TOPICAL REVIEW

Mechanisms contributing to persistently activated cell phenotypes in pulmonary hypertension

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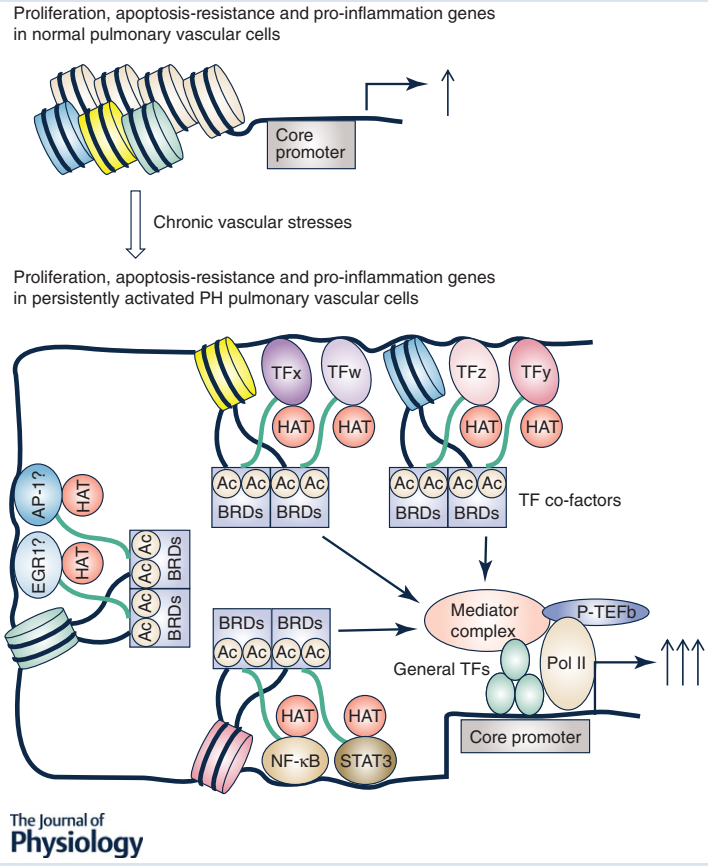

Cheng-Jun Hu received his PhD in Molecular Biology and Virology from Rush University, Chicago in 1999 and completed his postdoctoral training at the University of Pennsylvania. In 2007, he joined the University of Colorado Denver as an Assistant Professor. Dr Hu has extensive experience and expertise in the role of transcription factors, epigenetic regulators and chromatin structure in controlling gene expression in cancer cells. Due to parallel observations concerning how oncogene expression is controlled in cancer cells and how aberrantly expressed genes are regulated in PH pulmonary vascular cells, Dr Hu has been collaborating with Dr Kurt Stenmark's group to determine how gene expression is controlled in pulmonary vascular cells in chronic PH.

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Abstract Chronic pulmonary hypertension (PH) is characterized by the accumulation of persistently activated cell types in the pulmonary vessel exhibiting aberrant expression of genes involved in apoptosis resistance, proliferation, inflammation and extracellular matrix (ECM) remodelling. Current therapies for PH, focusing on vasodilatation, do not normalize these activated phenotypes. Furthermore, current approaches to define additional therapeutic targets have focused on determining the initiating signals and their downstream effectors that are important in PH onset and development. Although these approaches have produced a large number of compelling PH treatment targets, many promising human drugs have failed in PH clinical trials. Herein, we propose that one contributing factor to these failures is that processes important in PH development may not be good treatment targets in the established phase of chronic PH. We hypothesize that this is due to alterations of chromatin structure in PH cells, resulting in functional differences between the same factor or pathway in normal or early PH cells *versus* cells in chronic PH. We propose that the high expression of genes involved in the persistently activated phenotype of PH vascular cells is perpetuated by an open chromatin structure and multiple transcription factors (TFs) via the recruitment of high levels of epigenetic regulators including the histone acetylases P300/CBP, histone acetylation readers including BRDs, the Mediator complex and the positive transcription elongation factor (Abstract figure). Thus, determining how gene expression is controlled by examining chromatin structure, TFs and epigenetic regulators associated with aberrantly expressed genes in pulmonary vascular cells in chronic PH, may uncover new PH therapeutic targets.

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Abstract figure legend Hypothetical representation of chromatin structure, transcription factors (TFs) and TF co-regulators in normal (top panel), and persistently "activated" PH vascular cells (lower panel) of genes involved in proliferation, apoptosis-resistance and pro-inflammation. We posit that the persistently high expression of these genes in PH vascular cells is due to their "open" chromatin structure, allowing binding of multiple stress-related TFs and pioneer TF(s), which help maintain an active chromatin structure and high levels of gene expression by recruiting and maintaining high levels of TF co-factors including epigenetic regulators such as HATs, BRDs and the Mediator Complex (lower panel). Abbreviations: Ac, acetylation; EGR1, early growth response 1; p-TEFb, positive transcription elongation factor B; Pol II, RNA polymerase II.

Introduction

Pulmonary hypertension (PH) exists both as a primary pulmonary vascular disease, as in pulmonary arterial hypertension (PAH) or secondary to an underlying disease, such as chronic exposure to hypoxia as seen in respiratory diseases (COPD, sleep disordered breathing, and others) or chronic exposure to high altitude (Stenmark *et al.* 2009; Stenmark & Rabinovitch, 2010; Simonneau *et al.* 2013). Despite likely differences in the initiating vascular stresses and signalling pathways between primary and secondary forms of PH, there are shared consequences such as vasoconstriction and pathological remodelling of pulmonary vessels that increase pulmonary vascular resistance and stiffness, which stresses the right ventricle (RV), leading to progressive heart failure and death. At the cellular level, pathological remodelling of pulmonary vessels is due to endothelial dysfunction, as well as dysregulated proliferation, apoptosis and inflammatory signalling in all pulmonary vascular wall cells. Importantly, PH vascular cells established from animals with severe hypoxia-induced PH, as well as humans with end-stage idiopathic PAH, maintain *in vitro* their dysregulated or persistently activated cell phenotypes such as hyper-proliferation, apoptosis-resistance and pro-inflammation (Li *et al.* 2011; Pullamsetti *et al.* 2016, 2017; Stenmark *et al.* 2018). These activated phenotypes are likely due to a persistently high expression of genes such as *CCND1*, *CCNA2* (hyper-proliferation), *BCL2*, *BCL2L1* and *Survivin* (apoptosis-resistance), *CCL2*, *CXCL12*, *GM-CSF*, *IL6* and *VCAM1* (pro-inflammation), epidermal growth factor receptor (EGFR) ligands (*AREG*, *EREG*, *TGFA*), fibroblast growth factor receptor (FGFR) ligands (*FGF7*) and MET ligand (*HGF*) (Li *et al.* 2016; Pullamsetti *et al.* 2016, 2017)(authors unpublished observations). These persistently activated phenotypes and aberrant gene expression programmes are maintained *in vitro*, even after multiple passages and in the absence

