Translational Advances in the Field of Pulmonary Hypertension

Molecular Medicine of Pulmonary Arterial Hypertension
From Population Genetics to Precision Medicine and Gene Editing

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An improved understanding of the molecular causes and modifiers of pulmonary arterial hypertension (PAH) has emerged during the last several decades. However, the survival of patients with PAH remains poor, and there is no curative therapy (1–6). Further, specific molecular information to directly inform decision-making at the individual level regarding prognosis and therapeutic decision-making remains insufficient. The next decade and beyond should see major advances in our capacity to precisely define the factors involved in PAH, and all forms of pulmonary hypertension, using traditional and novel approaches to personalized care.

Traditional molecular medicine approaches have been invaluable in the expansion of our knowledge of PAH, including but not limited to the application of traditional genetic approaches, genetic epidemiology studies, molecular signaling and structural analyses, and the use of molecular pharmacology and gene modification techniques. But more recently, advanced experimental techniques and systems analyses have emerged to shed light on the genomic, transcriptomic, epigenomic, metabolomic, proteomic, and other domains of molecular activity, and their interactions, that contribute to PAH pathogenesis and progression (7). "Omics" and related advances are crucial, but must be integrated with alternative data sources. The resultant synergy of traditional and novel molecular approaches will facilitate the personalization of PAH risk, treatment, and cure.

We now know that in a minority of cases, a rare genetic variation (mutation) in a single gene creates a cellular and systemic milieu of PAH susceptibility. In fact, for prediction of PAH development, there is no greater risk than being the carrier of a mutation in one of the known "pulmonary hypertension (PH)–specific genes," such as bone morphogenetic protein receptor type 2 (*BMPR2*). And, about 20% of subjects thought to have idiopathic PAH (IPAH) actually have disease associated with mutations in this gene (8). For some families with a known *BMPR2* gene mutation, preimplantation genetic testing under the direction of experienced clinicians may be a viable option for future pregnancies, in women without PAH, to ensure that the mutation is not carried forward (9). In addition, for those individuals with a *BMPR2* mutation, and perhaps those without a mutation but reduced downstream signaling, mechanisms to enhance BMPR2 function and/or signaling by novel targeted approaches such as nonsense mutation read-through, manipulation of gene expression by alternative splicing, amplification of wild-type protein signaling, or other mechanisms are viable preventive and therapeutic possibilities on the horizon (10–14).

Yet, the presence of a mutation in *BMPR2* does not guarantee the development of clinical PAH as there is markedly decreased penetrance, suggesting that additional molecular and environmental factors modify the development and phenotypic expression, for example, age of onset of PAH among susceptible hosts (8). The factors modifying PAH penetrance are likely complex, incorporating multiple sources of variability including sex (females are threefold more likely to develop PAH than males [15]); wild-type expression of the nonmutated *BMPR2* allele (16); common and rare variations in other genes (17, 18); and epigenetic (19), biochemical (20), or other factors. It is also known that mutations in *BMPR2*, as well as in the hereditary hemorrhagic telangiectasia-associated PAH genes endoglin (*ENG*) and activin A receptor type II-like 1 (*ACVRL1*, *ALK1*) cause more severe PAH with shorter survival than seen in IPAH cases (21, 22). But, stratification of severity within genes according to genotype has not occurred because of the rarity of these mutations overall. Although rare, the prevalence of these mutations is significantly higher than that of other genes associated with PAH, such as caveolin-1 (*CAV1*), potassium channel two pore domain subfamily K member 3 (*KCNK3*), and eukaryotic translation initiation factor 2α kinase 4 (*EIF2AK4*) (23–25).

Mutations in *CAV1*, *KCNK3*, *TopBP1*, and *EIF2AK4* were discovered in PAH by the use of whole-exome sequencing, which is one of several more recent approaches employing next-generation sequencing technology (23–27). Although it can be

(Received in original form May 2, 2016; accepted in final form July 4, 2016)

Supported by NIH grants P01 HL108800 (E.D.A., J.W., J.E.L., and A.R.H.) and 1U01 HL125212-01 (A.R.H.).

