Repurposing FK506 to Increase BMPR2 Signaling and Improve Pulmonary Arterial Hypertension: A Fast Track From Cells to People

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To improve long-term survival in pulmonary arterial hypertension (PAH), new treatment approaches that target occlusive vasculopathy instead of vasoconstriction of pulmonary arteries are necessary. Identifying genes or pathways that unify different pathologies and etiologies in PAH is a crucial first step for drug development. The bone morphogenetic protein receptor 2 (BMPR2) pathway, originally described as the cause of familial PAH, has gained significant interest over the past few years as a potential master switch in PAH, and therefore presents a promising treatment target in PAH. FK506 (tacrolimus) was found in a high-throughput screen of US Food and Drug Administration (FDA)-approved drugs to significantly increase BMPR2 signaling. Low-dose FK506 was able to restore normal BMPR2 signaling and function in patient pulmonary artery (PA) endothelial cells, inhibit proliferation and induce apoptosis in PA smooth muscle cells, and prevent and reverse pulmonary hypertension (PH) in 3 experimental rodent models of PH. As an FDA-approved drug, it was possible to relatively quickly (in approximately 2 years) translate the basic science discoveries from the bench to the clinic. A Phase 2 proof-of-concept safety and tolerability trial is underway (*ClinicalTrials.gov Identifier: NCT01647945*) to evaluate the use of low-dose FK506 in PAH and to identify patients who might respond best to FK506 depending on their impairment in BMPR2 signaling.

Pulmonary arterial hypertension (PAH) is a devastating disease that affects patients of all ages, and if left untreated leads to right heart failure and death within 2 to 3 years.[1,2] The histopathology is characterized by occlusive vasculopathy of pulmonary arteries with dysfunction of endothelial and smooth muscle cells, as well as adventitial fibroblasts and inflammatory cells. Unfortunately, existing PAH drugs only dilate pulmonary arteries, while occlusive vasculopathy progresses unchecked until patients eventually need lung transplantation or die of the disease.[3-5] Therefore, there is a fundamental gap between current treatment for PAH and the need to reverse occlusive vasculopathy in PAH. Numerous molecular abnormalities have been described in PAH,[6] yet translation into the clinic has been hampered as respective therapies that target a candidate pathway propose only one treatment for one abnormality,[7] not taking into account different etiologies, pathologies, as well as multiple contributing factors in PAH such as the involvement of the immune system,[8] metabolism,[7] elastase activation,[9] and epigenetic mechanisms.[10-14]

THE IMPORTANCE OF BMPR2 SIGNALING AS A POTENTIAL "MASTER SWITCH" IN PAH

It has emerged that the bone morphogenetic protein receptor (BMPR2) pathway might link many of the "players" in PAH pathology, in that it either regulates or is regulated—is either a cause or a consequence of the above mechanisms[15-20]—which makes modulation of BMPR2 signaling a promising treatment approach.[21,22] Germline mutations causing BMPR2 loss of function have been described in about 80% of patients with hereditary PAH,[23,24] and mutations in other members of the transforming growth factor (TGF)-β superfamily have been found, such as aktivin-like kinase-1 (ALK1), endoglin, smad9, and caveolin-1, which also result in dysfunctional BMPR2 signaling.[21] Furthermore, even patients without a BMPR2 mutation have reduced BMPR2 expression in their pulmonary arteries at the time of lung transplantation.[25] There is evidence that patients with idiopathic or autoimmune-associated PAH with ongoing inflammation and increased levels of the cytokine IL-6 have lower BMPR2 expression in their blood, and it has been shown in cell culture that IL-6 reduces BMPR2 expression via a STAT3-miR17/92 mechanism.[16]

As further evidence that BMPR2 dysfunction might be the result of an altered immune system, it was shown that the viral proteins HIV-nef and HIV TAT, which are released by infected lymphocytes and macrophages during an HIV infection, decrease

