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Viruses in Pharmaceutical Research: Pulmonary Vascular Disease

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Abstract: The management and understanding of pulmonary arterial hypertension (PAH) has undergone something of a revolution in the last 10 years, with new pharmacological agents entering routine clinical practice and significantly improving outcomes. Nevertheless many patients ultimately progress, and additional new treatment approaches are needed. There is now greater understanding of the molecular and genetic basis for the development of PAH, specifically in regard to the role of bone morphogenetic protein receptor 2 (BMPR2) signaling and related pathways. The challenge is to determine whether these new discoveries can be exploited for new therapies. In this article the role of viruses as tools for gene delivery for pulmonary vascular disease is discussed. Gene delivery of BMPR2 has now been shown to ameliorate the development and progression of PAH in animal models, thereby identifying this approach as a therapeutic target.

Keywords: Adenovirus; pulmonary hypertension; bone morphogenetic protein receptor

Introduction

There is an ever-increasing body of knowledge concerning the association between genetic variability and disease processes. For medical science to best take advantage of this knowledge there is a need for further investigation to distinguish important causal links from epiphenomena. From there, many further steps are required to translate this new knowledge into therapeutic advances. This transition can be facilitated through studies of genetic manipulation which can identify new pathways for therapeutic development. These pathways may then be targeted through a variety of approaches including standard pharmaceuticals, biological agents or gene-based therapies. These gene manipulation studies can be approached using a wide range of strategies including the development of transgenic animals or exogenous gene delivery. In this latter regard, the capacity of viruses to efficiently deliver genes to cells in vitro and in vivo can be exploited.

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Pulmonary Arterial Hypertension

The last ten years has witnessed an enormous expansion of interest in pulmonary vascular disease, specifically around pulmonary arterial hypertension (PAH). PAH is a disease characterized by abnormal pulmonary vascular remodeling: endothelial and smooth muscle proliferation and intimal hyperplasia.[1,2] Physiologically, the disease is defined as a mean pulmonary artery pressure greater than or equal to 25 mmHg, with a normal pulmonary capillary wedge pressure (the latter to rule out left heart disease leading to secondary increases in pulmonary pressures).[3] PAH leads to cardiorespiratory death through progressive ventilation−perfusion mismatching and right heart failure. Much of the recent

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interest in PAH has been driven by the development of new pharmacological strategies that have had a significant impact on patients' quality of life and survival. Randomized controlled trials show benefits from the use of endothelin receptor antagonists, phosphodiesterase inhibitors and new prostacyclin analogues, and international guidelines using these agents alone or in combination have been devised.[4−7] However, despite all this, patients often ultimately continue to progress on therapy and may require lung transplantation or succumb to the disease.[8,9]

Genetics and PAH: Role of BMPR2

Concurrent with the development of pharmaceutical agents that focus primarily on downstream events in PAH pathogenesis, there have been important new discoveries concerning the genetic basis of the disease. The principal finding has been the discovery that heterozygous mutations in the gene for bone morphogenetic protein receptor type 2 (BMPR2) are linked to the development of hereditary PAH.[10,11] BMPR2 is a member of the TGFβ superfamily of receptors and has critical roles in embryongenesis and cellular proliferation (homozygous knockout in mice is a congenitally lethal mutation). A large number of mutations have now been described in connection with PAH. In essence these mutations lead to a downregulation of BMPR2 signaling. The mutations may result in the production of abnormal proteins which are degraded (lead to reduced expression via haploinsufficieny), or the mutations may lead to the production of proteins that retain the capacity for ligand binding but lack intracellular signaling, thereby leading to a dominant negative effect.[12,13] As knowledge of the mutations underlying hereditary PAH has grown, many apparent "sporadic" cases are being reclassified as hereditary. A number of conditional BMPR2 knockout mouse models have now been developed in which the animals are confirmed to have an increased propensity to develop PAH.[14−17]

In vascular smooth muscle, BMPR2 signaling leads to reduced cellular proliferation and induction of apoptosis and acts to retain smooth muscle cells in a contractile rather than secretory phenotype.[18] Thus, reduced BMPR2 signaling

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would fit with the abnormal cellular proliferation seen in PAH. In endothelium, however, there is some evidence that BMPR2 signaling has a protective effect against apoptosis.[19] Increased endothelial apoptosis is postulated to eventually lead to the emergence of apoptosis-resistant clones to account for the proliferative lesions seen in PAH. These proposed mechanisms remain speculative at this time. BMPR2 mutations alone are not sufficient to develop clinical disease as only around 20% of patients with mutations develop PAH (ie reduced penetrance).[20] Therefore, some additional, as yet undefined, "second hit" is required.

