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Acetyl-lysine erasers and readers in the control of pulmonary hypertension and right ventricular hypertrophy

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Abstract

Acetylation of lysine residues within nucleosomal histone tails provides a crucial mechanism for epigenetic control of gene expression. Acetyl groups are coupled to lysine residues by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs), which are also commonly referred to as “writers” and “erasers”, respectively. In addition to altering the electrostatic properties of histones, lysine acetylation often creates docking sites for bromodomain-containing “reader” proteins. This review focuses on epigenetic control of pulmonary hypertension (PH) and associated right ventricular (RV) cardiac hypertrophy and failure. Effects of small molecule HDAC inhibitors in pre-clinical models of PH are highlighted. Furthermore, we describe the recently discovered role of bromodomain and extraterminal (BET) reader proteins in the control of cardiac hypertrophy, and provide evidence suggesting that one member of this family, BRD4, contributes to the pathogenesis of RV failure. Together, the data suggest intriguing potential for pharmacological epigenetic therapies for the treatment of PH and right-sided heart failure.

Résumé

L'acétylation des lysines situées à l'intérieur des queues des histones nucléosomales constitue un mécanisme crucial de contrôle épigénétique de l'expression génique. Les groupes acétyles sont couplés aux lysines par les histone acétyltransférases (HAT) et sont enlevés par les histone déacétylases (HDAC), dont on dit qu'elles sont respectivement des « rédacteurs » et des « effaceurs ». En plus de modifier les propriétés électrostatiques des histones, l'acétylation des lysines crée souvent un site d'amarrage des protéines comportant un bromodomaine qu'on dit « lectrices ». Cet article de revue se concentre sur le contrôle épigénétique de l'hypertension pulmonaire (HP), et de l'hypertrophie et de l'insuffisance cardiaques ventriculaires droites qui lui sont associées. Les effets de petites molécules qui inhibent les HDAC dans des modèles précliniques d'HP sont soulignés. De plus, les auteurs décrivent le rôle récemment découvert des protéines lectrices BET (bromodomaine et domaine terminal) dans le contrôle de l'hypertrophie cardiaque, et présentent des indices qui suggèrent qu'un membre de cette famille, BRD4, contribue à la pathogenèse de l'insuffisance ventriculaire droite. Dans l'ensemble, les données suggèrent que les thérapies pharmacologiques épigénétiques possèdent un potentiel de traitement intéressant de l'HP et de l'insuffisance cardiaque localisée du côté droit. [Traduit par la Rédaction]

Keywords

epigenetics; RV hypertrophy; pulmonary hypertension; HDAC; bromodomain

Mots-clés

épigénétique; hypertrophie ventriculaire droite; hypertension pulmonaire; HDAC; bromodomaine

Introduction

Heart failure due to systolic and (or) diastolic ventricular dysfunction afflicts approximately 6 million Americans. The cost of treating heart failure in the United States is projected to rise to nearly \$100 billion annually by 2030 (Roger et al. 2012). Most preclinical studies of heart failure focus on the left ventricle (LV) of the heart, since LV failure causes death in the large populations of patients who suffer from conditions such as ischemic heart disease and resistant systemic hypertension. As a consequence, significantly more is known about the molecular mechanisms that govern LV failure compared to right ventricular (RV) failure.

In patients with pulmonary hypertension (PH), restricted blood flow through the pulmonary circulation increases pulmonary vascular resistance and often results in RV failure. The 5 year mortality rate for individuals with PH is ~50%, underscoring an urgent need for novel therapeutics (McLaughlin et al. 2009). Standards-of-care (SOC) for PH patients includes the use of vasoactive drugs, including endothelin receptor antagonists (ERAs), phosphodiesterase-5 (PDE-5) inhibitors, and prostacyclins (Wu et al. 2013). Many have postulated that more effective therapeutic strategies will be based on the combined use of vasodilators and agents that target distinct pathogenic mechanisms in PH, such as pulmonary vascular inflammation and fibrosis, as well as uncontrolled proliferation of smooth muscle cells, endothelial cells and fibroblasts in the lung vasculature (Humbert et al. 2004).

Crucially, maintenance of RV function is the key determinant of survival in patients with PH, and it does not appear that SOC therapy for LV failure (e.g., beta-blockers and angiotensin converting enzyme inhibitors) are effective for RV failure (Walker and Buttrick 2009). Consequently, it is clear that increased effort needs to be placed on elucidating pathogenic mechanisms in this chamber of the heart.