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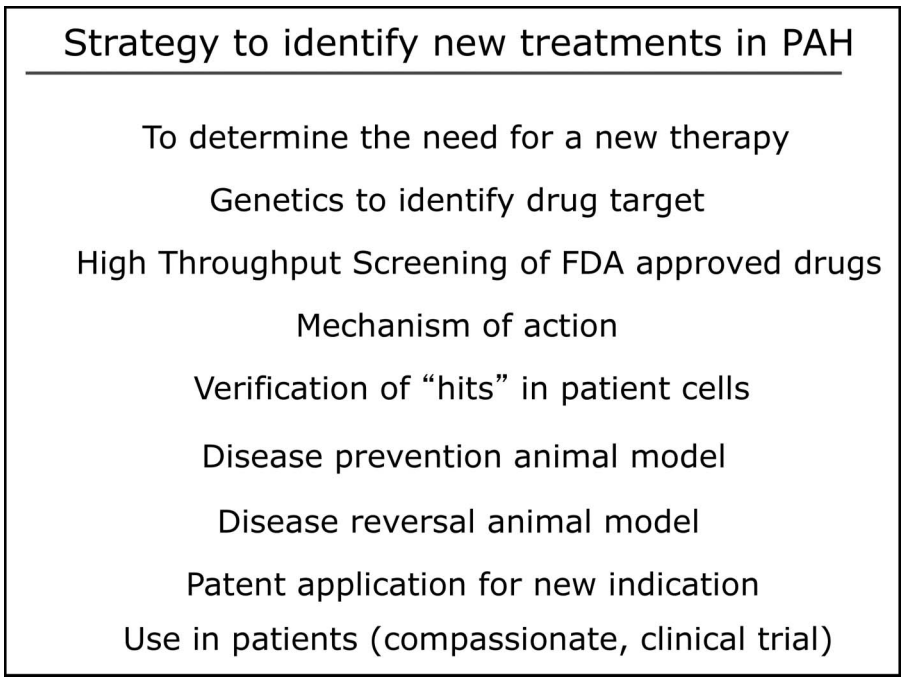

Figure 1: Strategy to identify new treatments in PAH. Figure 1 shows the drug development approach to finding new treatments in PAH—from the identification of a new drug target for PH based on a known genetic basis in PAH to the final testing of a repurposed drug in patients.

BMPR2 expression in pulmonary artery smooth muscle cells (PASMCs): HIV TAT by repressing the BMPR2 promoter[26] and HIV-nef potentially by interfering with the intracellular trafficking of BMPR2.[27] Macaques with infected chimeric simian/human immunodeficiency virus (SHIV) containing HIV-1-derived Nef protein develop severe pulmonary hypertension (PH) characterized by occlusive pulmonary vasculopathy mimicking the human disease.[28] The effect of an HIV infection on BMPR2 expression is even more pronounced in the presence of drugs such as cocaine,[29] commonly used by HIV-positive intravenous drug users. On the contrary, reconstitution of athymic rats with regulatory T cells prevents PH induced by the vascular endothelial growth factor (VEGF) receptor blocker SUGEN5416, and is associated with an increase in BMPR2 expression in pulmonary arteries.[17]

With regard to the role of BMPR2 in metabolism, recent data suggest that enhanced estrogenic activity decreases BMP signaling in a susceptible host, whereas altered BMP signaling modifies estrogenic activity.[30] BMPR2 is involved in the regulation of hepcidin gene expression and iron metabolism,[31] and most recently has been shown to be associated with insulin resistance[32,33] and impaired fatty acid oxidation in experimental PH.[34] In addition, metabolic reprogramming in PAH occurs downstream of BMPR2,[35] which is supported by studies in mice with a disease-causing mutation in BMPR2 that are more susceptible to oxidant injury in mitochondrial membranes compared to wild-type animals.[36] Furthermore, it was recently shown that the human neutrophil inhibitor elafin interacts with caveolin-1 to facilitate BMPR2 signaling and endothelial homeostasis,[37] linking elevated elastase activity to a reduced BMPR2 signaling. In addition, Drake et al showed that mutations in BMPR2 impaired normal micro RNA processing of miR-21 and miR-27a, which likely contributes to vascular cell proliferation in PAH.[38] These examples illustrate that BMPR2 signaling presents a critical pathway in PAH and a potential master switch linking different PAH pathologies and etiologies. We hypothesize that there is a threshold of BMPR2 signaling below which PAH develops, either due to a BMPR2 mutation, environmental factors, or modifier genes. Therefore, targeting the BMPR2 pathway could be a promising treatment approach in PAH (Figure 1).