of complex *in vivo* environments, indicating that the gene expression and cell phenotypes of vascular cells of chronic PH are stable and irreversible. Interestingly, all currently approved treatments for PH are based on the "vasoconstrictor hypothesis" of PH and are directed at either inhibiting vasoconstriction (endothelin receptors) or stimulating vasodilatation (prostacyclin and inhibition of phosphodiesterase 5 (PDE5)). Clearly treatments such as prostanoids, endothelin receptor antagonists, phosphodiesterase 5 inhibitors, soluble guanylate cyclase stimulants or, rarely, certain calcium channel blockers can improve patients' symptoms and extend life (Maron & Galie, 2016; Lau *et al.* 2017). However, though useful, these existing therapies do not halt or reverse PH since these treatments do not directly address the aberrant gene expression programmes responsible for persistently activated cell phenotypes. Thus, it is important to determine the molecular mechanisms that contribute to persistent activation of signalling pathways in cells of the chronically hypertensive vessel wall. Most current studies aimed at identifying therapeutic targets that may reverse the activated cell phenotypes have used an approach aimed largely at determining the initiating signals and their downstream effectors that are important in PH development. These studies have led to the generation of a large number of compelling PH treatment targets, which will be summarized. We will then summarize the success and failure of the clinical trials that target the factors or pathways that are important for PH development. Then, in the following sections, we will describe the data supporting a new hypothesis and its translational implications regarding mechanisms that contribute to the persistently activated cell phenotype that occurs in chronic PH. We specifically propose that alterations in chromatin structure and epigenetic regulators in chronic PH regulate the phenotypes of specific vascular cell types, distinct from the transcriptional mechanisms involved in disease onset.

Essential role of cell signalling ligands/receptors, signalling transducers, transcription factors, transcription factor co-factors and epigenetic regulators, in PH onset and development

We have learned a great deal about PH, particularly the pathways or factors that are important in PH onset and development. This knowledge has been derived from studies using epidemiological investigation, animal models and tissues/cells established from human PAH patients. Collectively, these studies support the well-accepted concept that PH is a multifactorial disease, which can be induced by numerous stimuli and pathological conditions that result in activation of numerous specific signalling pathways and resultant alterations of gene expression (Fig. 1). Below, we will briefly summarize these findings in the order of signalling transduction (from extracellular ligands to signalling transducers), TFs, TF co-regulators and epigenetic regulators.

Role of environmental or pathological stimuli in PH development. Environmental or pathological stimuli such hypoxia, mechanical stress, growth factors, chemokines, cytokines, oxidative stress and metabolic reprogramming can all lead to activation of specific signalling pathways resulting in pulmonary vascular cell proliferation, inflammatory response and pulmonary vessel occlusion (Hassoun *et al.* 2009; Stenmark & Rabinovitch, 2010; Schermuly *et al.* 2011; Pullamsetti *et al.* 2016, 2017). Some of these stimuli, such as growth factors, chemokines and cytokines initiate their function by binding to specific receptors located on the cell membrane. That increased expression of growth factors such as platelet-derived growth factor (PDGF), EGFR ligands, and transforming growth factor β (TGF-β) are important in initiating development of PH is evidenced by the studies demonstrating that the inhibitor of the EGFR/PDGF receptor downstream effector RAS/RHOB, Tipifarnib (Duluc *et al.* 2017), the TGF-β ligand trap, a soluble TGF-β type II receptor extracellular domain expressed as an immunoglobulin-Fc fusion protein (TGFBRII-Fc) (Yung *et al.* 2016) and direct PDGF inhibition with the tyrosine kinase inhibitor Imatinib (Pullamsetti *et al.* 2012) can all attenuate PH. In contrast, decreased signalling through the bone morphogenetic protein receptor type II (BMPR2) pathway also leads to PH development (Morrell *et al.* 2006; Guignabert *et al.* 2017; Orriols *et al.* 2017). The role of inflammatory cells, cytokines or chemokines in PH development has also been demonstrated as blocking bone-marrow-derived cell recruitment to the lung (Hayashida *et al.* 2005; Frid *et al.* 2006; Gambaryan *et al.* 2010), inhibition of the chemokine SDF-1 (Young *et al.* 2009), or its receptor CXCR4 (Yu & Hales, 2011), can also block or attenuate PH development (Rabinovitch *et al.* 2014; Pugliese *et al.* 2015). Both intracellular and extracellular redox status has also been shown to contribute to PH development. For instance, the levels of reactive oxygen species (ROS) are increased under hypoxia, due to increased production of ROS from mitochondria complex II and/or complex III (Paddenberg *et al.* 2003; Guzy *et al.* 2007). The role of ROS in PH is supported by the spontaneous development of PH in mice in which superoxide dismutase (*Sod1*) is deleted in smooth muscle cells (SMCs) (Nozik-Grayck *et al.* 2014) while hypoxia-induced PH is significantly attenuated in mice with overexpression of extracellular SOD (EC-SOD) in the lung (Nozik-Grayck *et al.* 2008).

The role of signalling transducers in PH development. Association of ligands such as growth factors, cytokines,

chemokines, or extracellular matrix to their receptors often leads to activation of intracellular and/or membrane-associated protein kinases, which results in signal transduction and signal amplification. Here, we primarily focus on the role of Ras–MEK–ERK and PI3K–Akt–mTOR signalling transducers in PH as these factors play critical roles in cell proliferation, survival and motility. Activation of Ras proteins, which, in turn, transduces signals through Raf, MEK and ERK, can be mediated by growth factors and extracellular matrix-mediated signals. Specific to PH endothelial cells, Ras can also be activated by BMPR2 silencing (Awad *et al.* 2016). The activation of Ras–MEK–ERK is well demonstrated in PH (Lane *et al.* 2005). The function of Ras–MEK–ERK in PH development is supported by the fact that *Raf-1* kinase inhibitor protein knockout mice exhibit more severe hypoxia-induced PH (Morecroft *et al.* 2011). The PI3K–Akt–mTOR pathway is often activated by receptor tyrosine kinases, G protein-coupled receptors and integrins. Multiple studies have documented the role of PI3K–Akt–mTOR in PH initiation including attenuated development of hypoxia-induced PH in rats when treated with PI3K or Akt inhibitors (Garat *et al.* 2013), or in mice with SMC-specific deletion of *Akt* (Tang *et al.* 2015) while knockdown of *PTen*, a negative regulator of Akt activation, leads to spontaneous PH (Nemenoff *et al.* 2008).