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Am J Respir Crit Care Med Vol 195, Iss 1, pp 23–31, Jan 1, 2017

Originally Published in Press as DOI: 10.1164/rccm.201605-0905PP on July 11, 2016
Internet address: www.atsjournals.org

applied to unrelated subjects, whole-exome (and whole-genome) sequencing may be particularly effective in the setting of familial disease. For example, as shown in Figure 1, a family with PAH in multiple family members with available DNA may be analyzed, as performed with the *CAV1* discovery. Briefly, relatively standardized approaches are employed to capture the exonic segments of each gene, which are then sequenced. Sequencing typically generates a raw sequence, which is subsequently evaluated and converted into a more mature final sequence of exons. This process is typically followed by careful analysis of the sequencing for common and rare genetic variations, which are differences from population databases of "normal" subjects, or subjects with other known diseases (e.g., Single Nucleotide Polymorphism Database, 1000 Genomes Project, and the human genome browser of University of California Santa Cruz). In most cases, when searching for rare variations (mutations), at this step common variants (e.g., more than 1% prevalence) are filtered out of the analysis. Careful analysis of the residual rare variants is then undertaken in a variety of ways, including confirmation of the prevalence in the population, using databases of variation (e.g., the Exome Variant Server [http://evs.gs.washington.edu/EVS/] and ExAC Browser [http://exac.broadinstitute.org/]); assessment of the various mutation types represented; and prediction of impact of the variations, using a variety of approaches (e.g., SIFT [http://sift.jcvi.org/] and PolyPhen-2 [http://genetics.bwh.harvard.edu/pph2/]).

In the case of the *CAV1* discovery (26), four patients with PAH in the same family were analyzed by whole-exome sequencing, providing the opportunity to search for shared heterozygous mutations in a family with autosomal dominant PAH. An initial detection of 54,540 genomic locations with variations was trimmed to 10,088 (18%) shared genomic locations, and then trimmed to 653 heterozygous variants shared by all four patients, and then trimmed to 52 shared mutations, which were ultimately narrowed to 16 shared mutations of interest after careful review of mutation type, functional impact, and conservation across species. Ultimately, 11 of the 16 candidate mutations were confirmed by standard sequencing (Sanger sequencing), and a fifth case of PAH in the family was used to filter the list down to three candidate mutations. From that list, careful analysis of the genes including tissue expression, conservation of the genetic site, mutation type, prediction of mutation impact, and other factors led to the identification of a *CAV1* mutation as the primary target. As is typically employed, additional case subjects and control subjects were screened for *CAV1* mutations to ultimately improve confidence of the finding.