INCREASING BMPR2 SIGNALING WITH FK506

We performed a high throughput screen of >3000 US Food and Drug Administration (FDA)-approved drugs and bioactive compounds using a reporter cell line where the BMP response element (BRE) from the Id1 promoter was linked to luciferase[22] to identify activators of Id1 expression and, respectively, BMPR2 signaling. FK506 (tacrolimus) was identified as the best BMPR2 activator. Even at low, sub-immunosuppressive doses (0.2-2 ng/mL), FK506 was able to bind to FKBP12, and by doing so removed the BMPR2 inhibitor FKBP12 from the type 1 receptors ALK1, ALK2, and ALK3, allowing for phosphorylation of the type 1 receptor and subsequent downstream smad dependent and independent signaling. In addition, FK506 inhibited the phosphatase calcineurin, which also facilitated phosphorylation of the type 1 receptor. Previous studies had shown that FKBP12 was necessary for SMURF-1 mediated degradation of type 1 and type 2 TGF-β receptors.[39] By removing FKBP12 from the type 1 receptor, FK506 is able to prevent SMURF-1 and smad7 mediated type 1 and type 2 receptor degradation, leading to an increase in BMPR2 protein

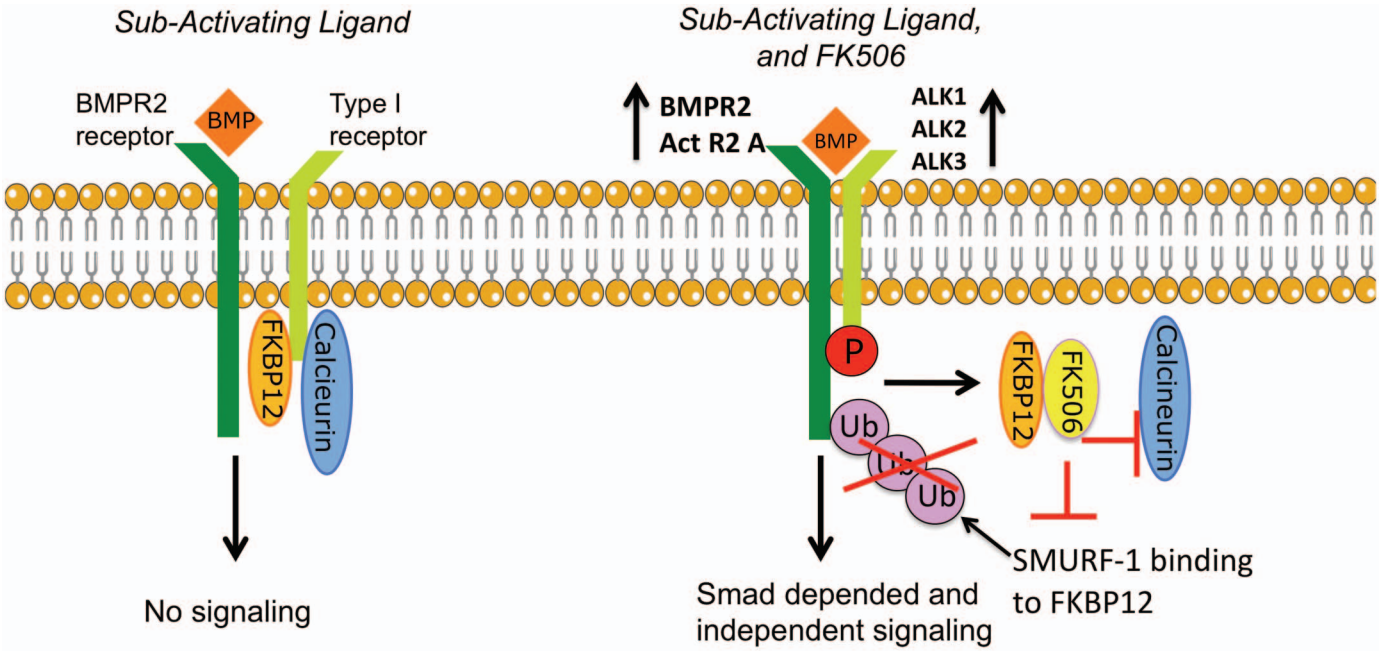

Figure 2: FK506 increases BMPR2 signaling through multiple avenues. The left panel shows that in the presence of a subactivating dose of the ligand BMP, phosphorylation of the type 1 receptor and subsequent downstream signaling is inhibited by binding of FKBP12 to the phosphorylation site of the type 1 receptor. Under the same conditions, yet with FK506 present (right panel), FK506 pulls FKBP12 away from the type 1 receptor. By doing so, it allows the type 1 receptors (ALK1, ALK2, ALK3) to become phosphorylated. Furthermore, binding of SMURF-1 to the receptor complex is prevented, as SMURF-1 requires FKBP12 to be present as a docking site. Consequently, SMURF-1 mediated degradation of the type 1 and 2 receptors is impaired, leading to an increased protein expression of BMPR2. As a third mode of action, FK506 inhibits the phosphatase calcineurin, facilitating phosphorylation of the type 1 receptor.

expression (unpublished data). FK506 was superior to rapamycin and cyclosporin in increasing BMPR2 signaling, due to its dual function as a calcineurin inhibitor and binder of FKBP12, as well as its ability to remove FKBP12 from all 3 type 1 receptors, ALK1, ALK2, and ALK3, which are implicated in BMP signaling (Figure 2).

FK506 PREVENTS AND REVERSES EXPERIMENTAL PH