Under normal circumstances BMPR2 expression in the lung is primarily seen on the pulmonary vascular endothelium, and is also present on smooth muscle and epithelium.[21] Importantly, there is now a significant body of work showing that BMPR2 expression is also downregulated in the setting of secondary pulmonary hypertension even in the absence of BMPR2 mutations (for example in congenital heart disease, and in the commonly used animal models of PAH due to hypoxic challenge or the endothelial toxin monocrotaline).[21,22] This therefore means that BMPR2 signaling likely has important relevance in a broad range of clinical settings beyond simply in cases of BMPR2 mutation alone.

Given all of the foregoing, there is a clear rationale to explore upregulation of BMPR2 signaling as an experimental approach to further understand the relevance of the pathway to disease, and to identify strategies that could exploit this genetic knowledge for the development of new treatments. To achieve this, in vivo studies are critical. One strategy to increase the expression of a membrane bound receptor in vivo is through gene delivery, and the efficiency of viruses as in vivo gene delivery vehicles makes them an attractive platform. Given the predominance of BMPR2 expression on pulmonary vascular endothelium, targeted gene delivery to this cell type is a rational approach.

Vector Engineering for Pulmonary Vascular Gene Delivery

Our group has focused primarily on the use the adenovirus (Ad) as a gene delivery platform. An early study comparing Ad vectors with nonviral gene delivery to pulmonary vessels using catheters had shown Ad to be around 100-fold more efficient.[23] This agent has generally good in vivo gene delivery characteristics, but a great deal of work has been done to improve upon this through strategies to retarget the virus to specific cells.[24] The degree of cell-specific targeting needed should be considered in light of the nature of the gene and gene product being investigated. For example, genes that express secreted proteins might achieve a useful effect in a locoregional context where actual cell-specificity is less critical. Thus, several studies have used untargeted Ad via the inhalational route (see below). For proteins that remain cell associated (such as receptors), more attention to cell-specificity is needed. For BMPR2 gene delivery to endothelium, good transduction of the endothelial cells themselves is required.

The native Ad infects cells primarily through an interaction between the viral knob domain and the Coxsackie and adenovirus receptor (CAR).[25] Therefore, strategies to retarget virus through manipulation of the viral knob domain have dominated Ad targeting research. More recently, it has been discovered that interactions between Ad capsid proteins and coagulation factors in the blood are largely responsible for uptake of virus into the liver.[26] This has been a critical development in our understanding because viral sequestration into the liver is the major limiting factor for the use of Ad via systemic injection for nonhepatic targets. Genetic modifications to viral capsid proteins have been performed which achieve some "untargeting" of the liver.[27,28] Such approaches in combination with cell-specific ligand targeting should see greater improvements in gene delivery efficiency.

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To achieve improved gene delivery to pulmonary vascular endothelium we developed an approach to target to angiotensin converting enzyme (ACE), which is highly expressed on pulmonary endothelial cells. Although ACE is expressed elsewhere, it is the accessibility and magnitude of ACE expression in the lung which make it an attractive target.[29] Further, it has been shown that ACE is upregulated in areas of vascular remodeling in the context of PAH.[30] For our targeting approach we developed a bispecific targeting molecule which contains the Fab fragment of an anti-Ad knob domain antibody linked to a monoclonal antibody against ACE (9B9).[31,32] The concept was to block knob−CAR interactions thereby reducing off-target transduction and increase pulmonary endothelial gene delivery. The antibody 9B9 has been shown to have excellent pulmonary targeting abilities.