The roles of TFs in PH development. The activities of transcription factors (TFs) are often regulated by signal transducers initiated from outside of the cell but they can also be modulated by intracellular signals. Regulation of TF activity by signalling molecules is typically mediated through post-translational modifications such as phosphorylation, acetylation and methylation, resulting in alterations of TF protein stabilization, translocation between cytoplasm and nucleus, alteration of TF binding affinity to its co-activators, and alteration of TF binding to DNA (Spitz & Furlong, 2012; Bhagwat & Vakoc, 2015). Multiple TFs have been implicated in PH development (Pullamsetti *et al.* 2016). For example, hypoxia-inducible factors (HIFs) are key regulators of the molecular response to hypoxia. The target genes of HIFs include genes controlling neovascularization, cell proliferation, migration, metabolism and others (Pawlus & Hu, 2013). Studies from multiple laboratories using mouse models have established a critical role of HIF2 in hypoxia-mediated PH in which global reduction (Brusselmans *et al.* 2003) or knockout of *Hif2* in

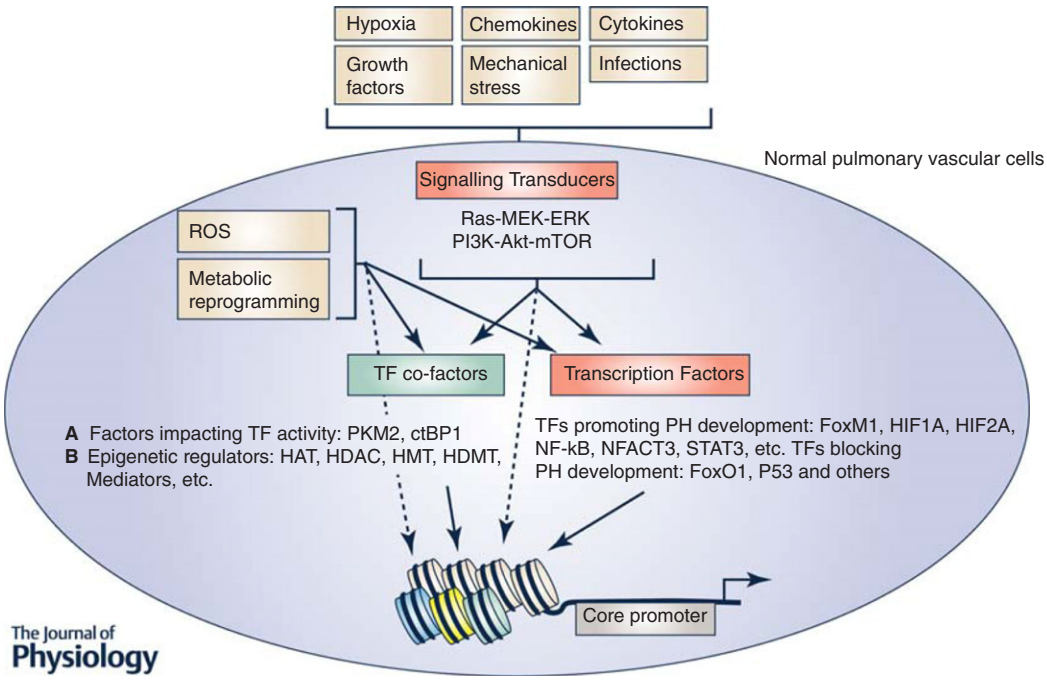

Figure 1. Role of cell signals, signalling receptors, signalling transducers, TFs and TF co-factors in altering chromatin structure and gene expression in normal pulmonary vascular cells
The traditional view is that microenvironmental signals impact gene expression by regulating the activities of TFs that regulate gene expression and disease progression. Studies in the last two decades support roles of extra- and intracellular signals in regulating the activities of TF co-factors (including epigenetic regulators) and nucleosome histone modifications, in addition to regulating TF activity, all of which together control chromatin structure and gene expression.

endothelial cells (ECs) (Bryant *et al.* 2016; Tang *et al.* 2018) or in pulmonary ECs (Cowburn *et al.* 2016) reduces or completely blocks the development of PH. Conversely, activation of HIF2 via inactivating mutation of Von Hippel-Lindau (*Vhl*) (Hickey *et al.* 2010) or deletion of *Phd2* (Dai *et al.* 2016; Kapitsinou *et al.* 2016; Wang *et al.* 2016; Tang *et al.* 2018), or activating mutation of *Hif2a* (Tan *et al.* 2013) leads to PH development under normoxic conditions. Increased expression of *FOXM1*, a transcription factor crucial for G1–S and G2–M cell cycle progression and ROS-induced DNA damage repair has been found upregulated in PH and blocking its expression prevents and reverses hypoxia-induced PH in rodents (Bourgeois *et al.* 2018; Dai *et al.* 2018). PH development is often associated with early and persistent perivascular inflammation in animal models of PH (Li *et al.* 2011; Stenmark *et al.* 2012) and persistent inflammation is also observed in most chronic forms of human PH (Tuder *et al.* 2013; Rabinovitch *et al.* 2014; Ghataorhe *et al.* 2017). Increased activation of inflammatory TFs such as STAT3 (Paulin *et al.* 2011*a*,*b*, 2012) and nuclear factor κB (NF-κB) (Sawada *et al.* 2007; Huang *et al.* 2008; Kimura *et al.* 2009; Hosokawa *et al.* 2013; Price *et al.* 2013; Farkas *et al.* 2014; Li *et al.* 2014) have been consistently observed in animal models and human PH. Further, STAT3 and NF-κB inhibition either block or attenuate PH development since these TFs not only sustain inflammatory responses but also promote cell proliferation, survival and metabolic reprogramming (Grivennikov & Karin, 2010). Further, inhibition of a TF called NFATc3, that is activated by increased levels of ROS, prevents hypoxia-induced PH in mice (Ramiro-Diaz *et al.* 2013).

Reduced activities of TFs such as p53 and FoxOs also promote PH development. p53 is necessary for responding to DNA damage and other stresses, and p53 activation often leads to inhibition of cell proliferation. Thus, it is not surprising that reduced p53 expression/activity contributes to PH development in which more severe PH is observed in *Tp53* knockout mice under chronic hypoxia (Mizuno *et al.* 2011) or in rats treated with a p53 inhibitor (Jacquin *et al.* 2015). The activities of FoxO TFs are often reduced by growth factors and inflammatory cytokine-mediated signalling pathways, leading to increased cell proliferation, survival and metabolic reprogramming. Indeed, both *in vitro* and *in vivo*, reduction of FoxO activity increases the severity of PH while restoration of FoxO activity can block or reverse PH (Savai *et al.* 2014).

