Bromodomain-Containing Protein 4
The Epigenetic Origin of Pulmonary Arterial Hypertension

Jolyane Meloche,* François Potus,* Mylène Vaillancourt, Alice Bourgeois, Ian Johnson, Laure Deschamps, Sophie Chabot, Grégoire Ruffenach, Sarah Henry, Sandra Breuils-Bonnet, Ève Tremblay, Valérie Nadeau, Caroline Lambert, Renée Paradis, Steeve Provencher, Sébastien Bonnet

Rationale: Pulmonary arterial hypertension (PAH) is a vasculopathy characterized by enhanced pulmonary artery (PA) smooth muscle cell (PASMC) proliferation and suppressed apoptosis. Decreased expression of microRNA-204 has been associated to this phenotype. By a still elusive mechanism, microRNA-204 downregulation promotes the expression of oncogenes, including nuclear factor of activated T cells, B-cell lymphoma 2, and Survivin. In cancer, increased expression of the epigenetic reader bromodomain-containing protein 4 (BRD4) sustains cell survival and proliferation. Interestingly, BRD4 is a predicted target of microRNA-204 and has binding sites on the nuclear factor of activated T cells promoter region.

Objective: To investigate the role of BRD4 in PAH pathogenesis.

Methods and Results: BRD4 is upregulated in lungs, distal PAs, and PASMCs of patients with PAH compared with controls. With mechanistic in vitro experiments, we demonstrated that BRD4 expression in PAH is microRNA-204-dependent. We further studied the molecular downstream targets of BRD4 by inhibiting its activity in PAH-PASMCs using a clinically available inhibitor, JQ1. JQ1 treatment in PAH–PASMCs increased p21 expression, thus triggering cell cycle arrest. Furthermore, BRD4 inhibition, by JQ1 or siBRD4, decreased the expression of 3 major oncogenes, which are overexpressed in PAH: nuclear factor of activated T cells, B-cell lymphoma 2, and Survivin. Blocking this oncogenic signature led to decreased PAH-PASMC proliferation and increased apoptosis in a BRD4-dependent manner. Indeed, pharmacological JQ1 or molecular (siRNA) inhibition of BRD4 reversed this pathological phenotype in addition to restoring mitochondrial membrane potential and to increasing cells spare respiratory capacity. Moreover, BRD4 inhibition in vivo reversed established PAH in the Sugen/hypoxia rat model.

Conclusions: BRD4 plays a key role in the pathological phenotype in PAH, which could offer new therapeutic perspectives for patients with PAH. (Circ Res. 2015;115:525-535. DOI: 10.1161/CIRCRESAHA.115.307004.)

Key Words: epigenomics ■ human BRD4 protein ■ microRNA ■ pulmonary hypertension ■ vascular remodeling

Pulmonary arterial hypertension (PAH) is an obstructive vascular pathology affecting the small pulmonary arteries (PAs). It is characterized by enhanced inflammation, vasoconstriction, and proliferation/apoptosis imbalance within the artery wall, leading to increased pulmonary vascular resistance, right ventricular (RV) failure and death.1 PAH is a rare disease with an estimated prevalence of 15 to 50 cases/million2 and its prevalence is thought to be highly underestimated1-6 because of lack of symptom specificity. Despite recent therapeutic advances using vasodilator therapies,1 most patients exhibit persistent poor exercise capacity and quality of life and their prognosis remains poor with a 3-year survival of 55% to 65%.4,6,7 As in cancer, PAH is associated with sustained DNA damage, which accounts for a poly(ADP ribose) polymerase 1-dependent downregulation of microRNA-204 (miR-204) and the activation of the nuclear factor of activated T cells (NFATs).8 The miR-204/NFAT axis affects mitochondrial function, bioenergetic profile and promotes the expression of oncogenes implicated in PAH, including B-cell lymphoma 2 (Bcl-2) and Survivin.8,10 This results in the proproliferative and antiapoptotic phenotype of PAH pulmonary artery smooth muscle cells (PASMCs).9