Recent studies have begun to highlight the role of epigenetics in the control of PH and RV failure. Histone tail acetylation is arguably the most well-characterized epigenetic mechanism for controlling gene expression. Acetyl groups are transferred to lysine residues by histone acetyltransferases (HATs) and removed by histone deacetylases (HDACs), which are also often referred to as “writers” and “erasers”, respectively. Lysine acetylation also creates binding sites for bromodomain-containing “reader” proteins such as bromodomain and extraterminal (BET) proteins (Fig. 1A). This review highlights recent, compelling findings made with HDAC inhibitors in animal models of PH and RV hypertrophy. Furthermore, we discuss the potential role of BET proteins in the control of cardiopulmonary disease.

Histone deacetylases (HDACs)

There are 18 HDACs that are encoded by distinct genes and are grouped into four classes on the basis of similarity to yeast transcriptional repressors. Class I HDACs (HDACs 1, 2, 3, and 8) are related to yeast RPD3, class II HDACs (HDACs 4, 5, 6, 9, and 10) to yeast HDA1, and class III HDACs (SirT1–7) to yeast Sir2. Class II HDACs are further divided into two subclasses, IIa (HDACs 4, 5, 7, and 9) and IIb (HDACs 6 and 10). HDAC11 falls into a fourth class (Gregoret et al. 2004). Coordination of a zinc ion in the catalytic domains of class I, II, and IV HDACs is required for catalysis (Fig. 1B). In contrast, class III HDACs (sirtuins) utilize nicotinamide adenine dinucleotide (NAD⁺) as a co-factor for catalytic activity. Class III HDACs will not be discussed further in this review, since they are not inhibited by the small molecule HDAC inhibitors that were used in the pre-clinical models of PH and RV hypertrophy (see below).

Class I, II, and IV HDACs

Class I HDACs, especially HDACs -1, -2, and -3, are thought to primarily reside in the nucleus, where they serve canonical roles in the control of gene expression through deacetylation of histone tails. These HDACs are present in large multi-protein complexes referred to as Sin3, NuRD, CoREST, and NCoR/SMRT, which are recruited to gene regulatory elements by sequence-specific DNA binding transcription factors (Cunliffe 2008). In general, HDAC1 and HDAC2 are found together in Sin3, NuRD, and CoREST complexes, while HDAC3 is a component of the NCoR/SMRT complex. However, it is important to emphasize that this is an oversimplification. For example, HDAC3 has also been found at the plasma membrane, where it regulates Src tyrosine kinase activity (Longworth and Laimins 2006), and in cardiac myocytes HDAC3 has been shown to co-localize with sarcomeres (Samant et al. 2011).

Relative to HDAC1, -2, and -3, little is known about the role of HDAC8 in the control of gene expression, and it is unclear whether HDAC8 is even able to deacetylate histones in vivo (Wolfson et al. 2013). However, HDAC8 has been shown to interact with the estrogen-related receptor alpha (ERR α) (Wilson et al. 2010) and CREB (Gao et al. 2009) transcription factors, suggesting a role for this HDAC in the control of gene expression. A recent proteomic study revealed HDAC8 association with a variety of nuclear proteins with known roles in the regulation of transcription, chromatin remodeling, and RNA splicing (Olson et al. 2014). HDAC8 can also be found in the cytoplasm, where it has been shown to interact with α -smooth muscle actin and promote smooth muscle cell contraction (Waltregny et al. 2005), and HDAC8 is able to deacetylate cytoplasmic heat shock protein 20 (HSP20) (Karolczak-Bayatti et al. 2011).

Class IIa HDACs (HDACs -4, -5, -7, and -9) have several unique features (McKinsey 2007). First, these HDACs have long (~500 amino acid) amino-terminal extensions, which harbor binding sites for transcription factors and cofactors. For example, all class IIa HDACs are able to bind to the myocyte enhancer factor 2 (MEF2) transcription factor by virtue of a conserved 18-amino acid MEF2 binding domain in their amino-terminal extensions (Lemercier et al. 2000; Lu et al. 2000a; Miska et al. 1999; Sparrow et al. 1999; Wang et al.