To investigate the utility of the targeting conjugate (designated Fab-9B9) a number of validation studies were performed. For all of these studies, the virus is simply preincubated with conjugate, and then the mixture is used for the application (whether in vitro or in vivo). Using CHO cells that were stably transduced to overexpress ACE we confirmed that Fab-9B9 significantly increased cell transduction in an ACE-specific manner. In vivo, injection of Ad-Fab-9B9 via the tail vein of rats significantly increased reporter gene expression in the lungs while reducing expression in nontarget organs such as the liver. Endothelial cell-specificity of gene delivery was confirmed at the electron microscopy level.[31]

Although we have found ACE to be a useful pulmonary-selective transduction target, a number of other vascular markers have been proposed or are being developed. The powerful strategy of using in vivo phage display has defined a number of peptides with pulmonary targeting capabilities that may potentially be adapted to viral gene delivery either through their incorporation into targeting adapters or by direct genetic incorporation of these sequences into the viral capsid.[33] As a proof-of-principle we evaluated an adenovirus that had an integrin-targeting RGD motif introduced into knob domain. Although not specific, this virus did have enhanced ability to transduce endothelial cells in culture, and upon systemic administration it showed a differential transgene expression profile compared to unmodified virus, which included enhanced gene expression in the lung.[34]

Technological improvements have also been made in regard to targeting adapters. Specifically, fusion proteins constructed by linking soluble CAR to targting motifs have achieved enhanced pulmonary gene delivery in mouse models.[35,36] Such recombinant techniques may be more readily progressed to develop molecules suitable for clinical use than chemically cross-linked antibodies.

Using gene delivery as an experimental platform provides an opportunity to control expression not only through transductional manipulation but also through transcriptional control. Thus, we investigated candidate endothelial-specific promoters and found that the flt-1 promoter had the desirable features of high endothelial cell activity and low activity elsewhere, particularly in hepatocytes.[37] Combination of transductional and transcriptional targeting has the capacity to substantially improve cell-specificity of gene expression (Figure 1).[38] As noted, further vector modifications to the capsid to reduce liver sequestration can also be incorporated into the system to potentially still further improve target versus off-target gene expression ratios: such an approach is currently under development. Vector platforms combining transductional and transcriptional variations provide a flexible mechanism for evaluating the consequence of target and off-target gene expression.

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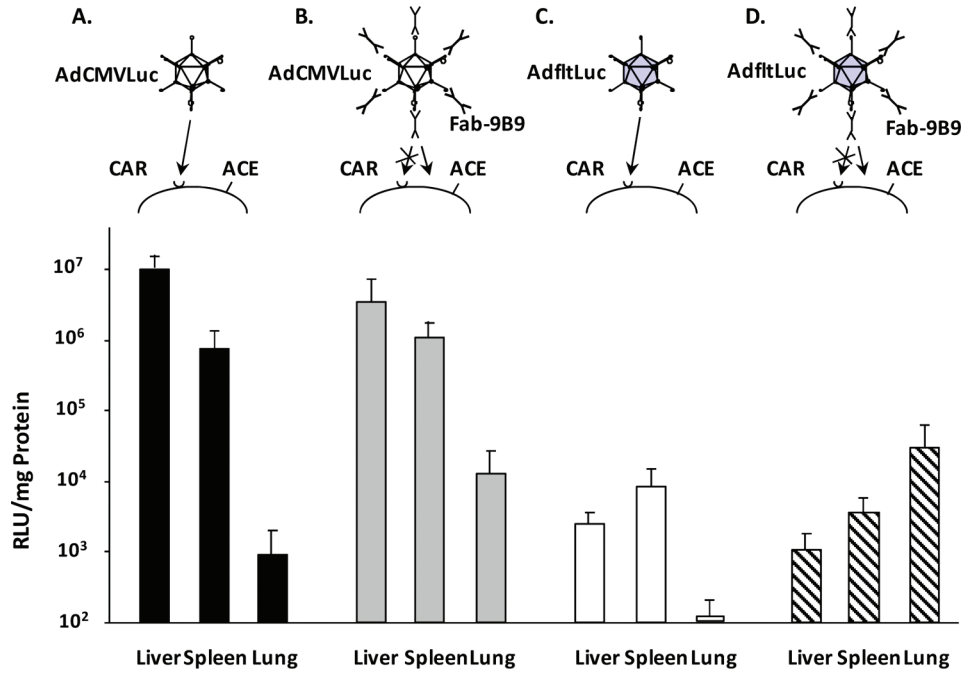