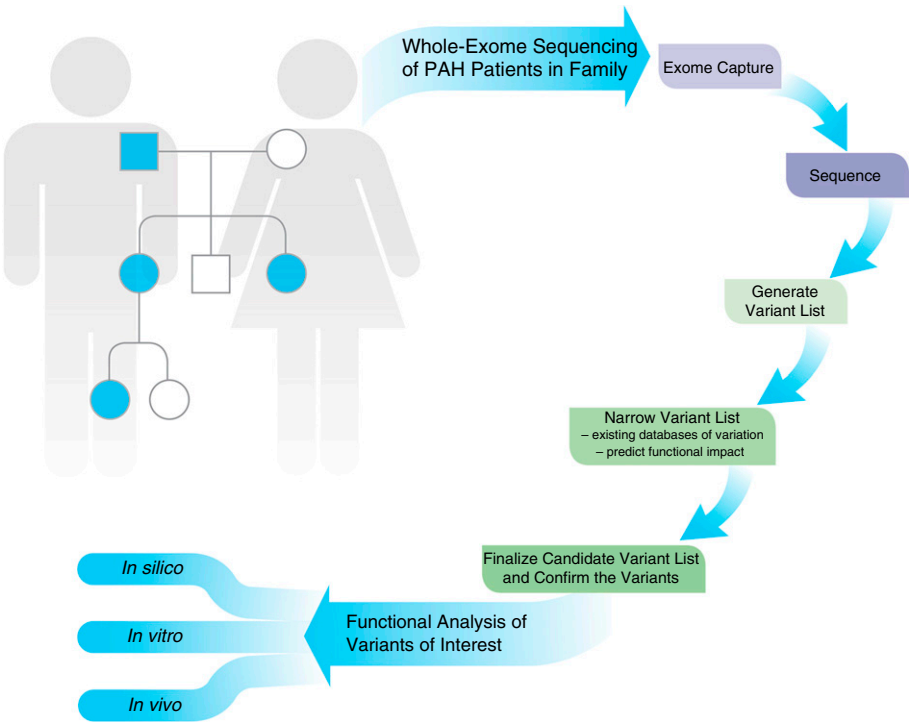

Figure 1. Whole-exome sequencing to identify rare variants relevant to pulmonary arterial hypertension (PAH) pathogenesis. A representative pedigree, similar to that employed for the discovery of a *CAV1* mutation in a family with heritable PAH, is represented, with four patients with PAH in the modified family pedigree. Whole-exome sequencing is performed, which involves a number of complicated laboratory and bioanalytical steps, to determine the variants of interest for confirmation. Ultimately, confirmed candidate genes and variants require biological studies to determine the true functional impact of the changes discovered. Finally (data not shown), additional case subjects and control subjects should be genotyped for mutations in the candidate genes of interest.

Although the true prevalence of *CAV1* mutations and mutations in other PH-specific genes has yet to be fully elucidated, at least two current programs underway are searching for common and rare variants (mutations) in large numbers of patients with PAH; therefore, the true prevalence of mutations in PH-specific genes will be elucidated soon, which may facilitate more detailed genotype–phenotype studies (National Biological Sample and Data Repository for PAH in the United States and BRIDGE-PAH [Biomedical Research Identification of Genetic Etiology of PAH] in the United Kingdom). In addition, in the near future, modifier genes that predispose to susceptibility to PAH or genes that promote resiliency may be known; these genes could be incorporated into a risk calculator to provide precise prediction as to which mutation carriers will develop disease and at what age. Concurrent with such a molecular breakthrough, novel approaches to detect early pulmonary vascular disease are needed to allow therapeutic intervention at the presymptomatic phase of disease (which is currently undetectable) (28).

Because mutations account for only a modest proportion of PAH cases, there is a critical need to identify the genetic and nongenetic factors involved in PAH pathogenesis, progression, and response to therapy, regardless of the presence or absence of a PH-specific gene mutation. Those same factors that appear to modify *BMPR2* penetrance, as noted previously,

may contribute in the setting of no PH-specific gene mutation, as well as additional variations such as common polymorphisms in the endostatin gene, endothelin-related genes, sex hormone–related genes, other mutations across the genome, or at the *CBLN2* locus (27, 29–35). In addition, a growing body of work implicates complicated molecular perturbations, such as disruptions in energy metabolism and mitochondrial dynamics involving both the pulmonary vasculature and right ventricle (5, 19). No set of variations occurs in isolation. To date, however, most molecular discoveries have been made in relative isolation without integration of multiple gene variants or molecular features, due to the limitations of traditional variant studies.

The complexity of cellular derangements that contribute to PAH requires a broad scientific approach that can be ultimately narrowed to the individual level. Achievement of this lofty goal requires integration of data from multiple sources involving traditional and novel molecular platforms, and physicians and scientists with a broad range of expertise. The components include well-defined human subject cohorts monitored longitudinally, "omics"-level data from those same subjects across multiple cell types, and the clinical and scientific expertise to determine disease onset, response to therapy, and disease progression. These resources will provide the necessary foundation for scientific approaches to advance understanding of the molecular complexity of PAH. Next, we present some of the more recent data on precision medicine in PAH and then discuss how modern gene-editing technology can be used to understand current and future discoveries.