1999). In muscle cells, binding of class IIa HDACs to MEF2 results in suppression of MEF2 target genes that govern cellular growth and differentiation (Lu et al. 2000*b*; McKinsey et al. 2000*a*). Second, class IIa HDACs undergo signal-dependent nuclear export upon phosphorylation of two serine residues within their amino-terminal extensions. Phosphorylation of these sites by kinases such as protein kinase D (PKD) or calcium/calmodulin-dependent protein kinase (CaMK) creates docking sites for the 14-3-3 chaperone protein (Backs et al. 2006, 2008; Grozinger and Schreiber 2000; Harrison et al. 2006; Kao et al. 2001; McKinsey et al. 2000*b*, 2001; Monovich et al. 2010; Vega et al. 2004). 14-3-3 binding masks the class IIa HDAC nuclear localization signal and activates a cryptic nuclear export signal in these HDAC isoforms. Thus, in response to PKD/CaMK signaling, class IIa HDACs are exported from the nucleus, freeing MEF2 to activate downstream target genes. Third, despite having conserved catalytic domains, class IIa HDACs are unable to deacetylate endogenous radiolabeled core histones purified from mammalian cells or synthetic peptides based on acetyl-histone H4 lysine-16 (Lahm et al. 2007). In fact, bona fide substrates of class IIa HDACs have yet to be identified, and class IIa HDAC catalytic activity can only be monitored using an artificial substrate (Heltweg et al. 2004). Notably, class IIa HDACs have a tyrosine residue in the catalytic active sites, while other HDACs (e.g., class I HDACs) have a histidine residue at this position. When this tyrosine is mutated to histidine in HDACs -4, -5, or -7, catalytic activity of the class IIa HDACs is increased 1000-fold. Bradner et al. (2010) have pointed out that despite the inability of class IIa HDACs to efficiently catalyze removal of acetyl groups, they readily associate with acetylated substrates. Thus, the authors suggest that class IIa HDACs could function as acetyl-lysine readers in a manner similar to bromodomain-containing proteins. This is a plausible hypothesis given the comparable binding affinities of class IIa HDACs and bromodomain proteins for acetylated targets and the roles of both classes of proteins in recruiting large complexes that regulate gene transcription. With regard to class IIa HDACs, HDAC4 and HDAC7 have been shown to interact with repressive HDAC3- SMRT/N-CoR complexes (Fischle et al. 2002).

A fourth unique feature of class IIa HDACs is that their catalytic activity (monitored using an artificial substrate) is largely resistant to commonly used HDAC inhibitors, including the compounds used in the preclinical PH models (see below) (Bradner et al. 2010). As such, when considering effects of HDAC inhibitors in these models, it is reasonable to de-emphasize the involvement of class IIa HDACs.

In contrast to class IIa HDACs, class IIb HDACs, particularly HDAC6, are highly sensitive to HDAC inhibitors such as trichostatin A (TSA) (Bradner et al. 2010). HDAC6 contains two deacetylase domains and resides primarily in the cytoplasm, where it is known to deacetylate tubulin, heat shock protein 90 (HSP90), and the F-actin binding protein, cortactin (Valenzuela-Fernandez et al. 2008). One well-documented role for HDAC6 is in the control of misfolded protein clearance via autophagy (Iwata et al. 2005; Lee et al. 2010; Pandey et al. 2007*a*, 2007*b*). Deacetylation of tubulin by HDAC6 promotes transport of misfolded protein aggregates (aggresomes) to lysosomes, where they are degraded; HDAC6 associates with ubiquitinated proteins within aggresomes through a carboxy-terminal ubiquitin-binding domain.

The role of HDAC6 in pulmonary vasculature has yet to be addressed. In the heart, we found that HDAC6 catalytic activity is elevated in RVs of rats exposed to chronic hypoxia or hypoxia plus the VEGF receptor inhibitor SU5416 (Lemon et al. 2011). Recently, we also demonstrated that HDAC6 null mice or wild-type mice treated with an HDAC6 inhibitor, Tubastatin A, are resistant to LV remodeling in response to angiotensin infusion (Demos-Davies et al. 2014). It will be interesting to determine whether HDAC6 plays a role in RV hypertrophy/failure.

The other class IIb HDAC, HDAC10, as well as the lone class IV HDAC, HDAC11, remain poorly characterized, in part due to the inability to effectively monitor their catalytic activity using in vitro assays (Bradner et al. 2010). Consequently, it is unclear whether existing HDAC inhibitors, including those used in PH models (below), target these HDAC isoforms.

HDAC inhibitors

HDAC inhibitors are typically grouped into four classes: hydroxamic acids (e.g., TSA and SAHA), short chain fatty acids (e.g., valproic acid [VPA]), benzamides (e.g., MS-275 and MGCD0103), and cyclic peptides (e.g., FK-228). Potency and selectivity profiles differ between and within these classes (Bradner et al. 2010). The robust zinc-chelating properties of the hydroxamic acid moiety produce potent (low nanomolar) “pan-HDAC inhibitors”, although these compounds only weakly inhibit class IIa HDACs (Bradner et al. 2010). In contrast, the short chain fatty acids such as VPA are weak (millimolar) HDAC inhibitors that possess selectivity towards class I HDACs -1, -2, -3, and -8 (Fass et al. 2010). Benzamide HDAC inhibitors are generally highly selective for HDACs -1, -2, and -3. The cyclic peptide FK-228 is an extremely potent HDAC inhibitor that is able to suppress the catalytic activity of all zinc-dependent HDACs tested, including class IIa HDACs (Bradner et al. 2010).