Figure 1. An illustrative example of improvements in pulmonary vascular gene delivery with vector development. Rats were injected with Ad vector containing the luciferase reporter gene; then luciferase expression (relative light units, RLU) was assessed in target and nontarget organs. Unmodified vector leads largely to liver and spleen transduction, with very little expression in the lung (A). Use of a targeting conjugate improves lung gene expression, but although liver expression is reduced it remains high (B). Use of the endothelial-specific flt-1 promoter reduces expression in nontarget organs, but expression is low in lung due to low transduction (C). Combined transductional and transcriptional control gives the most favorable lung selectivity (D). The system could potentially be further improved by capsid modifications to the vector to reduce liver uptake. Adapted with permission from Reynolds PN et al., *Nat. Biotechnol.* **2001**, *19*, 838−42 (ref. 38). Copyright 2001 Nature Publishing Group.

While the adenovirus has a number of favorable attributes for in vivo gene delivery, it also has several shortcomings. Beyond issues of transduction selectivity and efficiency is the issue of duration of gene expression. Ad delivered genes are only capable of transient gene expression due to the fact that the delivered genes are not integrated into the host DNA, and, especially with early generation vectors, there is an issue of low level expression of viral proteins leading to activation of immune responses and loss of transduced cells. To help address this latter problem, helper-dependent Ads have been developed that are deleted for all viral sequences except inverted terminal repeats and the packaging sequence.[39] These agents are much less immunogenic and have led to transgene expression for many months in various animal models. Although these agents have not been specifically assessed in pulmonary vascular disease, recent data does show that they have much less of a "collateral" stimulatory effect on endothelial cells transduced in vitro.[40] The adaptive immunological response to translated viral proteins is only part of the problem however. The outer coat of viral vectors is subject to attack from preformed antibodies that may have developed as a result of exposure to wild-type virions of the same serotype as that used to construct the vector. To help address this issue, vector platforms have been developed that use serotypes of adenovirus least commonly encountered through natural exposure. Even so, antibodies will be generated after first exposure to the new vector, and the transient gene expression achievable with adenovirus means that repeated delivery may be necessary to sustain a useful clinical effect. This challenge may be addressed perhaps by temporary immunosuppression at the time of vector administration or by switching serotypes of virus.

An alternate approach to achieving longer term transgene expression is to use a vector platform that achieves integration of the delivered gene into the host chromosome. Standard retroviral vectors are limited by their capacity to achieve transduction only in dividing cells and will not achieve efficient transduction of pulmonary vascular cells except perhaps theorectically in proliferating vascular lesions, although no reports illustrating this have been published. Lentiviral vectors are potentially a more attractive option as they are not limited to the transduction of proliferating cells. The development of targeting technology has been applied to lentivirus particularly in the context of endothelial cells in culture and for tumor vasculature but not at this stage for

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pulmonary vascular disease.[41−43] While this approach has an attraction, there remains a concern about insertional mutagenesis with integrating vectors, although newer advances with self-inactivating vectors may reduce this risk.[44]

All animal studies have been of short duration, a few weeks at best. The actual duration of transgene expression required to achieve a useful impact in PAH is not known. It is highly likely to be dependent on the actual gene being delivered, and thus no generalization should be made. It should be recalled in the context of BMPR2 that a mutation alone is not sufficient to cause clinical disease (reduced penetrance) and that a "second hit" of some sort is required. It is thus unknown whether even a temporary upregulation of BMPR2 signaling might interrupt a biological cascade and provide some lasting benefit.

Viruses have also been used to enhance the efficacy of nonviral vector platforms, specifically in the context of complexes of haemagglutinating virus of Japan (HVJ)-liposomes. This platform is not specifically targeted but has achieved positive physiological outcomes in animal models of PAH when used to deliver genes that have a "bystander" effect when administered by tracheal instillation or via hepatic transduction.[45−47] This approach also leads to transient expression.