The role of epigenetics in cancer and PAH is a fast-growing field of research. Epigenetics is defined as changes...
in gene expression without modifying the DNA sequence. The major epigenetic phenomena include DNA methylation, histone modifications, and miRs. Epigenetic erasers, writers, and readers are also crucial elements for epigenetic gene modulation. Recent research described the implication of the newly characterized epigenetic readers bromodomain and extra terminal (BET) domain family in a range of cancers.11–15 These BET domain proteins include bromodomain-containing protein 2 (BRD2), BRD3, BRD4, and bromodomain testis–specific protein. They bind to acetylated histone tails and other proteins through their tandem bromodomains 1 and 2. The bromodomain is a highly conserved motif of 110 amino acid residues forming 4 β-helices and is found in proteins that interact with chromatin (ie, transcription factors, histones acetylases, etc.). BET proteins regulate transcription of genes involved in many cell functions, such as cell cycle progression, apoptosis, inflammation, etc.16 BRD4 is the most characterized member of this family and its manipulation is a major focus of ongoing research in cancer.17 BRD4 is involved in the G2 to M phase and the M to G1 phase16 by interacting with positive transcription elongation factor b, a heterodimer of cyclin-dependent kinase 9 and cyclin T1, promoting postmitotic gene transcription. During interphase, BRD4 regulates signal-inducible gene transcription by recruiting this complex to the promoter’s region. BRD4 was also showed to promote elongation in a positive transcription elongation factor b-independent manner,18 by facilitating the progression of RNA polymerase II along the gene body via its histone–chaperone activity19 or its kinase activity.20 The main known targets of BRD4 are c-Myc, Bcl-2, as well as S-phase kinase-associated protein 2, p21, and p27.16,17 Interestingly, all these proteins are implicated in PAH pathogenesis.9,21–23 Thus, by regulating genes known to be deregulated in PAH notably through the miR-204 pathway, such as NFAT, Bcl-2, Survivin, and cell cycle regulator p21, BRD4 could contribute to the PAH-PASMC proliferation/apoptosis imbalance and trigger vascular remodeling. Although other BET proteins can also regulate gene transcription, we focused our research on BRD4 because it is one of the most characterized BET protein in cancer, it is a predicted target of miR-204 (TargetScan 6.2), and has binding sites on the NFAT promoter region.11

Methods

All experiments were performed with the approval of Laval University and the Institut Universitaire de Cardiologie et de Pneumologie de Québec Biosafety and Ethics Committees. Detailed Methods are available in the online Data Supplement.

Human Tissue Samples

Tissues were obtained from patients that had previously given signed consent. PAH and control small pulmonary arteries (<1000 μm diameter) were freshly isolated from explanted lungs or during lung resection for tumors from the noncancerous segments (Table; Online Table I).

Cell Culture and Treatments

PAH-PASMCs were isolated from 6 patients with PAH and 6 control cell lines were purchased. BRD4 was inhibited with a pharmacological (+)-JQ1 (JQ1), which is a pan-BET selective bromodomain inhibitor24 or by a specific siRNA (siBRD4). Furthermore, the implication of miR-204 in BRD4 regulation was assessed using an miR-204 antagonist and mimic.15

Proliferation and Apoptosis Measurements

Proliferation was measured using Ki67 staining and MTT assay. Apoptosis was assessed by AnnexinV and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stainings, as well as by mitochondrial membrane potential (ΔΨm). We measured spare respiratory capacity of cells to assess survival and their ability to respond to stress.25

Table. Clinical Characteristics of Patients With PAH and Control Donors

<table>
<thead>
<tr>
<th></th>
<th>Control (n=17)</th>
<th>PAH (n=11)</th>
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<tbody>
<tr>
<td>Sex: female (%)</td>
<td>11 (65)</td>
<td>8 (73)</td>
</tr>
<tr>
<td>Age, y</td>
<td>52±19</td>
<td>50±17</td>
</tr>
<tr>
<td>Subclass of PAH, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPAH</td>
<td>...</td>
<td>4 (36.4)</td>
</tr>
<tr>
<td>HPAH</td>
<td>...</td>
<td>3 (27.2)</td>
</tr>
<tr>
<td>APAH</td>
<td>...</td>
<td>4 (36.4)</td>
</tr>
<tr>
<td>Functional class, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>...</td>
<td>0</td>
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<tr>
<td>II</td>
<td>...</td>
<td>0</td>
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<tr>
<td>III</td>
<td>...</td>
<td>3 (27)</td>
</tr>
<tr>
<td>IV</td>
<td>...</td>
<td>8 (73)</td>
</tr>
<tr>
<td>PVR, dyn/s per cm²</td>
<td>...</td>
<td>777±225</td>
</tr>
<tr>
<td>RAP, mm Hg</td>
<td>...</td>
<td>11±6</td>
</tr>
<tr>
<td>mPAP, mm Hg</td>
<td>...</td>
<td>47±5</td>
</tr>
<tr>
<td>CO, L/min</td>
<td>...</td>
<td>4.2±0.9</td>
</tr>
<tr>
<td>CI, L/min/m²</td>
<td>...</td>
<td>2.4±0.5</td>
</tr>
<tr>
<td>S_{O2}, %</td>
<td>...</td>
<td>55±10</td>
</tr>
<tr>
<td>6MWD, m</td>
<td>...</td>
<td>248±87</td>
</tr>
<tr>
<td>Medication, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelin receptor antagonist</td>
<td>...</td>
<td>5 (45)</td>
</tr>
<tr>
<td>PDE5 inhibitor</td>
<td>...</td>
<td>6 (55)</td>
</tr>
<tr>
<td>Epoprostenol</td>
<td>...</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Medication not known</td>
<td>...</td>
<td>3 (27)</td>
</tr>
</tbody>
</table>

Values are mean±SD. Note that some patients take >1 type of medication. 6MWD indicates 6-minute walk distance; APAP, associated pulmonary arterial hypertension; CI, cardiac index; CO, cardiac output; HPAH, heritable pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension; mPAP, mean pulmonary arterial pressure; PDE5, phosphodiesterase-5; PVR, pulmonary vascular resistance; RAP, right atrial pressure; and S_{O2}, venous oxygen saturation.
Metabolic Activity Assay
Lactate dehydrogenase enzyme activity kit was used according to the manufacturer’s instructions (Abcam). Mitochondrial density and integrity were assessed by citrate synthase activity as previously described.26 In all cases, protein concentration was determined before detergent addition using a Bradford assay.

