safety and tolerability of FK506 in stable patients with PAH (clinicaltrials.gov, NCT01647945).

As in cancer, vascular remodeling in PAH is a state of apoptosis resistance of the distal PA cells. Several studies have demonstrated that a switch from

antiapoptotic glycolytic metabolism toward proapoptotic oxidative phosphorylation metabolism causes regression of vascular remodeling and PAH in several animal models (20, 64, 65). This has been achieved with the small-molecule metabolic modulator dichloroacetate, an inhibitor of the mitochondrial enzyme pyruvate dehydrogenase kinase. Dichloroacetate has been used in humans for more than 30 years, mostly in the treatment of inherited mitochondrial disorders, and is also currently being evaluated as a potential therapy in cancer (66–68). On the basis of these findings, a phase 1 RCT in subjects with advanced PAH was completed (clinicaltrials.gov, NCT01083524). Although results are still pending, a particular challenge faced for further developing this promising PAH therapy is securing financial backing for a clinical trial when using a repurposed and off-patent drug. In the apparent absence of a major return of financial investment, needed to lure venture capital support, it appears that exceptional dedication by committed investigators backed by governmental and foundational support may be required to advance this and other similarly promising PAH therapies.

We are entering an exciting era for new therapeutics in the treatment of PAH (69). Gene expression studies and high-throughput studies have emerged as powerful tools to identify novel therapeutic targets and biologically active compounds able to modulate dedicated pathways in PAH. This approach was employed for the identification of FK506 described previously, for example (62).

The use of inducible pluripotent stem cells (iPSCs) provides another exciting approach to drug discovery in PAH. Use of iPSCs to derive endothelial or smooth muscle cells from patients with PAH could certainly help us in further elucidating the genetic and molecular mechanisms of PAH and translating this into a platform for screening novel PAH-specific compounds. This approach could also be used to predict the pharmacological profile of a given compound in an individual patient, as these cells contain the patient's unique genetic and epigenetic markers. This could predict how various patients might respond to a given compound and help us move PAH treatment into personalized medicine.

However, various challenges exist, reinforcing the need for more robust experimental data with reduced false

positive signals, allowing the prioritization of the most promising compound. First, new approaches need to show benefit on top of optimized treatment with currently approved therapy; a barrier not faced in the original vasodilator trials. This makes the detection of any putative benefit potentially challenging, particularly if adverse interactions occur with existing therapies, or the benefit of the new compounds is best observable in treatment-naïve patients. Second, complex regulatory agency approvals are required to target novel pathways for PAH treatment. For all emerging PAH therapies, it is essential that regulatory authorities grant orphan drug status before proceeding to clinical trials to help advance the evaluation and development of such products. Third, some new therapies carry novel risks not encountered with approved agents, such as hypersensitivity reactions and immunosuppression. Fourth, the pathway to development for these novel drugs can be highly complex and not receive standard industry support. Therefore, investigator-initiated trials must provide a sufficiently convincing rationale to entice investigators who are accustomed to working with well-funded corporations that offer potentially more attractive compensation for examining already-trusted vasodilation pathways. Given these problems, it is heartening to note that clinician-scientists appear to be rising to the challenge, and the next decade should offer patients with PAH considerable hope for better cures.

Conclusions

A better translation of preclinical research findings into sustainable improvements in patient outcomes is clearly needed, but remains a substantial obstacle for many human diseases, including PAH. At present, research on PAH is characterized by significant discordance between preclinical findings and clinical results. Bridging this translational gap is thus vital and can be achieved only by rigorous use and characterization of human PAH tissues, a better design of preclinical studies, significant governmental and foundational support and funding, and close collaboration and significant commitment from clinical trial sites. ■

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

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