Two HDAC inhibitors, SAHA (vorinostat) and FK-228 (romidepsin), are approved for use in humans with cutaneous T-cell lymphoma. These compounds are generally well tolerated, with the most notable side effects being thrombocytopenia, nausea, and fatigue. VPA has been used in the clinic for decades as an anticonvulsant and mood stabilizer. In addition to being an HDAC inhibitor, VPA can influence ion channels, kinases, and other biochemical pathways (Terbach and Williams 2009). As such, in experimental settings, it is difficult to attribute effects of VPA solely to inhibition of HDAC activity.

It is hypothesized that isoform-selective HDAC inhibition will provide superior efficacy with a greater safety profile than pan- HDAC inhibition (McKinsey 2011). In this regard, recent medicinal chemistry efforts led to the discovery of compounds with even more selectively for certain HDAC isoforms. For example, compounds containing aryl substitutions on the benzamide warhead are highly selective for class I HDAC1 and HDAC2 over all other HDACs (Methot et al. 2008; Moradei et al. 2007; Wilson et al. 2008; Witter et al. 2008). Conversely, other scaffolds appear to be selective for HDAC3 (Chen et al. 2009; Chou et al. 2012), and several selective inhibitors of HDAC8 have been developed (Oehme et al. 2009; Suzuki et al. 2012). The first known HDAC6/class IIb-selective inhibitor, tubacin, was described in 2003 (Haggarty et al. 2003). More recent compounds, such as tubastatin A (Butler et al. 2010), exhibit enhanced selectivity for HDAC6 than tubacin. Finally, the first

potent and highly selective class IIa HDAC inhibitors were recently described (Lobera et al. 2013). Isoform-selective HDAC inhibitors are powerful chemical biological probes that enable dissection of the roles of distinct HDACs in various pathophysiological processes. Additionally, isoform-selective HDAC inhibitors could promote development of HDAC inhibitor-based therapies that are better tolerated by humans than vorinostat and romidepsin.

HDAC inhibitors in models of PH and RV hypertrophy

Beneficial effects of HDAC inhibitors on LV hypertrophy, fibrosis, and function have been described in multiple animal models and have been extensively reviewed elsewhere (McKinsey 2012; Xie and Hill 2013). In contrast, only five papers have addressed the role of HDACs in PH and RV remodeling (Fig. 2). Cho et al. (2010) tested VPA, which selectively targets class I HDACs, for efficacy in a rat model of monocrotaline (MCT)-induced PH and RV hypertrophy in ~4 week old Sprague Dawley (SD) rats. These same investigators tested VPA in a rat model of pressure-overload RV hypertrophy mediated by pulmonary artery banding (PAB), also in 4 week old SD rats. VPA was delivered via drinking water, concomitant with initiation of MCT or PAB, and RV hypertrophy was assessed after 3 weeks; increased histone acetylation in RVs of VPA-treated rats confirmed that the compound was efficiently exposed in the tissue. VPA effectively reduced RV hypertrophy in both studies, as determined by echocardiographic assessment of RV wall thickness and quantification of RV mass at the time of necropsy. Echocardiography also revealed that VPA improved RV systolic function (ejection fraction) in animals subjected to MCT or PAB. However, it should be noted that echocardiographic evaluation of RV function is notoriously difficult to assess (Jiang et al. 1997), and thus pressure-volume analyses with an invasive catheter should also be performed in the future. In addition, the authors did not address whether VPA reduced PH in MCT treated rats, which would obviously influence the RV hypertrophic response. Nonetheless, these studies were the first to implicate HDACs in the control of RV hypertrophy.

Bogaard et al. (2011) performed an exhaustive study with TSA in male SD rats subjected to PAB. These investigators used a protocol in which the initial constriction of the pulmonary artery was mild; however, animal growth over the subsequent 6 weeks resulted in a progressive increase in RV systolic pressure and significant RV hypertrophy. This procedure does not result in RV failure (Bogaard et al. 2009). Four weeks after PAB surgery, animals were given TSA via intraperitoneal injection for five days per week for two weeks. TSA did not suppress RV hypertrophy, and strikingly, treatment with this pan-HDAC inhibitor worsened RV function, as revealed by reduced cardiac output and RV dilatation. TSA treatment in the setting of PAB was also associated with exaggerated RV fibrosis, increased numbers of apoptotic cells in the RV, and capillary rarefaction in the RV.

The authors hypothesize that the deleterious effects of TSA on the RV are due in part to its ability to reduce capillary density in this chamber of the heart. Indeed, HDAC inhibitors have been shown to elicit anti-angiogenic effects through suppression of VEGF signaling in cancer models (Deroanne et al. 2002; Mottet and Castronovo 2010). To provide mechanistic insight into the observed reduction in RV capillary density, the authors performed in vitro studies with cultured human cardiac microvascular endothelial cells (HCMVECs) and

discovered that TSA reduced expression of several angiogenic factors in these cells, including vascular endothelial growth factor (VEGF) and angiopoietin-1. Using RNAi to knockdown expression of individual HDACs in MCMVECs, it was determined that class II HDACs, particularly HDAC6, promote VEGF mRNA expression in these cells. Importantly, suppression of class I HDAC expression failed to reduce VEGF levels.