The role of TF co-factors in PH development. A TF co-factor (co-activator or co-repressor) is a type of protein that itself has no DNA binding activity, but can interact with other general or sequence-specific TFs to modify the ability of TFs to regulate gene expression. Broadly speaking, TF co-factors can be divided into two types, one with activity on chromatin structure, called epigenetic regulators, and another type functioning on TF activity only (Kornberg, 2001; Cosma, 2002; Ries & Meisterernst, 2011). We first focus on two non-epigenetic TF co-factors: PKM2 and C-terminal binding protein-1 (CtBP1). Both of these factors are controlled by the metabolic state of the cell, which in all pulmonary vascular wall cells is known to change in both acute and chronic forms of PH (Sutendra & Michelakis, 2014; Stenmark *et al.* 2015; Plecita-Hlavata *et al.* 2016, 2017; D'Alessandro *et al.* 2018). The metabolic adaptation, often referred to as 'Warbug-like" leads to increased glycolysis and increased fatty acid oxidation, but reduced oxidative phosphorylation in mitochondria. The reduced oxidative phosphorylation in mitochondria is the result of reduced input of acetyl-CoA to TCA, and/or increased mitochondria fission (D'Alessandro *et al.* 2018). *PKM2* is one of the splicing isoforms of a gene called pyruvate kinase muscle type, a gene that plays an important role in glycolysis (Wong *et al.* 2015; Dayton *et al.* 2016; Dong *et al.* 2016). However, in addition to its role in glycolysis, PKM2 can serve as a HIF1 co-activator by promoting HIF1's role in activating HIF target genes through its binding to and phosphorylation of HIF1α protein (Luo & Semenza, 2011; Luo *et al.* 2011). PKM2 also has other functions including activating cell proliferation via phosphorylation of the cell cycle regulator BUB3 and regulating chromatin structure by phosphorylating histones (Dong *et al.* 2016). Indeed, inhibition of PKM2 activity directly, or of its upstream activator or downstream effectors reduce the "activated" phenotypes of PH vascular cells (Caruso *et al.* 2017; Zhang *et al.* 2017). Different from PKM2, CtBPs function as transcriptional corepressors (Kuppuswamy *et al.* 2008; Wang *et al.* 2012; Blevins *et al.* 2017). CtBPs repress gene expression by binding to an inhibitory TF and recruiting histone-modifying enzymes that add repressive histone marks and remove activating histone marks (Byun & Gardner, 2013). In PH fibroblasts, CtBP activity is increased, due to increased free NADH (increased NADH/NAD^+ ratio). Increased CtBP activity enhances cell proliferation and apoptosis resistance by decreasing expression of cell cycle inhibitors such as p15 and p21 and pro-apoptosis genes such as *NOXA* and *PERP* (Li *et al.* 2016). Importantly, normalizing metabolic activity via metabolic inhibitors such as 2-deoxyglucose (2DG) or directly reducing CtBP1 expression reduces PH fibroblast proliferation and apoptosis resistance (Li *et al.* 2016).

The role of epigenetic regulators in PH development. Another type of TF co-factors in eukaryotic cells are chromatin or epigenetic regulators that function in gene expression by controlling chromatin structure (Shlyueva

et al. 2014; Voss & Hager, 2014). These factors include histone post-translational modifying enzymes such as histone acetylases (CREB-binding protein (CBP) and p300), readers of histone modifications such as bromodomain (BRD) proteins, Brahma-associated factor (BAF) complex, Mediator complexes and others (Fig. 1). All of these epigenetic regulators can be recruited by TFs, but can also be additionally recruited by other chromatin-associated proteins including histones (see below). CBP and its paralogue p300 are histone acetyl-transferases (HATs) that acetylate histones at both promoters and enhancers as well as numerous non-histone proteins including TFs (Spange *et al.* 2009; Slingerland *et al.* 2014). HATs are often recruited to chromatin by TFs. BRD4 is a member of the BET (bromodomain and extra-terminal domain) family proteins that are characteristic of two tandem bromodomains (BDs) located in the N-terminus. The BDs of BET proteins recognize acetylated-lysine residues in nucleosomal histones and other proteins such as TFs (Filippakopoulos *et al.* 2012). BRD proteins can activate gene transcription by recruiting positive transcription elongation factor (P-TEFb), Mediator, and other chromatin remodelling complexes including BAF complex (Jang *et al.* 2005). Mediator is a large multiprotein complex >1 MDa in size and >30 nm in length. Besides interacting with BRD proteins, different TFs bind different Mediator subunits. Thus, Mediator complex can act as a bridge mediating interaction between TFs and components of the general TFs (GTFs)/RNA polymerase II (RNA Pol II). Additionally, Mediator also activates gene transcription by recruiting the P-TEFb to activate elongation activity of RNA Pol II (Allen & Taatjes, 2015). BAF complexes (not shown in Abstract figure), which belong to the SWI/SNF family of ATPase-dependent chromatin remodelling complexes, are also involved in chromatin structure changes through their effects on movement of nucleosome position relative to specific DNA sequence, ejection of nucleosome, and exchange of classic core histones with variant histones (Halliday *et al.* 2009; Reisman *et al.* 2009). BAF complexes can be recruited by TFs, BRDs and acetylated histones since BAF complex contains multiple bromodomain-containing proteins (Halliday *et al.* 2009; Reisman *et al.* 2009).

Recently, new work has led to an appreciation of the important role epigenetic regulators play in PH initiation as several epigenetic regulators such as histone deacetylases (HDACs), and double bromodomain proteins (BRDs), have been shown to exhibit increased expression in PH vascular cells (Zhao *et al.* 2012; Meloche *et al.* 2015, 2017). Furthermore, HDAC and BRD inhibitors have been shown to prevent or reverse PH (Zhao *et al.* 2012; Meloche *et al.* 2015, 2017).

Can we reverse the persistently "activated" phenotypes of PH vascular cells, based on potential targets uncovered in PH initiation studies?