Physiological Outcomes of Pulmonary Vascular Gene Delivery

Having developed a vector platform that achieved good pulmonary endothelial gene delivery, we then embarked upon physiological studies. Given the uncertainties about the potential impact of BMPR2 gene delivery, we wished to first evaluate our vector approach using a gene already established to have a therapeutic impact in PAH models. Part of the rationale also was that it is known that first generation Ad vectors have pro-inflammatory properties and that there was at least a potential risk that the impact of the virus itself in endothelium could confound the interpretation of the effects of our gene under investigation. In this first round of studies we used an Ad vector containing the gene for endothelial nitric oxide synthase (eNOS). Rats were injected with either a control vector (AdCMVCEA), an eNOS vector (AdCMVeNOS) or the eNOS vector plus the Fab9B9 targeting conjugate and then placed in a hypoxic chamber (10% oxygen) for three weeks. Vascular pressures were then measured.

Chronic hypoxia produced a significant increase in right ventricular systolic pressure (RVSP) compared to the normoxic group (Figure 2). Animals given AdCMVCEA demonstrated similar hypertensive response in RVSP compared to the hypoxic control group. However, Ad CMVeNOS treatment in chronic hypoxia significantly attenuated RVSP changes compared to AdCMVCEA animals. Moreover, when AdCMVeNOS was targeted to the pulmonary endothelium (AdCMVeNOS+Fab-9B9) a further significant reduction in RVSP was seen compared to the untargeted AdCMVeNOS (unpublished observations).

This study not only illustrated that our vector approach did not confound the therapeutic impact of a gene with established efficacy in PAH but also showed that the targeting approach actually improved the outcome. This improvement was consistent with the impact we had seen using targeted vascular gene delivery in a model of systemic hypertension.[48]

Given this result we then progressed our studies of our primary gene of interest, BMPR2. A vector containing the BMPR2 gene under the control of the CMV promoter was constructed. The BMPR2 gene contained a myc tag to facilitate immunohistochemical detection of the delivered versus native receptor. In vitro validation studies confirmed that transduction achieved BMPR2 expression by Western blot, that this was membrane localized by immonhistochemistry, and that the receptor was functional as determined by a SMAD signaling assay (one of the downstream mediators of BMPR2 signaling).[49]

Gene delivery of BMPR2 was first evaluated in the rat hypoxia model. Rats were injected with either a control

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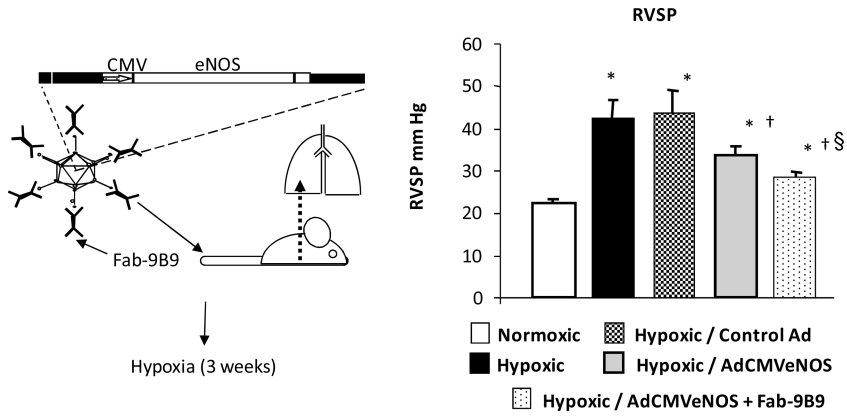

Figure 2. Strategy to evaluate endothelial gene delivery in the rat hypoxia model. Rats injected with vector or controls as shown, then exposed to hypoxia (10% oxygen) for three weeks. Right ventricular systolic pressure (RVSP) among the five treatment groups. Values are mean ± SE, $n = 5-6$ per group. $^{*}P < 0.05$ for normoxia vs all hypoxia treatments. $^{\dagger}P < 0.05$ for hypoxia-AdCMVCEA vs hypoxia-AdCMVeNOS and hypoxia-AdCMVCEA vs hypoxia-AdCMVeNOS+Fab-9B9. $^{\S}P < 0.05$ hypoxia-AdCMVeNOS vs hypoxia-AdCMVeNOS+Fab-9B9, i.e. untargeted vs targeted eNOS.