PAH and the Concept of Precision Medicine

Although some patients do well on current therapies for prolonged periods, PAH remains a deadly disease and a significant proportion of patients rapidly decline despite appropriate and aggressive therapy (2, 36). With the notable exception of calcium channel blocker therapy, our current treatment recommendations are based on clinical markers of disease severity, matching more severe disease to prostaglandin and combination therapy, while recommending treatment with oral therapies for patients with preserved right ventricular function and more mild disease (37). The current U.S. Food and Drug Administration (FDA)-approved PAH therapies offer many choices to tailor to patients' preferences and disease severity, but also create uncertainty, as we cannot easily predict which patients may benefit from which treatment class. Clinicians at the bedside know that certain patients often have dramatic responses to one particular class of medications, while they have no response to another. The only way to identify these responses, presently, is by trial and error. Unfortunately, when ineffective medications are tried, disease often progresses, and thus valuable time is lost. Although modern molecular techniques and next-generation sequencing can and should be used to understand disease etiology, it can also be used to predict drug responsiveness. Given the persistent high mortality in PAH and the current inability to predict drug responsiveness, a key use of molecular investigations should be to match an individual patient to the drug most likely to be effective, called "precision medicine" (38).

The concept of precision medicine is not new in PAH, as we have long known that patients who meet specific criteria for hemodynamic response to inhaled nitric oxide during right heart catheterization have long-term favorable responses to calcium channel blocker therapy (39). Although detailed phenotypic assessments and classification are a major part of the broader PH field, analysis of differential treatment response according to PAH subtype remains an underused precision opportunity; for example, last year Rhee and colleagues used individual participant data from phase 3 placebo-controlled randomized controlled trials of therapies for PAH submitted to the FDA for drug approval to demonstrate that PAH treatment may be less effective in connective tissue disease–associated PAH compared with IPAH (40). Careful phenotyping offers great opportunity to fine-tune the selection of therapies for the appropriate subtype of patient.

In addition, "omics" studies may be employed to better understand disease processes, and ultimately support the development and selection of therapeutics for certain individuals. A growing body of literature supports the concept that irregularities in microRNA activity and related networks promote PAH pathophysiology, which creates exciting opportunities for precision therapeutics design (19, 41–44). Rhodes and colleagues analyzed pulmonary artery endothelial cells from control subjects and subjects with IPAH, using RNA sequencing to determine novel pathways perturbed in relation to reduced BMPR2 signaling (45). We identified a peripheral blood signature of RNA expression patterns that faithfully identifies patients with a PAH subphenotype responsive to calcium channel blocker therapy, suggesting that peripheral blood can be used to identify drug responsiveness in PAH (46). Using whole-exome sequencing, genetic variants that may underlie this subphenotype were also uncovered (47). In another example of precision pharmacogenomics, Benza and colleagues explored genetic variants in endothelin-1 (ET-1) signaling that predict good responses to ET-1 receptor antagonists (34). These different and equally successful approaches highlight potential ways forward for our field in using modern molecular medicine techniques to improve patient care in PAH. The traditional scientific approach used by Benza and colleagues tested the hypothesis that variants in ET-1 receptor or signaling will underlie different responses to endothelin receptor antagonist therapy. They used genome-wide association study data to generate a candidate list of variants in ET-1 signaling and then performed targeted genotyping for the candidates. One of their candidate variants (rs11157866) predicted response to endothelin receptor antagonism. Although successful in this study, this methodology may miss important molecular associations as one is limited by the current knowledge of molecular disease etiology.

One example of a different strategy for molecular prediction of drug responses is an unbiased, discovery-based approach to precision medicine in PAH. This methodology uses technology such as next-generation sequencing, RNA sequencing or microarray, and proteomics or metabolomics to measure thousands of chemicals, proteins, transcripts, or genes. Rather than having an *a priori* idea of which findings are important, those with the strongest association with disease are selected for further study, without the requirement

for biologic plausibility in all cases. The challenge of this approach is that it may identify compounds unimportant to the disease or may identify so many potential targets that prioritization requires advanced bioinformatics and confirmation of functional consequences. However, if one wants solely to identify strong predictors of drug responsiveness, the unbiased discovery approach may offer the best hope for finding molecules or genes that fulfill this requirement. Considering the urgency of patients with progressive disease treated by ineffective therapy, the specific functions of these genes, proteins, or metabolites may matter little in the short term if they are strong predictors of response to a particular therapy. The hard work of figuring out how these molecules are relevant to disease could be sorted out after confirming their predictive value. Of course, this approach necessitates serial acquisition of samples in advance and over time from patients exposed to therapeutics.