There is a large body of compelling data, including some that was described above, supporting critical roles for factors ranging from membrane receptors, signalling transducers, TFs, non-epigenetic TF co-factors, and epigenetic regulators in PH development (Fig. 1). Clearly, the alterations of these factors or pathways are required in PH development, and are at least in part, responsible for changing the gene expression programme that gradually transforms the normal pulmonary vascular cells to the activated PH vascular cells (Abstract figure). Importantly, the role of many of these factors has been evaluated with regard to both PH prevention and PH reversal in animal models. Further, the function of these factors, in some cases, has also been demonstrated in one or multiple human PH vascular cells *in vitro*. Thus, these studies provided a large list of potential new PH treatment targets (Lythgoe *et al.* 2016; Wilkins, 2018). However, despite all these efforts, there has been little success in new therapies targeting structural remodelling or the activated phenotypes of the PH vascular cells (Lythgoe *et al.* 2016; Wilkins, 2018). For example, drugs such as Terquride (a serotonin antagonist), statins and vaso-active intestinal peptide (VIP), all with very promising effects in pre-clinical animal models (Said *et al.* 2007; Morecroft *et al.* 2010; Wright *et al.* 2011), all failed to meet their primary endpoint in clinical trials. Further, the tyrosine kinase inhibitor Imatinib, though shown to improve haemodynamics in many patients, has not been licensed because of unacceptable side effects (Lythgoe *et al.* 2016). More recent reports indicate that an inhibitor for ASK1 (apoptosis signal-regulating kinase 1) also failed to meet the primary endpoint in a clinical trial (Wilkins, 2018), again despite the demonstrated critical role of ASK1 in PH animal models and in human PH vascular cells (Welsh *et al.* 2001; Mortimer *et al.* 2007; Church *et al.* 2015; Budas *et al.* 2018). Additionally, Eiger BioPharmaceuticals announced that it has halted clinical development of Ubenimex for PAH due to lack of efficacy to treat PH in the Phase 2 LIBERTY study although leukotriene B4, the target of Ubenimex, plays an important role in PH in animals (Tian *et al.* 2013). Collectively, failure of these clinical trials indicates a significant challenge in developing new PH treatments. The reasons for these disappointing findings could be multiple, including a need to improve the selection of patients in clinical trials and the poor fidelity of animal models of PH for the human PH disease, all of which have been reviewed by Lythgoe *et al.* (2016). We believe that another factor usually not considered, but that could also contribute to the failure of translation

from animal studies to humans, is that most PH targets tested have been uncovered from studies in the early stages of PH development. We think that factors or pathways that are essential in PH development may or may not be critical in established disease due to extensive changes in chromatin structure, between normal pulmonary vascular cells and vascular cells in chronic PH patients (Stenmark *et al.* 2018). There are multiple examples in a variety of cancers that support the hypothesis that pathways/factors that play major roles in cancer development play no or only minor roles in maintaining the transformed phenotypes of established cancers. The best studied example is *KRAS* whose mutation drives multiple early onset lung tumours. Interestingly, these lung cancers often become KRAS independent, if the phenotype of the cancer switches from epithelial to mesenchymal (Singh *et al.* 2009). Further, although mutations of *EGFR* predispose to the development of lung cancer, most of the *EGFR* mutated lung cancers are resistant to EGFR inhibition (Ware *et al.* 2013). Also, both HIF1 and HIF2 are required for initiation of clear cell renal cell carcinoma (ccRCC) (Schonenberger *et al.* 2016). However, in the later stage of human ccRCC tumours, both HIF1 and HIF2 are dispensable (Shen *et al.* 2011; Murakami *et al.* 2017).

Below, we will examine the importance of chromatin structure and epigenetic regulators in maintaining the activated phenotypes of PH vascular cells. Because the cancer paradigm is often invoked in explaining the cellular changes observed in severe chronic PH, we will start this section by summarizing the extensive epigenetic changes observed in cancer cells and reported success of developing epigenetic regulators as promising cancer treatment targets in human cancers.

Future research in "transcriptional addiction" of PH vascular cells: roles of chromatin structure, multiple transcription factors and epigenetic regulators in persistent activation of PH vascular cells

Transcriptional addiction as a promising cancer therapeutic strategy. Research performed in the last 20 years makes it clear that mutated signalling and TFs that initiate cancer development often end in changes of chromatin structure and gene expression in cancer cells (Lee & Young, 2013; Sur & Taipale, 2016; Bradner *et al.* 2017) since kinases can alter chromatin structure by: (1) controlling the activities of epigenetic regulators; (2) controlling chromosomal histone phosphorylation; and (3) controlling the levels of epigenetic regulators on chromatin by regulating the activities of TFs (Badeaux & Shi, 2013; Morgan & Shilatifard, 2015; Sur & Taipale, 2016) (Fig. 1). For example, expression of oncogenes such as *MYC* are much higher in pancreatic and colorectal cancers, as well as in T cell leukaemia, *versus* their normal control cells (see Fig. 6*B* of Hnisz *et al.* 2013). Interestingly, detailed analysis of *MYC* enhancers in myeloid leukaemia cells indicates that the *MYC* gene is regulated by at least five active enhancers (E1–E5) that cover a large region (more than 100 kB) of DNA and each enhancer exhibits high levels of active histone modification marks (H3K27Ac and H4K8Ac), high binding densities of epigenetic regulators (BRD4 and p300) and multiple TFs (PU.1, FLI, ERG, CEBPα, CEBPβ and MYB) (Roe *et al.* 2015) (Fig. 2), all of which are marks of open chromatin structure. Further, all the BRD4-occupied sites overlap with binding sites of one or more TFs and most BRD4-enriched regions exhibit binding of several TFs (Fig. 2), indicating BRD4 protein is commonly required for different TFs to regulate gene expression. The role of epigenetic deregulation and chromatin structure changes in cancer gene expression is also supported by the fact that almost every cancer cell type contains mutation of genes involved in epigenetic regulation (Koschmann *et al.* 2017). All these studies support a critical role of chromatin structure and epigenetic regulators in cancer development and maintenance (Badeaux & Shi, 2013). Thus, epigenetic regulators such as histone methyltransferases and de-methylases, histone acetylases and deacetylases, and the BET proteins (BRD2, BRD3 and BRD4) are attractive targets for therapeutic intervention in cancers (Barbieri *et al.* 2013; Heerboth *et al.* 2014; Slingerland *et al.* 2014; Wee *et al.* 2014; Cai *et al.* 2015; McGrath & Trojer, 2015; Jones *et al.* 2016). Clearly, like cancer, there is a "transcriptional addiction" in PH vascular cells, reflected in persistently high expression of genes involved in hyper-proliferation, apoptosis-resistance and pro-inflammation (Li *et al.* 2016; Pullamsetti *et al.* 2016, 2017). We have preliminary data to support a hypothesis that high expression of genes involved in the persistently activated pro-inflammatory phenotype of PH vascular cells, at the chromatin level, are maintained by an open chromatin structure and multiple TFs, via the recruitment and maintenance of high levels of epigenetic regulators such as histone acetylate P300/CBP, histone acetylation readers including BRDs, Mediator complex and positive transcription elongation factor (Abstract figure). Thus, it will be important to perform chromatin immunoprecipitation-sequencing (ChIP-Seq) occupancy profiles of histone modifications, TFs and epigenetic regulators in PH vascular cells, as has been done for oncogenes in cancers (Fig. 2).