Increasing BMPR2 signaling by low-dose FK506 (blood level 0.2 ng/mL) prevented the development of hypoxia-induced PH in mice with a conditional endothelial deletion of BMPR2 by improving endothelial health and preventing loss of small vessels.[22] Low-dose FK506 was also able to reverse established severe PH in rats exposed to the endothelial toxin monocrotaline by reducing medial hypertrophy. Furthermore, low-dose FK506 reversed severe PH and occlusive vasculopathy in rats where PH was induced by the VEGF receptor blocker SUGEN5416, followed by 3 weeks exposure to 10% hypoxia and 5 weeks normoxia. This animal model refined by Abe et al[40] mimics the clinical disease very well, as it is characterized by severe PH accompanied by an occlusive vasculopathy of small pulmonary arteries.

FK506 IN CLINICAL USE FOR PAH

FK506 has been used for more than 20 years as a potent immunosuppressive agent in solid organ transplantation. Low-dose FK506 is used in certain autoimmune diseases such as psoriasis and rheumatoid arthritis, again for its anti-inflammatory properties.[41] Furthermore, FK506 was found to increase endothelial expression of ALK1 and endoglin.[42] As loss-of-function mutations in these genes are observed in PAH associated with hemorrhagic hereditary telangiectasia (HHT), FK506 could also be of potential benefit in patients with this disorder. In fact, FK506 was given following liver transplant in a patient with HHT who had multiple arteriovenous malformations, and it was noted that internal and external telangiectases, epistaxes, and anemia disappeared, suggesting that the mechanism of action of FK506 involved partial correction of endoglin and ALK1 haploinsufficiency.[42]

We have treated 3 end-stage PAH patients with low-dose FK506 with very promising results (unpublished data). Subsequently, a Phase 2 proof-of-concept safety and tolerability trial was initiated at Stanford University to test whether low-dose FK506 treatment is feasible in PAH, and to identify patients who might benefit most from low-dose FK506 depending on their BMPR2 impairment (*ClinicalTrials.gov Identifier: NCT01647945*). One goal of this trial is to develop a "BMPR2 signature" in the blood of PAH patients at baseline and follow-up to first prove that the BMPR2 pathway is targeted by the study drug, and second to define and develop criteria that might reflect which patient subgroup potentially responds best to therapy—valuable information for the design of a subsequent efficacy trial.