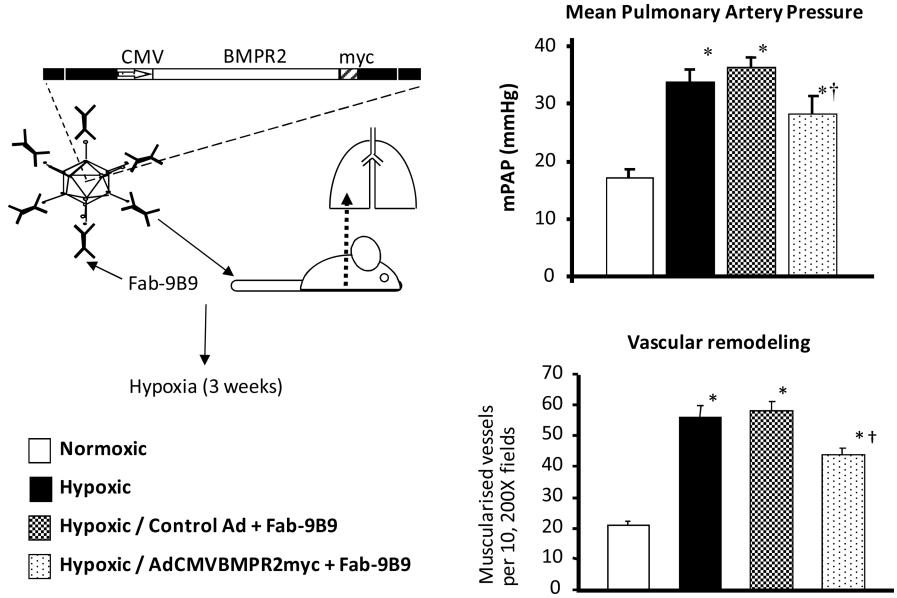

Figure 3. Rats were administered targeted vector via tail vein injection prior to hypoxic challenge for 3 weeks. BMPR2 led to reduced pulmonary artery pressure and mucularization of distal vessels. $^{*}p < 0.05$ versus normoxic, $^{\dagger}p < 0.05$ versus hypoxic Ad control. Adapted with permission from Reynolds AM et al., *Am. J. Physiol.* **2007**, *292*, L1182−92 (ref 49). Copyright 2007 the American Physiological Society.

vector or AdCMVBMPR2myc each together with Fab-9B9 then placed in a hypoxic chamber for three weeks, along with uninjected rats.

As before, chronic hypoxia produced a significant increase in RVSP mean pulmonary artery pressure (mPAP) compared to the normoxic group. Animals given control Ad+Fab-9B9 had similar hypertensive responses in RVSP and mPAP compared to the hypoxic control group. AdCMVBMPR2myc+Fab-9B9 treatment significantly attenuated RVSP and mPAP changes compared to control Ad+Fab-9B9 animals (Figure 3).[49]

Chronic hypoxia induced marked right ventricular hypertrophy, as assessed by RV weight over septum + left ventricle (S+LV), compared to normoxic animals. The degree of hypertrophy was unchanged in control Ad+Fab-9B9 treated animals but was significantly reduced by BMPR2 gene delivery by approximately 20%.[49]

Histological analysis of lung sections from the rats showed that BMPR2 gene delivery was associated with reduced muscularization of distal vessels and reduced cellular proliferation. Because it has been proposed that endothelial BMPR2 signaling may protect against endothelial apoptosis in vivo, we assessed apoptosis by staining for cleaved caspase 3, but in fact found increased apoptosis with BMPR2 gene delivery. We further assessed the impact of BMPR2 gene delivery on endothelial apoptosis using cultures of human pulmonary microvascular endothelial cells, but again found increased apoptosis. In vivo, even when assessing hypoxia-

induced endothelial apoptosis at early time points we could not find a protective effect of BMPR2. Thus, the mechanism by which our BMPR2 gene delivery is acting to reduce vascular remodeling requires further study but may relate to reductions in endothelially derived mediators or possibly an impact on endothelial to mesenchymal transition (EndMT) .