Mechanisms that can drive "transcription addiction" of PH vascular cells. We believe that various components of transcriptional and epigenetic control play a crucial role in controlling gene expression in chronic disease such as PH, in which mutations of epigenetic regulators are rarely reported. In addition, we also posit that the

components that control gene expression in normal and persistently activated PH vascular cells are different. Thus, determining the mechanisms controlling gene expression in persistently activated PH vascular cells may uncover new molecular mechanisms and may form the basis on which novel PH therapeutic targets will be developed.

The genomic DNA in eukaryotic cells is packaged in the nucleosome, which consists of two copies of each histone protein (H2A, H2B, H3 and H4) and 146 base pairs of superhelical DNA wrapped around this histone octamer. The nucleosome structure creates a problem for TFs and RNA polymerase to access the DNA, but also provides an opportunity for regulated gene expression. It is now accepted that the level or rate of gene transcription in eukaryotic cells is determined by interplay among *cis*-acting regulatory DNA elements, which includes the core promoter, proximal promoter regions as well as those that act over large genomic distances, such as enhancers (Spitz & Furlong, 2012), and *trans*-acting factors including gene-specific TFs, epigenetic regulators, general TFs and RNA polymerase II. Thus, it is important to address all three components (chromatin structure, TFs and epigenetic regulators) that control gene expression in PH vascular cells.

Determine the chromatin structure of genes involved in the persistently activated cell phenotypes of PH vascular cells. Enhancers, composed of dense clusters of TF binding motifs, are cell type-specific and highly regulated. Thus, enhancers are critically important in controlling a subset of eukaryotic genes, called regulated genes, that are often involved in development, cell identify and functional phenotypes (Shlyueva *et al.* 2014; Smith & Shilatifard, 2014; Heinz *et al.* 2015). Enhancer DNA can exist in an active (accessible to TF binding) or inactive (inaccessible) status. Tools now exist for annotating the status of the enhancers on a genome-wide scale by measuring levels of histone modifications, TF and epigenetic regulator binding and chromatin accessibility. These approaches have shown that the functional enhancer landscape is largely unique to each cell type and maintained by lineage-specific TFs and epigenetic regulators. However, new evidence reveals how acute or chronic signalling events can lead to reprogramming of enhancer configurations (Brown *et al.* 2014; Lavin *et al.* 2014). Studies have uncovered multiple mechanisms involved in reprogramming enhancers during development and disease progression. Regulation of gene expression is inherently associated with alterations in chromatin architecture because TFs often recruit co-activators such as p300 to acetylate histones at the enhancer at which the TF binds, but such histone acetylation often extends to neighbouring nucleosomes, leading to larger active DNA regions and more active enhancers. These transient histone modifications to larger DNA regions are heritable if cells are proliferating (Probst *et al.* 2009). It is well accepted that PH development involves extensive vascular cell proliferation at least at the peak stage(s) of PH development, thus transient increased expression of genes involved in hypoxia response, inflammation, growth factor signalling and others, in a combination of cell proliferation, may lead to a more "open" chromatin structure of

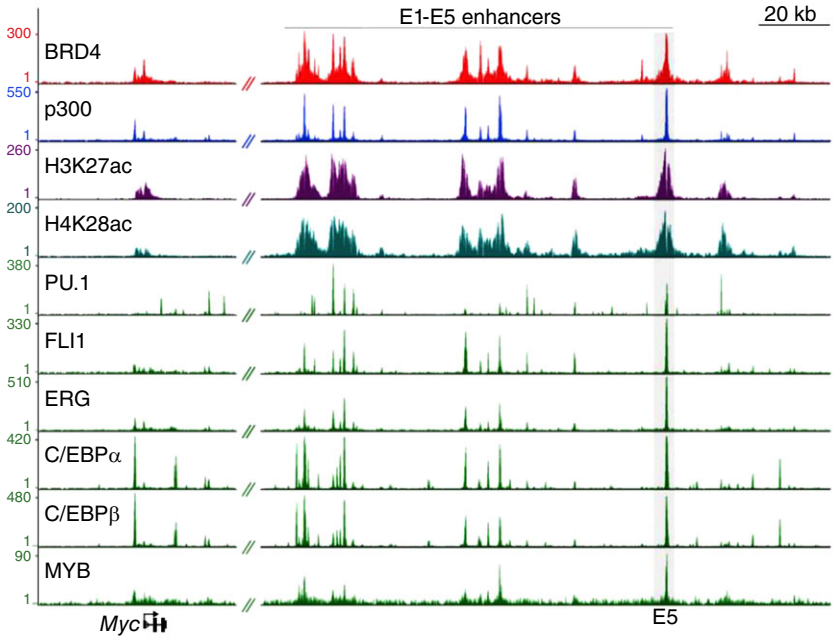

Figure 2. ChIP-Seq occupancy profiles of epigenetic regulators BRD4 and p300, active histone modification marks H3K27Ac and H4KAc, and TFs PU.1, FL1, ERG, C/EBPα, C/EBPβ and MYB at the *MYC* locus in myeloid leukaemia cells
Note there are at least 5 distinct enhancers for *MYC* gene expression in a more than 100 kb regulatory region. Each enhancer exhibits co-existence of high histone acetylation and high binding densities of several TFs and epigenetic regulators (Roe *et al*. 2015). Importantly, although TFs exhibit unique binding densities to each enhancer, epigenetic regulators exhibit a similar binding pattern to each active enhancer, suggesting that epigenetic regulators are commonly required for different TFs to regulate gene expression.