THE ADVANTAGES OF DRUG REPOSITIONING IN PAH—FROM CELLS TO PEOPLE

Recycling is good for the environment and for drug development, as well. There are multiple advantages to repurposing existing drugs for the treatment of PAH. New drug development is a

long, expensive, and complicated process starting with research to determine targets for treatment. Once a promising compound has been identified, preclinical research is done, which often involves pharmacokinetic and toxicology studies, testing of the compound in model systems of disease such as PASMCs as well as in animal models of PAH. Once a compound has completed preclinical testing, it moves into human clinical trials. The purpose of a clinical trial is to test a new drug or other intervention for safety and effectiveness in treating the disease in question before it can be prescribed to patients. The institutional principal investigators and review boards, the Data Safety Monitoring Board, and the steering committees are charged with oversight of the trial, whereas the FDA is involved in evaluation and approval of new therapies in PAH. The average drug development time once a lead compound has been identified is about 15 years, including preclinical research, development, pivotal trials, clinical research, and post-approval studies. The average cost for drug development is between $500 million and $1 billion, and is relatively more expensive for rare diseases such as PAH. Identifying and testing drugs that are already FDA approved shortens the development duration significantly. In addition, drug development can be considerably less expensive with repurposed drugs, which might result in better affordability for patients.

As repurposed drugs were originally developed for different indications, the mode of action often involves several different pathways instead of a single one. This is a potential advantage, as seen in the case of low-dose FK506 and PAH. While FK506 increases BMPR2 signaling, it also mildly inhibits the nuclear factor of activated T-cells (NFATc) activation. Although not the major effect, as least in our studies of experimental PH, the potential effect on NFATc signaling might be beneficial in PAH. Cyclosporin was found to inhibit NFATc2, restore the potassium channel Kv1.5 expression, decrease proliferation, and increase apoptosis of PASMCs, and was able to decrease established PH in the monocrotaline rat model.[43]

Changing the paradigm of drug development from novel compounds for PAH to repurposing existing drugs offers the advantage of a known safety and toxicity profile, resulting in shorter dose-finding studies and accelerating the use of a potential disease-modifying drug in patients.