Regardless of methodology, identified molecules or genes of significance to PAH can be used for multiple purposes. Most urgently, they can be used to predict responses to already FDA-approved therapies in PAH. Next, they can potentially be used to improve clinical trial design in PAH (Figure 2). At present, data from clinical trials of new therapeutics are analyzed in aggregate, so that variability in individual patient response is generally not presented. If we find clinical "superresponder" patients in new drug trials, "omics" techniques could be applied to identify molecular markers of these patients. Future trials could enroll only patients with this excellent response, thereby saving money and time and potentially maximizing therapeutic benefit for enrollees (48). For instance, in the case of the drug imatinib, if superresponders were identified in clinical trials and specimens available for analyses (49), next-generation sequencing may have been able to find genetic predictors of this response to select patients in whom the risk-to-benefit ratio of this therapy is warranted. Preselecting patients for study trial participation has already proven successful in cystic fibrosis (50), and it should be forthcoming in PAH.

Thus there are many potential ways to use the broad "omics" data that will be generated through application of modern molecular medicine in PAH. An additional

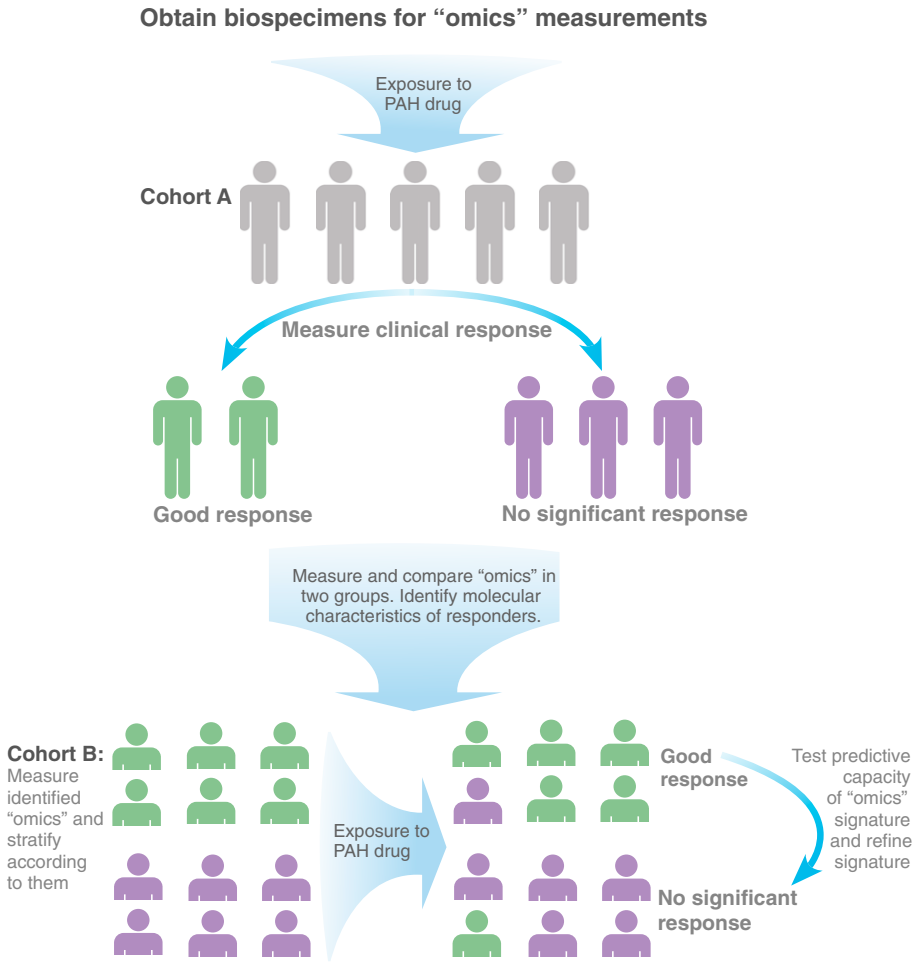

Figure 2. The identification of genes, pathways, and molecules relevant to pulmonary arterial hypertension (PAH) can be used for multiple purposes, including the improvement of clinical trial design. Subjects exposed to a given therapeutic could be compared in a number of different ways, including according to therapeutic response. Comprehensive approaches to explore the shared variations among those who respond and those who do not respond may support the determination of a predictive signature of response to help refine patient selection for a given therapeutic.

critical step will be to take the identified genes, pathways, and molecules and use them to expand our understanding of disease etiology, which is critical to developing curative therapy. This will require the application of traditional and novel molecular techniques, such as gene editing.