Supplemental data from Bogaard et al. (2011) described effects of VPA in the rat PAB model. Notably, although VPA appeared to cause a mild increase in RV inner diameter during diastole, it did not increase fibrosis or reduce capillary density; details regarding VPA dosing regimen were not provided. Since VPA only inhibits class I HDACs, these results suggest that isoform-selective HDAC inhibition will be better tolerated in the setting of PH and RV hypertrophy than nonselective pan-HDAC inhibition with compounds such as TSA.

More recently, De Raaf et al. (2014) assessed TSA in the Sugen/hypoxia (SU-Hx) model of severe angioproliferative PH. A breakthrough in PH research was provided by the discovery that combining hypoxia with the VEGF receptor inhibitor, SU5416, in rats results in progressive and severe PH characterized by occlusive neointima and complex plexiform lesions reminiscent of those found in lungs of patients with PH (Nicolls et al. 2012; Taraseviciene-Stewart et al. 2001). De Raaf et al. treated male Sprague Dawley rats with a single dose of SU5416 and then housed the animals in 10% O₂ for four weeks. Some animals received an intraperitoneal injection of TSA (450 mg/kg) for five days/week for the duration of the study, while other animals received vehicle control. In this study, TSA did not reduce RV hypertrophy, improve RV function, or alter pulmonary vascular remodeling. There was no evidence of worsening RV function with TSA treatment in the SU-Hx model. Although the results raise questions regarding the utility of HDAC inhibitors for PH treatment, it should be noted that TSA has an exquisitely short half-life in vivo, and that the authors failed to provide pharmacodynamics data to prove that TSA effectively inhibited cardiac and lung HDAC activity in SU-Hx rats. Additional studies with structurally distinct HDAC inhibitors should be conducted in the SU-Hx model.

We performed studies with the benzamide class I HDAC inhibitors, MGCD0103 and MS-275 (Cavasin et al. 2012), which are highly selective for class I HDACs -1, -2, and -3 (Bradner et al. 2010). Ten week old male SD rats were subjected to hypobaric hypoxia and treated with vehicle control, MGCD0103 or MS-275 every other day for three weeks. Both class I HDAC inhibitors were well tolerated and were shown to effectively suppress class I HDAC catalytic activity in RV and lung. Both compounds blunted hypoxia-induced PH as well as pulmonary vascular smooth muscle cell proliferation and medial thickening. Importantly, exhaustive pressure-volume analyses of rat RVs after three weeks of class I HDAC inhibitor treatment failed to reveal any decrement in RV contractile function. Likewise, biochemical and histological analyses of RVs failed to uncover evidence of HDAC inhibitor-mediated apoptosis. These findings further suggest that class I HDAC isoform-selective inhibitors will be well tolerated in the setting of PH.

Unexpectedly, class I HDAC inhibition only modestly decreased RV hypertrophy (Cavasin et al. 2012). Concerning this finding, it is important to note that RV hypertrophy per se is not harmful, since it serves to reduce wall stress in the face of elevated after-load. It will be

important to determine if class I HDAC inhibitors suppress the transition from RV hypertrophy to chamber dilation and pump failure, which will require longer term studies.

Together with Zhao and colleagues, we also showed that VPA and the pan-HDAC inhibitor, SAHA, can reverse existing PH and RV hypertrophy in male SD rats exposed to normobaric hypoxia (Zhao et al. 2012). Rats were housed under hypoxic conditions for two weeks prior to HDAC inhibitor treatment, via drinking water, for two additional weeks; immunoblotting confirmed that HDAC inhibitor treatment increased histone acetylation in the lungs. Reduced PH in HDAC inhibitor-treated rats was again associated with diminished muscularization of pulmonary arterioles and induction of anti-proliferative genes in the lungs. HDAC inhibitor treatment was well tolerated by the rats. However, it should be noted that RV function was not evaluated in these studies, and thus an adverse effect of pan-HDAC inhibition with SAHA on the RV cannot be ruled out.