these genes. The second way of TF-mediated enhancer reprogramming is mediated by a subset TFs called 'pioneer factors'. These pioneer TFs are particularly important in creating brand new active enhancers due to their ability to engage silent, closed enhancers (Zaret & Carroll, 2011; Adam *et al.* 2015). Studies have identified about 100 pioneer TFs in development and cancer research. While clearly functionally important, so far the pioneer TF concept has not been introduced into the PH research. While TF-mediated enhancer reprogramming is well accepted at least in development and cancer research, new evidence supports a direct role of cell signalling in reprogramming enhancers by kinase-mediated phosphorylation of histones and/or epigenetic regulators (Badeaux & Shi, 2013). Also, the activity of chromatin regulators can be altered by other mechanisms such as metabolic intermediates and redox stress (Berger & Sassone-Corsi, 2016; Kreuz & Fischle, 2016; Reid *et al.* 2017). There is extensive literature demonstrating alterations in growth factor-mediated signalling, reprogramming of cellular metabolic and redox state during PH progression (Merklinger *et al.* 2005; Schermuly *et al.* 2005; Plecita-Hlavata *et al.* 2016; Zhang *et al.* 2017), which could directly impact chromatin structure in PH vascular cells. Further, demethylation of H3K4me3 and H3K27me3, two critical histone modification events, is mediated by oxygen and 2-oxoglutarate dependent dioxygenase enzymes such as demethylase KDM6B/JMJD3, whose function can be inhibited by oxygen deprivation (hypoxia) (Hancock *et al.* 2015; Prickaerts *et al.* 2016). All these studies provide ample support for the hypothesis that there are significant differences in chromatin structure between normal and PH vascular cells. Thus, it is essential to determine the chromatin structure in control and PH vascular cells. Such studies may also provide a molecular explanation of why targeting TFs that are important in PH initiation may or may not be sufficient in PH treatment. For example, in a normal vascular cell (Fig. 3, cell A), TFs X and Y are critical in expression of this gene, but TFs X and Y become less and less important in activation of this gene in both cells B and C (Fig. 3) in which this gene's expression is regulated by additional TFs, including ubiquitous TFs that have no role in regulating this gene in cell A (Fig. 3), due to more open chromatin structure in cells B and C. Chromatin structure analysis will also allow us to identify the set of TFs that are potentially associated with these newly activated enhancers, using motif analysis.

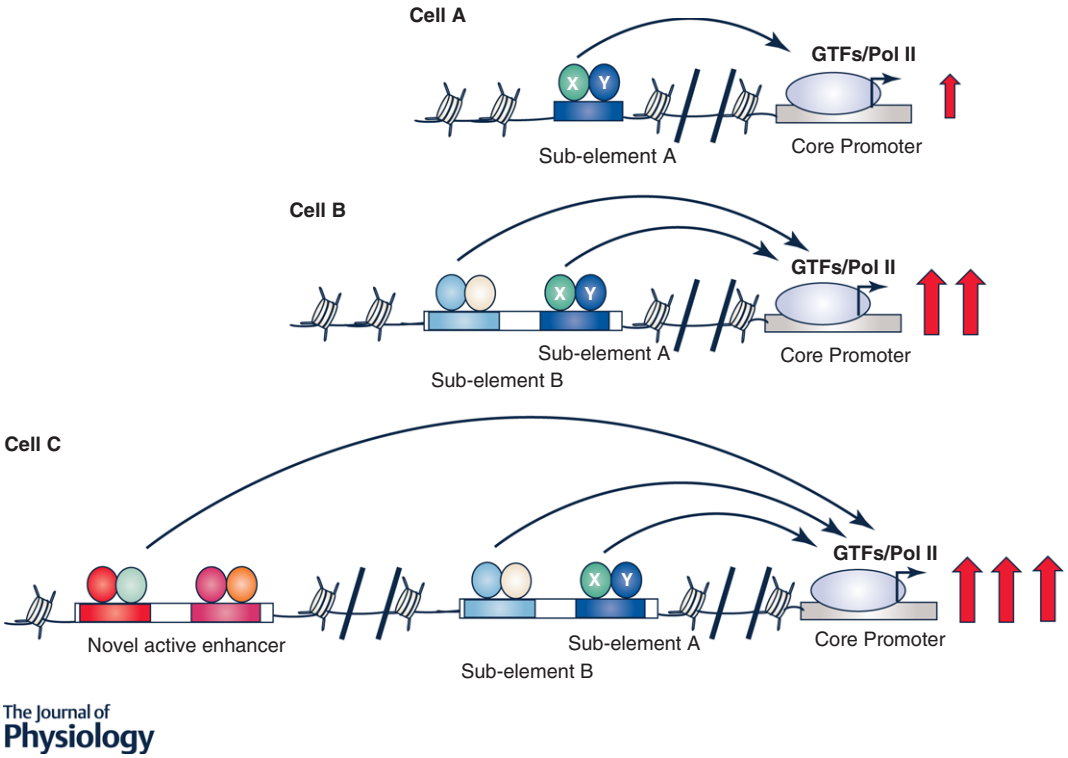

Figure 3. Hypothetical representation of chromatin structure determines the functional importance of TFs
TFs X and Y play a critical role in expression of this gene in cell A by binding to sub-element A. But a more "open" chromatin structure, allowing other TFs to bind to the regulatory elements of this gene, diminishes the contribution of TFs X and Y in expression of this gene in cells B and C.

Determine the identities and function of the TFs that are associated with the genes involved in persistently activated cell phenotypes of PH vascular cells. TFs play an indispensible role in the control of chromatin structure, gene expression, response to specific signals and thus disease initiation. Due to alterations of the endogenous as well as extracellular microenvironment between normal and diseased tissues/organs, the set of TFs active in normal cells and diseased cells often only partially overlap. Even though the expression and activity of a specific TF may be maintained in established disease, global changes in chromatin accessibility may reprogramme the TF binding profile, thus its function. Further, TF binding to DNA often depends on its partner(s), thus changes in expression/activity of TF binding partners may also alter the function of a specific TF in established disease. TFs can be broadly divided into two types. One is pioneer TFs or lineage-specific TFs that are important in creating and maintaining cell identity as well as cellular functional phenotypes (Adam *et al.* 2015). Another type of TF are stress TFs such as HIF, STAT3, NF-κB and activator protein 1 (Ap-1) that are activated in response to specific signals. Pioneer and stress TFs often work together to regulate gene expression in which pioneer TFs establish the competency for stress TFs to further activate gene expression. TF expression and their activities in diseased cells are rarely studied, due to the misconception that epigenetic changes that are introduced in disease progression are sufficient to maintain gene expression. In fact, studies have shown that the maintenance of chromatin structure and gene expression patterns requires participation of both TF activity and epigenetic regulators (Spitz & Furlong, 2012). Thus, it is critical to determine the set of TFs that function in PH vascular cells to increase our understanding of PH-activated phenotypes and to provide potential therapeutic targets for PH disease (Bhagwat & Vakoc, 2015). TFs that are critically important for control and PH vascular cells can be profiled by checking their gene expression (using RNA-Seq) and their binding profile in genomic DNA (using ChIP-Seq of TF). The functions of TF can be determined using TF specific inhibitors and/or siRNA-mediated knockdown or CRISPR/cas9-mediated gene knockout in PH vascular cells. We must emphasize the importance of studying the function of TF in all of the PH vascular cells involved in the disease process. It is well accepted that enhancer landscape is often unique to each cell type, suggesting that different enhancers (Fig. 4), and thus different TFs, could be utilized in different cell types for the same gene.

