Addressing Complexity in Pulmonary Hypertension

The FoxO1 Case

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Pro-proliferative and inflammatory signaling converge on FoxO1 transcription factor in pulmonary hypertension

Savai et al


“...We think Android is very fragmented and becoming more fragmented by the day. We think integrated will trump fragmented every time...”

Steve Jobs (on iPhone)

Pulmonary arterial hypertension (PAH) is a mysterious killer that, like cancer, is characterized by tremendous complexity. A myriad of apparently unrelated molecular abnormalities have been described but, perhaps because of this complexity, the progress in PAH drug development suffers from poor bench-to-bedside translation.¹ Savai et al,² attacked the master transcription factor forkhead box O1 (FoxO1), arguing that targeting more proximal hubs that integrate many mechanisms in the PAH pathogenesis cascade will be more effective than attacking individual distal targets. It is not the first time that this has been attempted in PAH. Master transcription factors like nuclear factor of activated T-cell c2 (NFATc2),³ hypoxia inducible factor 1α (HIF-1α),⁴ and signal transducer and activator of transcription 3 (STAT3)⁵ have effectively been targeted in PAH models. Attempts to integrate this fragmented field, like the metabolic⁶ and inflammatory⁷ theories of PAH, have emerged.

The Fox-containing proteins are a diverse family of ≥100 transcription factors that can integrate many cellular signals.⁸ Their diversity ensures their ability to fine tune gene transcription in health, development, and common diseases like cancer. The most studied, that is, the FoxO family, are bona fide tumor suppressors and are almost ubiquitously expressed.⁸ FoxO factors mediate G1 phase cell-cycle arrest and G2/M cell-cycle phase transition; they are also important in inflammation, metabolism, and vascular homeostasis (Figure). FoxOs are inhibited by phosphorylation that creates a binding motif for the 14-3-3 scaffolding proteins, causing FoxO1 exclusion from the nucleus, promoting their cytoplasmic ubiquination and degradation.⁸ Acetylation also attenuates FoxO1 transcriptional activity.⁸

Savai et al² showed that FoxO1 levels are decreased in the media of small pulmonary arteries in PAH, along with the expression of many FoxO1 target genes. Signals important in PAH, like platelet-derived growth factor-BB, insulin growth factor-1, interleukin-6, and tumor necrosis factor-α, increased FoxO1 phosphorylation, promoting its nuclear exit and degradation. FoxO1 inhibition in vitro by AS1842856 increased pulmonary artery smooth muscle cell (PASMC) proliferation. Mice with smooth muscle cell–specific genetic ablation of FoxO1 developed spontaneous PAH. However, activation of FoxO1 using a constitutively active adenoviral construct (Ad-FoxO1-AAA) with mutated phosphorylation sites, or treatment with the FoxO1 nuclear export inhibitor psammaplyse A, inhibited PAH-PASMCs proliferation, migration, and induced apoptosis. Gene therapy with Ad-FoxO1-AAA reversed rat monocrotaline-induced PAH, decreasing right ventricular (RV) systolic pressure, RV hypertrophy, and vascular remodeling. Paclitaxel, an anti-neoplastic agent, reduced FoxO1 phosphorylation and increased its nuclear accumulation in vitro, normalizing the phenotype of PAH-PASMCs in terms of proliferation and apoptosis resistance. Paclitaxel treatment by intravenous injections or tracheal aerosolizations reduced RV hypertrophy and vascular remodeling in rat monocrotaline and hypoxia+SU5416 models.

How do these findings relate to the theories that also attempt to address complexity, like the inflammatory and metabolic theories of PAH?

FoxO1 and the Inflammatory Theory of PAH

Inflammation is an increasingly recognized feature of PAH.⁷ The inflammatory milieu in PAH is composed of activated resident and recruited macrophages, dendritic, T, B and mast cells.⁷ FoxO1 is highly expressed in the immune system and plays a key role in promoting T-cell quiescence and peripheral tolerance.⁹ FoxO1 resides in the nucleus of quiescent T cells and relocates to the cytosol after T-cell receptor stimulation. Conditional deficiency of FoxO1 causes spontaneous activation of T cells, expansion of B cells, and autoantibody production (Figure). Thus, inhibition of FoxO1 is in keeping with the activation of immune cells involved in PAH, but on the other hand, activation of FoxO1 may deregulate inflammation and immunity by suppressing overall T- and B-cell function.