Targeting pulmonary artery smooth muscle cells with class I HDAC inhibitors

The beneficial action of HDAC inhibitors in PH models is related, in part, to their ability to block abnormal proliferation of pulmonary vascular cells. The class I HDAC inhibitor, MGCD0103, reduced hypoxia-induced medial thickening of lung arterioles through a mechanism involving suppression of smooth muscle cell proliferation (Cavasin et al. 2012). In vivo and in cultured pulmonary artery smooth muscle cells (PASMCs), MGCD0103 stimulated expression of anti-proliferative factors such as FoxO3a and the cyclin-dependent kinase (CDK) inhibitor, p27 (Cavasin et al. 2012). Consistent with this, in a separate study both SAHA and VPA were shown to stimulate the p21 CDK in lungs of chronically hypoxic rats, and this was associated with reduced pulmonary arteriolar muscularization (Zhao et al. 2012). HDAC inhibitors also reduced expression of the anti-apoptotic protein BCL-2 in these rats, suggesting that HDAC inhibition blocks pulmonary vascular remodeling by concomitantly suppressing cell proliferation and promoting cell death.

Galletti et al. (2014) inhibited class I HDACs in PASMCs derived from rats with monocrotaline-induced PH. These investigators found that class I HDAC inhibition or siRNA-mediated knockdown of HDAC1 and HDAC2 blocks PASMC proliferation in association with suppression of AKT phosphorylation and reduction of cyclin D expression. This same group found that sodium butyrate, which selectively inhibits class I HDACs, blocks PDGF-induced PASMC proliferation (Cantoni et al. 2013). Work by an independent group demonstrated that apicidin potently blocks proliferation of PASMCs from newborn sheep, further illustrating a key role for class I HDACs in the control of pulmonary vascular cell growth (Yang et al. 2013).

Targeting pulmonary artery adventitial fibroblasts with class I HDAC inhibitors

Additional studies with cultured cells have begun to reveal the molecular mechanisms by which HDAC inhibitors impact pulmonary vascular cells. Pulmonary artery cells from

calves with PH due to chronic hypoxia have been invaluable for these studies. Cells with high proliferative capacity can be isolated from both the adventitia (PH-fibroblasts) and media (R-cells) of the bovine vessels. Strikingly, these cells maintain a hyper-proliferative phenotype over many passages in culture, suggesting a role for epigenetics in this process since, by definition, epigenetic events are heritable. In comparison, cells from control animals lose proliferative capacity after several passages. We found that class I HDAC expression and activity is dramatically elevated in PH-fibroblasts and R-cells and, selective class I HDAC inhibitors (VPA and apicidin) block the ability of both cell types to proliferate (Li et al. 2011; Zhao et al. 2012). Once again, the anti-proliferative action of class I HDAC inhibitors in PH-fibroblasts and R-cells was associated with induction of FoxO3a and p21. Importantly, HDAC inhibitors were able to block pulmonary vascular cell proliferation at doses that did not alter cell viability.

More recently, efficacy of HDAC inhibitors in PH-fibroblasts was linked to induction of microRNA-124 (miR-124) (Wang et al. 2013). MiR-124 had previously been shown to have anti-proliferative actions in glioblastoma cells (Silber et al. 2008). In cultured PH-fibroblasts (both human and bovine), miR-124 expression was found to be dramatically down-regulated. Upon treatment with class I HDAC inhibitors, miR-124 was derepressed, resulting in reduced expression of the miR-124 target, polypyrimidine tract binding protein 1 (PTBP1), and reversal of the hyper-proliferative and pro-inflammatory phenotype of PH-fibroblasts (Wang et al. 2013). PTBP1 was found to down-regulate expression of p21 and FoxO3a in PH-fibroblasts. Interestingly, miR-124 was also found to directly regulate expression of the potent inflammatory chemokine, MCP-1 (CCL2). Together, this work defined a class I HDAC/miR regulatory circuit that controls aberrant proliferation and pro-inflammatory effects of pulmonary vascular cells in response to cues for PH.

PH-fibroblasts also promote pro-fibrotic signaling and likely contribute to vascular stiffening in the setting of PH (Li et al. 2011). HDAC inhibitors have long been known to have potent anti-fibrotic actions (Bush and McKinsey 2010), and we recently demonstrated that class I HDAC inhibition with MGCD0103 blocks angiotensin-dependent cardiac fibrosis (Lishnevsky and Haudek 2014; Williams et al. 2014). It is possible that a portion of the beneficial action of class I HDAC inhibitors in models of PH is due to suppression of fibrosis in the lung vasculature.