References

1. Barst RJ, Rubin LJ, Long WA, et al; Primary Pulmonary Hypertension Study Group. A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. *N Engl J Med.* 1996;334(5):296-301.
2. Benza RL, Miller DP, Barst RJ, Badesch DB, Frost AE, McGoon MD. An evaluation of long-term survival from time of diagnosis in pulmonary arterial hypertension from the REVEAL Registry. *Chest*. 2012;142(2):448-456.
3. Chin KM, Kim NH, Rubin LJ. The right ventricle in pulmonary hypertension. *Coron Artery Dis.* 2005;16(1):13-18.
4. Stacher E, Graham BB, Hunt JM, et al. Modern age pathology of pulmonary arterial hypertension. *Am J Respir Crit Care Med.* 2012;186(3):261-272.
5. Rich S, Pogoriler J, Husain AN, Toth PT, Gomberg-Maitland M, Archer SL. Long-term effects of epoprostenol on the pulmonary vasculature in idiopathic pulmonary arterial hypertension. *Chest*. 2010;138(5):1234-1239.
6. Pullamsetti SS, Schermuly R, Ghofrani A, Weissmann N, Grimminger F, Seeger W. Novel and emerging therapies for pulmonary hypertension. *Am J Respir Crit Care Med.* 2014;189(4):394-400.
7. Paulin R, Michelakis ED. The metabolic theory of pulmonary arterial hypertension. *Circ Res.* 2014;115(1):148-164.
8. Tian W, Jiang X, Tamosiuniene R, et al. Blocking macrophage leukotriene b4 prevents endothelial injury and reverses pulmonary hypertension. *Sci Transl Med.* 2013;5(200):200ra117.
9. Cowan KN, Heilbut A, Humpl T, Lam C, Ito S, Rabinovitch M. Complete reversal of fatal pulmonary hypertension in rats by a serine elastase inhibitor. *Nat Med.* 2000;6(6):698-702.
10. Bogaard HJ, Mizuno S, Hussaini AA, et al. Suppression of histone deacetylases worsens right ventricular dysfunction after pulmonary artery banding in rats. *Am J Respir Crit Care Med.* 2011;183(10):1402-1410.
11. Caruso P, Dempsie Y, Stevens HC, et al. A role for miR-145 in pulmonary arterial hypertension: evidence from mouse models and patient samples. *Circ Res.* 2012;111(3):290-300.
12. Courboulin A, Paulin R, Giguère NJ, et al. Role for miR-204 in human pulmonary arterial hypertension. *J Exp Med.* 2011;208(3):535-548.
13. Kim GH, Ryan JJ, Marsboom G, Archer SL. Epigenetic mechanisms of pulmonary hypertension. *Pulm Circ.* 2011;1(3):347-356.
14. Zhao L, Chen CN, Hajji N, et al. Histone deacetylation inhibition in pulmonary hypertension: therapeutic potential of valproic acid and suberoylanilide hydroxamic acid. *Circulation*. 2012;126(4):455-467.
15. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature*. 2008;454(7200):56-61.
16. Brock M, Trenkmann M, Gay RE, et al. Interleukin-6 modulates the expression of the bone morphogenic protein receptor type II through a novel STAT3-microRNA cluster 17/92 pathway. *Circ Res.* 2009;104(10):1184-1191.
17. Tamosiuniene R, Tian W, Dhillon G, et al. Regulatory T cells limit vascular endothelial injury and prevent pulmonary hypertension. *Circ Res.* 2011;109(8):867-879.
18. Casalena G, Daehn I, Bottinger E. Transforming growth factor-β, bioenergetics, and mitochondria in renal disease. *Semin Nephrol.* 2012;32(3):295-303.
19. Austin ED, Cogan JD, West JD, et al. Alterations in oestrogen metabolism: implications for higher penetrance of familial pulmonary arterial hypertension in females. *Eur Respir J.* 2009;34(5):1093-1099.
20. Hamid R, Cogan JD, Hedges LK, et al. Penetrance of pulmonary arterial hypertension is modulated by the expression of normal BMPR2 allele. *Hum Mutat.* 2009;30(4):649-654.
21. Austin ED, Loyd JE. The genetics of pulmonary arterial hypertension. *Circ Res.* 2014;115(1):189-202.
22. Spiekerkoetter E, Tian X, Cai J, et al. FK506 activates BMPR2, rescues endothelial dysfunction, and reverses pulmonary hypertension. *J Clin Invest.* 2013;123(8):3600-3613.
23. Deng Z, Morse JH, Slager SL, et al. Familial primary pulmonary hypertension (gene PPH1) is caused by mutations in the bone morphogenetic protein receptor-II gene. *Am J Hum Genet.* 2000;67(3):737-744.
24. Thomson JR, Machado RD, Pauciulo MW, et al. Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family. *J Med Genet.* 2000;37(10):741-745.
25. Atkinson C, Stewart S, Upton PD, et al. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation*. 2002;105(14):1672-1678.
26. Caldwell RL, Gadipatti R, Lane KB, Shepherd VL. HIV-1 TAT represses transcription of the bone morphogenic protein receptor-2 in U937 monocytic cells. *J Leukoc Biol.* 2006;79(1):192-201.
27. Sehgal PB, Mukhopadhyay S. Dysfunctional intracellular trafficking in the pathobiology of pulmonary arterial hypertension. *Am J Respir Cell Mol Biol.* 2007;37(1):31-37.
28. George MP, Brower A, Kling H, et al. Pulmonary vascular lesions are common in SIV- and SHIV-env-infected macaques. *AIDS Res Hum Retroviruses*. 2011;27(2):103-111.
29. Dalvi P, O'Brien-Ladner A, Dhillon NK. Downregulation of bone morphogenetic protein receptor axis during HIV-1 and cocaine-mediated