Conversely, FoxO1 promotes macrophage activation stimulating the expression of interleukin-1β and toll-like-receptor 4 signaling in mature macrophages.¹⁰ Thus, FoxO1 activation may increase macrophage activation in the pulmonary circulation, promoting PAH. For example, paclitaxel upregulates...
several circulating inflammatory mediators in vitro and in vivo, suggesting a paclitaxel-induced FoxO1 nuclear retention in macrophages, at least in cancer.

Although FoxO1 integrates incoming inflammatory signals in PASMCs, the output is diverse, with some aspects of it being beneficial, whereas others potentially detrimental in PAH. Although some unwanted effects on circulating cells may be limited by selective delivery of such therapies (ie, inhaled approach), the effects on immune cells within and around the pulmonary arteries will remain a concern.

**FoxO1 and the Metabolic Theory of PAH**

Metabolism is a critical driver of PAH pathogenesis. Pulmonary and extrapulmonary tissues exhibit mitochondrial suppression and a compensatory upregulation of glucose uptake and cytoplastic glycolysis, in a manner similar to cancer. PASMCs, endothelial cells, RV cardiomyocytes, skeletal muscle, and activated T cells exhibit this switch that best suits the rapid energy requirements of proliferative/hypertrophic cells, including the suppression of mitochondria-dependent apoptosis.

FoxO1 is progluconeogenic (Figure) and highly expressed in insulin-responsive tissues, where insulin inhibits FoxO1 transcriptional activity via nuclear exclusion. Insulin activates the phosphatidylinositol-3-kinase, which is associated with insulin receptor substrate 1 to 2, promoting an Akt-dependent FoxO1 phosphorylation. Akt, which is activated in PAH, also phosphorylates other substrates that enhance glucose uptake, increase glycolysis, and decrease mitochondria-dependent apoptosis (through hexokinase 2 translocation on the outer mitochondrial membrane). Although gluconeogenesis mostly occurs in liver and kidney, FoxO1 inhibition in lung vessels is compatible with the overall glycolytic phenotype and Akt activation in PAH.

Multiple hypertrophic factors inactivate FoxO1 in cardiomyocytes. FoxO1 overexpression represses cardiac growth at least in part through inhibition of the calcineurin/NFATc2 pathway. NFATc2 regulates mitochondria, inhibiting the transcription of many mitochondrial proteins and inducing the expression of antiapoptotic members of the BCL2 (B-cell CLL/lymphoma 2) family, suppressing mitochondrial-dependent apoptosis in PAH. Phospho-FoxO1 (Thr24) is reduced in the hypertrophied RV cardiomyocytes of PAH rats, leading to an increase in pyruvate dehydrogenase kinase 4 expression and subsequent mitochondrial suppression through pyruvate dehydrogenase inhibition. This may be opposed to the fact that Akt and NFATc2 are activated in RV hypertrophy (suggesting that FoxO1 is inhibited). This potential conflict may be because the RVs studied in these reports were not necessarily separated as compensating versus decompensating. For example, FoxO1 may have opposing effects during RV hypertrophy progression and play a role in the transition from a compensated to decompensated RV function.
and failure need to be studied to determine whether they will be beneficial on RV hypertrophy or not.