Transcriptional regulation of LV cardiac hypertrophy by BET proteins

Positive transcription elongation factor b (P-TEFb) consists of a complex of cyclin-dependent kinase 9 (CDK9) and cyclin T1 or cyclin T2. CDK9-mediated phosphorylation of serine-2 of the heptad repeat in the carboxy-terminal domain (CTD) of RNA polymerase II (Pol II) promotes productive transcriptional elongation. Although it had long been recognized that P-TEFb plays a critical role in the regulation of cardiac hypertrophy (Sano et al. 2002; Sano and Schneider 2004), the mechanism for targeting P-TEFb to pro-hypertrophic genes in response to pathological stress in the heart remained unknown until recently. Our group and the team led by Dr. Saptarsi Halder (Case Western Reserve University) recently discovered that recruitment of P-TEFb to target genes during cardiac hypertrophy is governed by bromodomain and extraterminal (BET) acetyl-lysine reader

proteins (Anand et al. 2013; Spiltoir et al. 2013). The BET family consists of four family members (BRD2, BRD3, BRD4, and the testis-specific BRDT), all of which have two tandem N-terminal acetyl-lysine binding motifs, termed bromodomains (Fig. 3A). Of the BETs, BRD4 is the most extensively characterized and is the family member that has been shown to specifically interact with the P-TEFb complex. BRD4 associates with acetylated nucleosomal histones via its bromodomains and recruits CDK9 to transcriptional start sites through a carboxyterminal P-TEFb-interacting domain (Bisgrove et al. 2007; Jang et al. 2005; Yang et al. 2005). In so doing, BRD4 functions to promote transcriptional elongation by triggering phosphorylation of the Pol II CTD (Fig. 3B).

Remarkably, neither BRD4 nor other BET proteins had previously been studied in the heart. Endogenous BRD4 protein expression was found to be induced in the LV during cardiac hypertrophy (Spiltoir et al. 2013), and BRD4 was recruited to a select set of genes in response to hypertrophic stimuli (Anand et al. 2013; Spiltoir et al. 2013). Gene expression profiling and genome-wide ChIP-sequencing studies revealed that BRD4 promotes transcriptional pause release of Pol II in response to pathological stress in the heart, which is consistent with its ability to associate with P-TEFb (Anand et al. 2013). These studies defined BRD4 as a nodal effector of LV cardiac remodeling.

Strong validation of a role for BET proteins in the control of LV hypertrophy was provided by the chemical biological probe, JQ1. JQ1 is a small molecule that selectively binds BET bromodomains and displaces these acetyl-lysine reader domains from chromatin, resulting in suppression of downstream phosphorylation of Pol II (Filippakopoulos et al. 2010). In a mouse pressure-overload model, JQ1 dramatically blocked the development of LV hypertrophy, interstitial fibrosis, and systolic dysfunction (Anand et al. 2013; Spiltoir et al. 2013). Based on these findings, BRD4 represents an attractive therapeutic target for LV failure (Halдар and McKinsey 2014). Further studies with JQ1 and other BET inhibitors in additional pre-clinical models of LV dysfunction are clearly warranted.

BRD4 protein expression is induced during RV hypertrophy in the setting of PH

To begin to address the potential role of BRD4 in the control of RV remodeling, we assessed BRD4 protein expression in the rat SU-Hx model of severe PH. Male Sprague Dawley rats were given a single injection of SU5416 and housed in a hypobaric chamber, which simulated an altitude of 18 000 feet above sea level and a 10% O₂ environment. After three weeks in the chamber, rats were placed at Denver altitude for an additional month. At study endpoint, rats treated with SU5416 and hypoxia had massive RV hypertrophy associated with severe PH (Fig. 4A and data not shown). Immunoblotting was performed with RV homogenates to assess BRD4 protein expression. As shown in Fig. 4B, BRD4 levels were dramatically elevated in hypertrophic RVs from SU-Hx rats. Thus, similar to the LV, BRD4 expression is enhanced during RV hypertrophy. It will be interesting to determine whether JQ1 is able to block RV hypertrophy in the setting of PH. Furthermore, given the role of aberrant vascular cell proliferation in the pathogenesis of PH (Morrell et al. 2009) and the

potent anti-proliferative actions of JQ1 (Shi and Vakoc 2014), it is reasonable to predict that JQ1 will also suppress pulmonary vascular remodeling.

Conclusions

Epigenetic regulation of PH and RV hypertrophy is a largely untapped area of investigation that holds tremendous promise for therapeutic intervention (Pullamsetti et al. 2014). Initial findings with HDAC inhibitors in models of PH and RV hypertrophy highlight this potential and also underscore the need for more exhaustive research to define the mechanistic underpinnings of HDAC inhibitor action in the lung and right side of the heart. With regard to clinical translation, it will be important to determine whether a favorable therapeutic index can be achieved with an HDAC inhibitor in the setting of PH. In this regard, it is reasonable to speculate that isoform-selective HDAC inhibitors will prove to be safer than pan-HDAC inhibitors such as TSA. The demonstration that TSA actually worsened RV function in the setting of PAB (Bogaard et al. 2011) is consistent with this hypothesis.