pulmonary smooth muscle hyperplasia: implications for HIV-related pulmonary arterial hypertension. *Arterioscler Thrombosis Vasc Biol*. 2013;33(11):2585-2595.
30. West J, Austin E, Fessel JP, Loyd J, Hamid R. Rescuing the BMPR2 signaling axis in pulmonary arterial hypertension. *Drug Discov Today*. 2014;19(8):1241-1245.
31. Mayeur C, Leyton PA, Kolodziej SA, Yu B, Bloch KD. BMP type II receptors have redundant roles in the regulation of hepatic hepcidin gene expression and iron metabolism. *Blood*. 2014; 124(13):2116-2123.
32. Brunner NW, Skhiri M, Fortenko O, et al. Impact of insulin resistance on ventricular function in pulmonary arterial hypertension. *J Heart Lung Transplant*. 2014;33(7):721-726.
33. Zamanian RT, Hansmann G, Snook S, et al. Insulin resistance in pulmonary arterial hypertension. *Eur Respir J*. 2009;33(2):318-324.
34. Hemnes AR, Brittain EL, Trammell AW, et al. Evidence for right ventricular lipotoxicity in heritable pulmonary arterial hypertension. *Am J Respir Crit Care Med*. 2014;189(3):325-334.
35. Fessel JP, Hamid R, Wittmann BM, et al. Metabolomic analysis of bone morphogenetic protein receptor type 2 mutations in human pulmonary endothelium reveals widespread metabolic reprogramming. *Pulm Circ*. 2012;2(2):201-213.
36. Fessel JP, Flynn CR, Robinson LJ, et al. Hyperoxia synergizes with mutant bone morphogenic protein receptor 2 to cause metabolic stress, oxidant injury, and pulmonary hypertension. *Am J Respir Cell Mol Biol*. 2013;49(5):778-787.
37. Nickel NP, Kaschwich M, Diebold I, Li C, Spiekerkoetter EF, Rabinovitch M. The human neutrophil inhibitor elafin interacts with caveolin-1 to facilitate BMPR-2 signaling and endothelial homeostatis associated with reversal of experimental pulmonary hypertension. *Am J Respir Crit Care Med*. 2014;189:A3638.
38. Drake KM, Zygmunt D, Mavrakis L, et al. Altered MicroRNA processing in heritable pulmonary arterial hypertension: an important role for Smad-8. *Am J Respir Crit Care Med*. 2011;184(12): 1400-1408.
39. Yamaguchi T, Kurisaki A, Yamakawa N, Minakuchi K, Sugino H. FKBP12 functions as an adaptor of the Smad7-Smurf1 complex on activin type I receptor. *J Mol Endocrinol*. 2006;36(3): 569-579.
40. Abe K, Toba M, Alzoubi A, et al. Formation of plexiform lesions in experimental severe pulmonary arterial hypertension. *Circulation*. 2010; 121(25):2747-2754.
41. Motomura H, Matsushita I, Seki E, Mine H, Kimura T. Inhibitory effect of tacrolimus on progression of joint damage in patients with rheumatoid arthritis. *Int J Rheum Dis*. 2013 Nov 20. In press.
42. Albiñana V, Sanz-Rodriguez F, Recio-Poveda L, Bernabéu C, Botella LM. Immunosuppressor FK506 increases endoglin and activin receptor-like kinase 1 expression and modulates transforming growth factor-β1 signaling in endothelial cells. *Mol Pharmacol*. 2011;79(5): 833-843.
43. Bonnet S, Rochefort G, Sutendra G, et al. The nuclear factor of activated T cells in pulmonary arterial hypertension can be therapeutically targeted. *Proc Natl Acad Sci U S A*. 2007;104(27): 11418-11423.