FoxO1 enhances the expression of antioxidant genes, including manganese superoxide dismutase, which is epigenetically suppressed in PAH (Figure).\(^{18}\) FoxO1 also mediates the effects of insulin on peroxisome proliferator-activated receptor gamma, coactivator 1\(\alpha\) (PGC-1\(\alpha\)) promoter activity.\(^{9}\) PGC-1\(\alpha\) promotes mitochondrial biogenesis/function, at least in part through the induction of the mitochondrial deacetylase sirtuin 3 (SIRT3) that activates several mitochondrial enzymes. Both PGC-1\(\alpha\) and SIRT3 are downregulated in PAH, suggesting that FoxO1 activating therapies could restore their expression.\(^{6,17}\)

**Challenges in the Translation of FoxO1-Activating Therapies in Patients**

FoxO1 is important for angiogenesis and neovascularization,\(^{18}\) and thus its deficiency could explain angiogenic defects in PAH: plexiform lesions, decreased angiogenesis in skeletal muscle, and the decompensating (but not compensating) RV. However, FoxO1 activation represses the expression of the endothelial nitric oxide synthase (Figure),\(^{18}\) a potential concern that should be evaluated in PAH.

Savai et al showed that FoxO1 is phosphorylated in response to PAH proproliferative signals; but it remains to be established whether these signals converge to an Akt-dependent FoxO1 phosphorylation. In that case, FoxO1 activation may not offer more than drugs that inhibit the Akt axis. Several FoxO1 nucleus-excluding stimuli tested by Savai et al, act through tyrosine kinase receptors, but their inhibition in clinical PAH has been problematic because of unexpected adverse effects.

Interestingly, although FoxO1 is excluded from the nucleus in PAH on proinflammatory and proproliferative signals, NFATc2, HIF-1\(\alpha\), and STAT3 enter the nucleus to promote proliferation. But while FoxO1 acetylation attenuates its transcriptional activity, NFATc2, HIF-1\(\alpha\), and STAT3 acetylation increases their transcriptional activity (Figure).\(^{19}\) Therefore, targeting acetylation by inhibiting acetylases (like CBP/p300) or increasing deacetylase activity (like SIRT1) could be a better way to activate FoxO1 and simultaneously inhibit STAT3, HIF-1\(\alpha\), and NFATc2. Nuclear acetylation is associated with suppression of mitochondrial function and the translocation of pyruvate dehydrogenase (which produces acetyl-CoA from pyruvate) into the nucleus,\(^{20}\) further links FoxO1 inactivation to the metabolic theory of PAH (Figure). Like acetylation, could targeting phosphorylation comprehensively be a better way to bring FoxO1 in the nucleus and remove HIF-1\(\alpha\), NFATc2, and STAT3? For example, HIF-1\(\alpha\), NFATc2, and STAT3 phosphorylation (and nuclear entry) can be mediated by signals arising from suppressed mitochondria, such as GRIM-19, P-GSK-3\(\beta\) (phospho-glycogen synthase kinase 3\(\beta\)), decreased \(\alpha\)-ketoglutarate levels, and Akt can also be activated by mitochondrial suppression.\(^{6}\) Could reversal of this mitochondria suppression normalize all of these important transcription factors’ nuclear levels comprehensively?

FoxO1 seems to be an important target of paclitaxel, despite its original description as a microtubule stabilizer. But FoxO1 can create a feedback loop inhibiting the Akt pathway by repressing the expression of tribble 3, a pseudokinase capable of binding Akt, and inhibiting its phosphorylation.\(^{21}\) This could be a mechanism of resistance to paclitaxel and other FoxO1-activating therapies.

**Conclusions**

FoxO1, like the other transcription factors discussed above, is involved in many molecular events, particularly in inflammation and metabolism. Although targeting proximal signaling hubs in complex diseases like PAH is important, we have to anticipate adverse effects because of the multitude of downstream effects. There may be ways that acetylation and phosphorylation of FoxO1, STAT3, HIF1\(\alpha\), and NFATc2 can be comprehensively attacked (for example via normalization of mitochondria-derived signals) to more effectively inhibit the proliferative and antiapoptotic signals in PAH. The work by Savai et al points to several avenues for future research. Although there are challenges in the clinical translation of FoxO1-activating therapies, it is important to note that the authors’ group is effective in addressing translational challenges and advancing preclinical findings to clinical trials.

**Disclosures**

None.

**References**


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