Gene Editing

A set of powerful new technologies that allow precision editing of genomes has been developed. These have multiple potential applications in pulmonary hypertension research and therapy, from understanding the specific variants arising from "omics" studies to designing cells for targeted drug delivery applications, to the repair of mutations. Two relatively new technologies allow targeting of specific nucleotides: TALEN and CRISPR; each of these has multiple related technologies (51). TALEN, and the related technologies of zinc finger nucleases (52) and MegaTAL (53), use modular protein-based sequence recognition, whereas CRISPR uses RNA-guided sequence recognition. Although variations on these technologies are likely to develop over time, the core technologies are unlikely to change.

Transcription activator–like (TAL) effectors were derived from the plant pathogen *Xanthomonas* in 2007 (54), with

a nuclease function for gene editing added in 2010 (55). The combination, TAL effector–like nucleases (TALENs), consist of a modular array of TAL recognition sequences fused to a *Fok*I nuclease (56). These are inserted in pairs, one for each strand, and work as a dimer to create double-stranded breaks in specific DNA sequences.

The components of the other major method of making targeted cuts in the genome, CRISPR–Cas9, are also derived from bacteria and archaea, in which they are part of a viral defense system (57). It consists of clustered regularly interspaced short palindromic repeats (CRISPR), which bind a guide RNA and an associated endonuclease (Cas9). Binding specificity is thus dependent on RNA–DNA interaction strength. The main advantage of CRISPR–Cas9 over the TALEN-based technology is its speed of production and extremely low cost; its disadvantages, as discussed later, are a likely inherently lower specificity, and the legal challenges that have not been resolved as of this writing (58).

Fundamentally, TALEN and CRISPR both cause site-specific cleavage, which allows gene editing through two mechanisms: homologous recombination (HR) and nonhomologous end joining (NHEJ). When TALEN or CRISPR make the site-specific double-stranded break, DNA repair mechanisms are employed to repair the break. If a piece of DNA matching the sites flanking the cleavage site is available, the cell can use HR to repair the damage. If no such DNA is available, the cell will use NHEJ.

In NHEJ, although the DNA is rejoined, there is often a deletion or insertion of a small number of nucleotides. If the cleavage site was in the middle of a coding sequence or essential regulatory element, this will serve to destroy gene expression or function. Without addition of a homologous recombination template, then, TALEN and CRISPR are capable only of truncation or knockout, not precision edits. However, the efficiency is relatively high—successfully transfected cells will have DNA insertions or deletions at the target site in 10–60% of cells (59).

Homologous recombination is much more powerful. In HR, the cell is provided with a DNA template to repair the damage caused by TALEN or CRISPR, which precisely matches the surrounding area, aside from the specific gene edits desired. Historically, this technique has been used for decades to create precision gene-edited mice. However, without use of TALEN or CRISPR to drive local DNA repair mechanisms, the efficiency was extraordinarily low (less than 1 in 1,000,000 cells) and even then required DNA arms that precisely matched the surrounding sequence with total lengths approaching 10,000 bp. This was possible only in specific strains of clonal mice for which libraries of DNA were available. With TALEN and CRISPR, the efficiency is dramatically higher (between 1 in 100 and 1 in 1,000 cells), and the size of the homology arms can be much smaller (fewer than 1,000 bp on each side) (60).

In PAH research, the usefulness of these techniques is clear. In the past, we were limited to viral or plasmid-based expression of mutations, in which regulation and expression levels were generally completely nonphysiological. With these new techniques, we can easily and rapidly create cell lines with the precise mutations arising from our "omics," sequencing, and genome-wide association study approaches, to allow testing of the effects individually and in combination with the exact alterations identified in our studies. Moreover, because all of these variations can be created on the same background line, they are perfectly controlled.