The impact of BMPR2 gene delivery on hypoxia-induced PAH implies that targeting this pathway could be of benefit in PAH-related disorders other than those simply due to BMPR2 mutations. However, to strengthen the case further, we evaluated the impact of BMPR2 gene delivery after first inducing PAH by hypoxia, and also extended the studies to the monocrotaline-induced PAH model which has an inflammatory basis. Targeted BMPR2 was injected after first inducing PAH with three weeks of hypoxia, then subjecting the animals to a further three weeks of hypoxic challenge. In separate studies we induced PAH with monocrotaline, then administered vector ten days later and assessed the animals ten days after that.[50] In the hypoxia model BMPR2 treatment (compared to control viral vector) resulted in a 29% fall in total pulmonary vascular resistance, and a 20% increase in cardiac output. In the monocrotaline model BMPR2 treatment resulted in a 38% fall in total pulmonary vascular resistance and a 22% increase in cardiac output. In both models, histological indices of vascular remodeling were reduced.[50]

Our work with BMPR2 contrasts with that of McMurtry et al., who also developed an Ad vector containing the BMPR2 route but administered it by aerosol.[51] In so doing they demonstrated vascular smooth muscle gene delivery. However, no therapeutic physiological response was achieved. Due to the differences in methodology, the basis of the differences to our work is not clear but it obviously raises the possibility that endothelial BMPR2 expression in this context may be more important in disease pathogenesis than is smooth muscle expression. Potentially, using viral vectors could allow one to compare and contrast the impact on disease of targeting distinct populations. At this time we have not been able to reproduce vascular smooth muscle gene delivery via the aerosol route (we see only epithelial transduction; unpublished observations). Beyond BMPR2 gene delivery, delivery of genes coding for BMP ligands (e.g. BMP2, BMP7) may also be a useful approach. Although this has not yet been reported in a PAH model system, Ad-mediated BMP-2 gene delivery has been shown to reduce smooth muscle proliferation in a carotid artery injury model.[52]

The discussion around BMPR2 gene delivery reflects the original notion of gene therapy as an approach to treat inherited genetic diseases via the replacement of a defective gene. The concept of gene therapy has long since moved beyond this to a concept of using "genes as medicines". The possibility of using BMPR2 upregulation in settings other than those of BMPR2 mutation would fall into that category. There are several other approaches that have evaluated gene delivery to modify downstream events in vascular remodeling. Apart from our work, all other studies using Ad have administered the vector by inhalation. Tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) was shown to improve MCT-induced PAH but actually to worsen PAH due to hypoxia.[53,54] These studies provide an important note of caution in trying to extrapolate findings from model systems to the human clinical context. McMurtry et al. delivered the gene for a dominant negative survivin molecule which reduced cellular proliferation and showed significant improvements in established PAH in the MCT model.[55] Improvements in the MCT model have also been seen with adenoviral-mediated gene delivery of extracellular superoxide dismutase.[56] Benefits have been seen in the hypoxia model with gene delivery of potassium channel Kv1.5.[57] Using a pulmonary fibrosis model Farkas et al. showed reduced PAH with administration of the gene for vascular endothelial growth factor (VEGF).[58] This was associated with reduced endothelial cell apoptosis but also led to increased fibrosis.

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Conclusion

The studies discussed illustrate the utility of viral vectors to explore and potentially develop new therapies based on genetic discoveries. Using these vectors as tools of discovery is highly useful, although as noted one must always be cognizant of the impact of the vector itself and ensure appropriate controls are in place. Further technological improvements are already taking place which will improve upon these tools. Improvements include the development of more consistent targeting adapter molecules (e.g. fusion proteins between recombinant CAR and targeting moieties including single chain antibodies), the development of viruses with direct genetic incorporation of targeting ligands into the capsid structure, and capsid modifications to reduce hepatic sequestration and helper-dependent Ads. Advances in other viral vector platforms, particularly lentivirus, are also encouraging. Although our work to date has made use of rat models, some of the later targeting techniques are applicable to mice and thus expand the possibilities of examining the impact of gene delivery in transgenic animals. For gene therapy to progress to the point of clinical application for pulmonary vascular disease, new advances in vector technology will need to be incorporated.

Despite the challenges yet to be overcome to see viral gene delivery vectors become useful clinically in PAH, even in their present form they are useful tools in exploring the mechanisms underlying pulmonary vascular disease.

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