Determine the identities and the function of the epigenetic regulators that are associated with the genes involved in persistently activated cell phenotypes of PH vascular cells. Besides chromatin structure and TFs, a third component that is critically important in gene regulation is epigenetic regulators. Several groups including ours have reported the potential for HDAC inhibitors (HDACi) to reverse hypoxic PH and to have beneficial effects on cardiac fibrosis (Cavasin *et al.* 2012; Zhao *et al.* 2012; De Raaf *et al.* 2014; Williams *et al.* 2014).

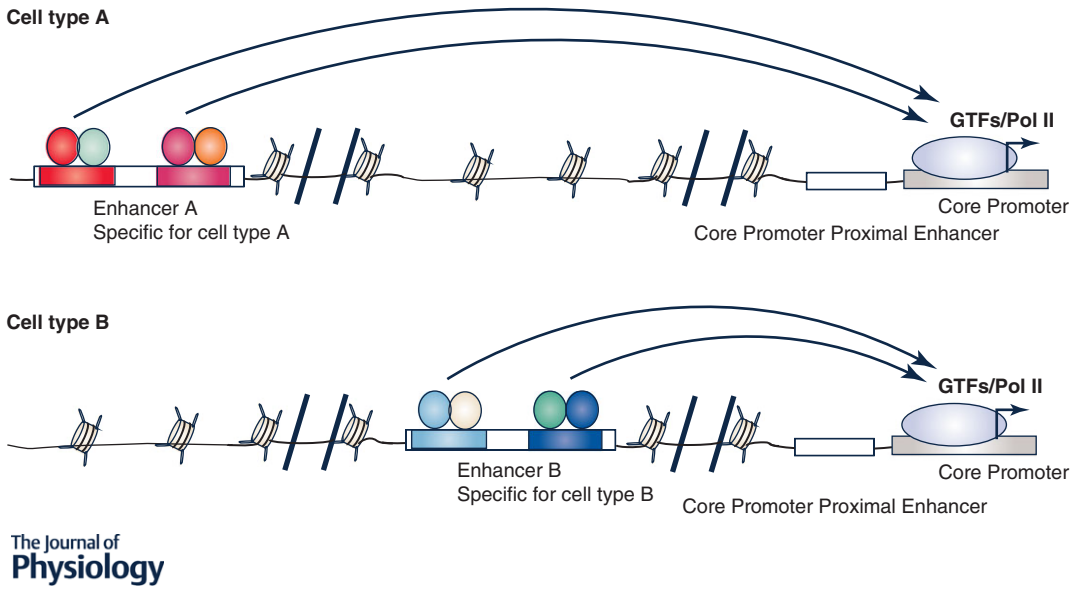

Figure 4. Enhancers are often cell type specific
Enhancer A is important in controlling this gene in cell type A while enhancer B is critical in regulating the same gene in cell type B. Thus, the TFs that regulate the same gene could be totally different between cell type A and cell type B. This hypothetical model suggests that targeting a specific TF that is effective in reducing a specific gene in one cell type may have no role in reducing the same gene in another cell type.

Recently, the Bonnet group has presented evidence for increased BRD4 levels in the SMCs not only in remodelled pulmonary arteries but also in the coronary artery vasculature (Meloche *et al.* 2015, 2017). Importantly, these investigators demonstrated that the BRD inhibitor (BRDi) JQ1 could not only mitigate the hyper-proliferative pulmonary hypertensive SMC phenotype *in vitro* but could reverse the vascular remodelling observed in the Sugen/hypoxia model (Meloche *et al.* 2015). However, most HDACi and BRDi studies have not addressed the downstream target(s) of the inhibitors. Progressing to clinical trials without understanding the downstream effectors of these inhibitors is premature as we need more information on their prospective gene targets in different cells and at different disease stages (Andrieu *et al.* 2016). Thus, determining the binding profile and function of epigenetic regulators such as HATs (p300), BRDs (BRD4) and HDACs in controlling the persistently activated phenotypes of PH cells such as SMCs, fibroblasts (Fibs), ECs and macrophages is essential. Our preliminary data indicate that inhibitors for epigenetic regulators produce more potent effects on all phenotypes in all PH vascular cells than does inhibition of a single transcription factor, due to their common requirement for gene transcription, independent of the signal types, TFs and cell types. Proteins such as BRDs are particularly interesting, because different from histone modifying enzymes such as HDAC, HAT, methyl-transferase and demethylase, BRD proteins are only involved in gene transcription while the histone-modifying enzymes also regulate the activities of non-histone proteins (Choudhary *et al.* 2009; Spange *et al.* 2009), which have functions beyond gene transcription and make it challenging to understand the downstream targets of these enzymes.

Conclusion

A root problem in the vascular remodelling observed in chronic PH is the presence of "persistently activated" cell phenotypes with aberrant gene expression. It is likely that currently approved treatments do not directly target this problem. In this review we raise the possibility that one factor that contributes to these failures is that factors/pathways important in PH initiation and development may or may not be good treatment targets later in the disease, due to alterations of chromatin structure in "persistently activated" PH cells. These changes in chromatin can result in distinctly different functional responses to a signalling pathway or TF in normal or early PH cells *versus* cells in chronic PH. We provide evidence that another approach to uncover novel therapeutic targets for established PH is to determine the molecular mechanism(s) controlling gene expression in chronic established PH. Our central hypothesis is that high expression of genes involved in the persistently activated phenotype of PH vascular cells are maintained by an open chromatin structure and multiple TFs via the recruitment and maintenance of high levels of epigenetic regulators such as histone acetylases P300/CBP, histone acetylation readers including BRDs, Mediator complex and positive transcription elongation factor (Abstract figure). The evidence provided for this hypothesis comes at present largely from studies in the cancer field. The data shown regarding how aberrant gene expression is controlled by chromatin structure, TFs and epigenetic regulators may provide potential therapeutic targets for PH treatment. We emphasize that in PH it is not easy to extrapolate the findings from one cell type to other disease-involved cell types or findings from one pathway to other pathways. Thus, we believe effective treatments for PH must target the phenotypes of excessive proliferation, apoptosis-resistance, pro-inflammation, in all or at least most cell types of PH vascular cells such as SMCs, Fibs, ECs and inflammatory cells. Thus, studies of "transcriptional addiction" in PH vascular cells should be performed in all the aforementioned cells and in their most "activated" phenotypic state. Due to cross-talk among PH vascular cell types, at least some of the studies will need to be performed in co-culture systems to integrate protein and metabolic cross-talk. There is reason for excitement regarding potential new treatment options but more knowledge is needed before we proceed.

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Additional information

Competing interests

None declared.

Author contributions

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