Further, there are several therapeutic possibilities derived from gene editing. These make use of the strong evidence that several varieties of circulating cells, including endothelial progenitor cells and a variety of bone marrow–derived cells, are found in the vicinity of the pulmonary vascular lesions and may play a role in disease etiology or progression (61). Closest to the clinic is use of cell-based therapies for drug delivery. In the PHACeT (Endothelial Nitric Oxide Synthase Gene–Enhanced Progenitor Cell Therapy for PAH) trial, endothelial progenitor cells were transfected with endothelial nitric oxide synthase (eNOS) and introduced into patients (62). Short-term hemodynamic benefits were not sustained at 3 months but there were persistent improvements in 6-minute walk test distance. Most importantly, the transient transfection strategy resulted in detectable eNOS expression for only about 1 week. One might imagine that using gene-editing approaches would result in longer term delivery of eNOS, and would be safer than other stable gene therapy approaches such as viral integration, because potentially dangerous random integration effects are removed. The safety and some efficacy of this initial trial provide promise for future targeted delivery of eNOS, or potentially other proteins.

Another possibility, however, is correction of known deleterious mutations. There is evidence from several groups that bone marrow cells alone are sufficient to drive PAH, including our finding that control bone marrow–derived stem cells can reduce the PAH phenotype when transplanted into lethally irradiated *Bmpr2* mutant mice (63, 64). This suggests the future possibility of autologous bone marrow transplantation after edited correction of stem cells as a therapeutic possibility; that is, correction of the mutation from the stem cells of a *BMPR2* mutation carrier with PAH could be a therapeutic approach (Figure 3) (64). However, this is technically challenging for a variety of reasons, including the need for high efficiency, the need to avoid off-target effects, and the need for high speed to avoid genomic instability or terminal differentiation of stem cells.

The need for high efficiency is a particularly difficult problem as homologous recombination has an efficiency of at best 1%. Some sorting or selection technology must be employed to identify the cells that have been correctly edited, ideally without leaving any sorting markers embedded in the cell. Transposase-based approaches such as Piggybac have been used for successful footprint-free gene editing in, for instance, correction of cystic fibrosis mutations in human induced pluripotent stem cells (65), and so the problem is solvable, but becomes difficult when combined with the need for rapid editing.

Because CRISPR–Cas9 uses RNA–DNA interactions for its specificity, it inherently has difficulty with off-target effects (66). Numerous methodologies to reduce these off-target effects have been proposed, and can reduce these effects under carefully controlled circumstances (67).