It will also be essential to determine if HDAC inhibitors are able to reverse existing PH and RV dysfunction, and the rat SU-Hx model of progressive PH is ideally suited to address this question. With regard to class I HDAC inhibition, MGCD0103 and MS-275 inhibit HDAC1, HDAC2, and HDAC3, and thus it will be important to determine if these HDAC isoforms function redundantly in the control of PH and RV remodeling, or if selective inhibition of HDAC1, -2, or -3 is sufficient to confer benefit. These experiments are now feasible due to the recent discovery of even more selective HDAC inhibitors that can be used as precise chemical biological probes (McKinsey 2011). Concerning molecular mechanisms of HDAC inhibitor action, we know that class I HDACs regulate genes that are important for proliferation of pulmonary vascular cells (Cavasin et al. 2012; Galletti et al. 2014; Li et al. 2011; Zhao et al. 2012). However, it is also important to emphasize that thousands of proteins in all cellular compartments are subject to reversible lysine acetylation (Choudhary et al. 2009; Lundby et al. 2012), and thus HDAC inhibitors may function non-epigenetically to control acetylation of proteins that regulate signaling events that contribute to the pathogenesis of PH and RV failure.

Finally, we provide the first evidence to suggest that BET acetyllysine binding proteins are involved in RV remodeling (Fig. 4). The dramatic increase in BRD4 protein expression in hypertrophic RVs from SU-Hx rats implies a role for this reader protein in transcriptional control of RV failure through recruitment of P-TEFb and subsequent phosphorylation of Pol II. With the availability of highly selective small molecule inhibitors of BET bromodomains, such as JQ1, it will be possible to rapidly validate/invalidate a role for these proteins in PH and RV hypertrophy in vivo.

We are still in the early stages of research on epigenetic regulation of pathological cardiopulmonary remodeling. With the advent of novel genetic and chemical biological tools to accurately interrogate the functions of specific epigenetic writers, erasers, and readers, we are likely to see rapid and profound advances in this field in the near future.

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Fig. 1. Regulation of histone acetylation. (A) Lysine residues within nucleosomal histone tails are acetylated by histone acetyltransferases (HATs) and deacetylated by histone deacetylases (HDACs), which are referred to as writers and erasers, respectively. Acetylation creates docking sites for bromodomain and extraterminal (BET) reader proteins. (B) HDACs fall into four classes. Class II is further subdivided into class IIa and class IIb HDACs. All of the zinc-dependent HDACs are depicted.

Activity of HDAC Inhibitors in PH Models

Pan HDAC Inhibitors		Class I HDAC (HDAC1/2/3) Inhibitors		
TSA	SAHA	Valproic Acid	MGCD0103	MS-275
rat PAB ¹ (no efficacy)	rat Hypoxia ³	rat MCT ⁴ rat PAB ¹ rat Hypoxia ³	rat Hypoxia ⁵	rat Hypoxia ⁵
rat SU-Hx ² (no efficacy)				

1: Bogaard et al., 2011 2: DeRaaf et al., 2014 3: Zhao et al., 2012 4: Cho et al., 2010 5: Cavaasin et al., 2012

Fig. 2.
Efficacy of HDAC inhibitors in models of PH and RV hypertrophy. Pan- and class I HDAC inhibitors have been tested in a variety of rodent models of PH and RV hypertrophy. With two exceptions (TSA in the rat pulmonary artery banding [PAB] model and TSA in the rat SU5416 plus hypoxia [SU-Hx] model), HDAC inhibitors lowered pulmonary arterial pressure and exerted beneficial effects in the heart. References are indicated.

Fig. 3. BET acetyl-lysine binding proteins. (A) BRD2, BRD3, BRD4, and testis-specific BRDT comprise the bromodomain and extraterminal (BET) family of proteins. BET proteins contain conserved N-terminal acetyl lysine binding bromodomains (BD1 and BD2) and an extraterminal domain (ET). BRD4 and BRDT are able to interact with the positive transcription elongation factor b (P-TEFb complex) via a carboxyl-terminal motif (CTM). (B) BRD4 bromodomains read histone acetylation marks in gene regulatory elements. Through the CTM:P-TEFb association, BRD4 directs CDK9-mediated phosphorylation of RNA Pol II, resulting in transcriptional elongation. JQ1 is an acetyl-lysine mimetic that blocks binding of BRD4 to acetylated histones.

Fig. 4.

BRD4 protein expression is induced during RV hypertrophy. Male Sprague Dawley rats were given a dose of the VEGF receptor inhibitor SU5416 and housed for three weeks in a hypobaric chamber simulating an altitude of 18 000 feet above sea level and a 10% O₂ environment; these rats are referred to as SU-Hx rats. Rats were subsequently placed at Denver altitude for an additional month. Control rats (Normoxia) were housed in “sea level” chambers for four weeks followed by Denver altitude for four weeks. SU-Hx rats had significant RV hypertrophy, as evidenced by a ~2-fold increase in RV/LV + septum ratio (A). RV hypertrophy coincided with a dramatic increase in levels of BRD4 protein in the RV (B).