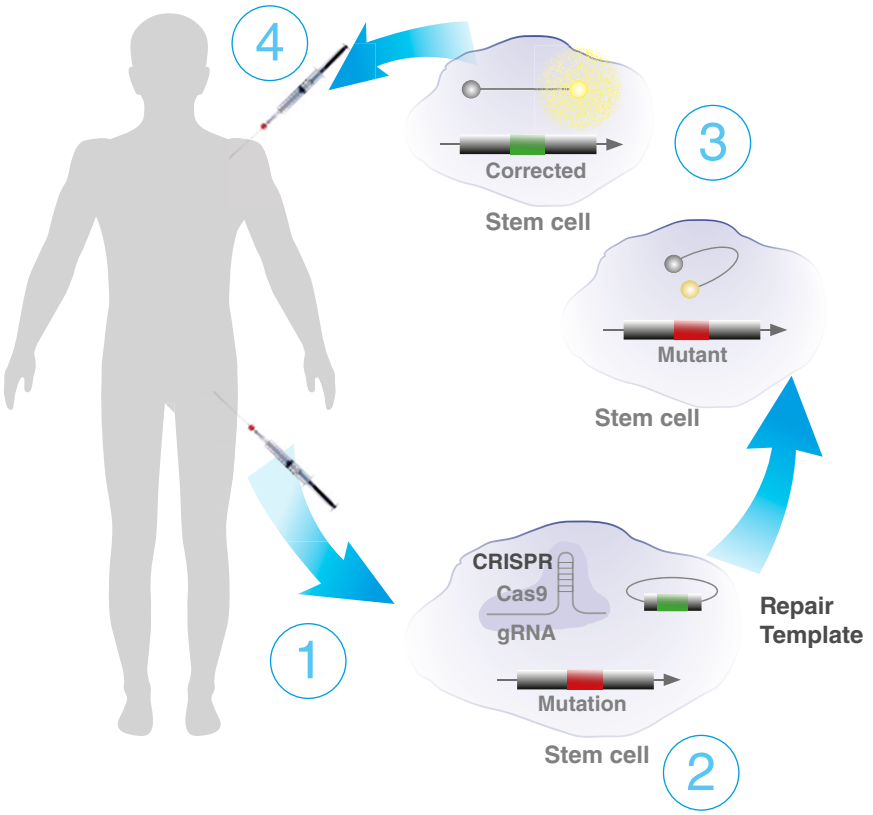

Figure 3. Schematic of the use of gene-editing techniques to repair a known gene mutation as a therapeutic approach, in this case demonstrating the manipulation of a patient's own stem cells. (*1*) CRISPR–Cas9 can be used with homologous recombination to repair a bone morphogenetic protein receptor type 2 (*BMPR2*) mutation for autologous transplant in patient bone marrow. (*2*) Bone marrow is extracted from the patient and sorted for stem cells. Stem cells are transfected with CRISPR–Cas9 guided to the mutation site, with homologous recombination DNA containing corrected sequences. (*3*) Small-molecule nonhomologous end-joining blockers can be used to increase the efficiency of homologous recombination. (*4*) Molecular probes that fluoresce only when bound to the corrected RNA can be used to sort for cells that have had their mutations corrected. Corrected stem cells are reintroduced into the patient. This process is still a hypothetical approach to correct stem cells with a *BMPR2* mutation that may promote pulmonary arterial hypertension; there are a number of technical barriers that must be overcome as described in text. Cas9 = CRISPR-associated protein 9; CRISPR = clustered regularly interspaced short palindromic repeats; gRNA = guide RNA.

Nonetheless, in actual practice, there are substantial fidelity and specificity issues, as demonstrated by the detailed analysis done in one study in which CRISPR was used to edit human preimplantation embryos (68). Although TALENs theoretically could have off-target cleavage, this is much less of an issue for current-generation TALENs. Several studies using TALEN for gene editing have failed to find any evidence of off-target mutations (69, 70).

Finally, gene editing must be done in such a way as to preserve the primitive, long-term repopulating cell populations in hematopoietic stem and progenitor cells, which will require either improved understanding of the signaling factors needed to preserve the stem state, or greatly increased speed (at present, editing by homologous recombination takes months). A number of groups are working on these issues, for instance, by delivering the gene-editing components through viruses (71). This could also hold promise for the targeted correction of somatic lung cells with spontaneous mutations due to increased mutagen sensitivity, which may be a feature of PAH (72–74). The use of gene editing has yet to reach the bedside, but this rapidly evolving field may provide great opportunities for therapeutic approaches in the near future.

Conclusion

It is an exciting new era for PH-focused clinicians, researchers, and patients. PH-specific mutations identify individuals with substantial risk of disease, and provide an opportunity to provide more in-depth information about disease severity and progression *a priori*. However, much work remains to broaden our understanding of how rare variations in specific genes associate with PAH in some but not all cases. For example, the clinical value of a mutation in a PH-specific gene is currently limited by the reduced penetrance and broad age at onset across a lifetime—there is a critical need to identify the additional susceptibility and resiliency factors that modify disease penetrance and expression.

It is likely that those factors will be involved in other forms of pulmonary hypertension, in terms not only of pathogenesis, but also sensitivity to therapeutics, performance of the right ventricle under stress, and other functions. Traditional and novel technologies must be employed and merged to facilitate a broader understanding of these molecular and other factors that coalesce to promote and perpetuate the PAH state. Also, we must recognize that a "one size fits all" approach to therapeutics must be abandoned in favor of a more detailed molecular understanding that facilitates the precise selection of treatment approaches for each individual. This will require investigators to incorporate sample collection into future clinical trials consistent with the need to determine molecular or clinical characteristics that segregate responders and nonresponders. Exciting new approaches are on the horizon, such as gene-editing techniques, with tremendous potential to accelerate progress at the bench, and bedside, with roles such as cell-based drug delivery and mutation correction. The era of precision medicine is truly here (38); it is time for prevention and treatment strategies that make individual variability the priority in PH. ■

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