Animal Models
Sugen/hypoxia rat model was used, as previously described.8 Rats were randomly distributed between groups (5–10 animals/group). After PAH establishment, animals were treated with JQ1, its vehicle (dimethyl sulfoxide), siBRD4, or siRNA negative control (siSCRM). All rats underwent closed-chest right heart catheterization to assess mean PA pressure (mPAP), RV systolic pressure, RV cardiac output, and total pulmonary resistance as previously described.27 All hemodynamic measurements were performed blinded to the condition.

Statistical Analysis
Values were expressed in fold changes or mean±SEM. Unpaired Student t test was used for comparisons between 2 groups and 1-way ANOVA followed by a Holm–Sidak multiple comparisons was used for >2 groups when n≥25 per group. We performed nonparametric analyses for groups with n<5 (Mann–Whitney test for 1 or 2 groups or Kruskal–Wallis test for >2 groups). If the variances of different groups were not equal and depended on the mean of the data, statistical analyses were performed on log transformation of the data. Probability values <0.05 were considered statistically significant. Statistical analyses were performed using Prism 6 (GraphPad Software Inc).

Results
BRD4 Is Overexpressed in Human PAH
We first measured BRD4 expression in human lungs and distal PAs by immunoblot and demonstrated that it is significantly increased in PAH patients’ lungs (>2-fold, Figure 1A), as well as in distal PAs (7-fold increase, Figure 1B) compared with tissues from control subjects. We further confirmed that BRD4 overexpression is present in isolated PAH-PASMCs from patients with PAH compared with control cell lines; n=6 control and n=6 PAH. BRD4 expression was measured by qRT-PCR in control cells treated with miR-204 antagoniR (antagomiR-204, 200 nmol/L) or its negative control (antagomiR-ctrl, 200 nmol/L) for 48 hours and measured by immunoblot after restored miR-204 levels in PAH-PASMCs (mimic-204, 200 nmol/L); n=3. Protein expression was normalized to total protein content. *P<0.05 and **P<0.001.
BRD4 Expression Is Regulated by miR-204

To assess the mechanism regulating BRD4 overexpression in PAH, we investigated the role of the miR-204 on its expression since BRD4 is a predicted target of miR-204 (TargetScan 6.2), which is downregulated in PAH.10 To determine if miR-204 downregulation is accountable for BRD4 overexpression in PAH, we used a bidirectional approach using our isolated PAH-PASMCs and control cells. We first used a loss of function analysis and treated control PASMCs with an miR-204 antagomiR or its negative control (antagomiR-ctrl). Indeed, control cells treated with antagomiR-204 displayed subsequent increased BRD4 mRNA levels (Figure 1D), suggesting a possible interaction between miR-204 and BRD4 mRNA as when miR-204 is absent, BRD4 is expressed. We also restored miR-204 levels in PAH-PASMCs using a synthetic miR-204 mimic (mimic-204). We measured the miR-204 mimic effect on BRD4 protein level because not all miRs will lead their targeted mRNA to degradation. We found that BRD4 protein levels were decreased in PAH-PASMCs on treatment with mimic-204 compared with them treated with the negative control mimic (Figure 1D), thus supporting our hypothesis that miR-204 is responsible for BRD4 overexpression in PAH.

BRD4 Inhibition Reverses the Oncogenic NFAT, Bcl-2, Survivin Upregulation, and Increases p21 Levels

In this study, we demonstrated the implication of BRD4 on NFATc2 and Bcl-2 regulation using JQ1, a pharmacological inhibitor of BET proteins with a higher affinity for BRD4,24 and a specific siRNA against BRD4 to further confirm our hypothesis. JQ1 is a competitive BET inhibitor, and thus does not affect BRD4 expression (Online Figure IIA). However, BRD4 siRNA at 20 nmol/L decreases BRD4 expression, as shown in Online Figure IIB. Using both these inhibitory techniques, we showed that BRD4 inhibition decreases at least by half NFATc2 and Bcl-2 mRNA levels (Figure 2A and 2B), suggesting that BRD4 may directly regulate their expression. We also assessed NFATc2 activity level (nuclear translocation assay) after JQ1 or siBRD4 treatment (Online Figure IIC). Furthermore, we showed that BRD4 inhibition dramatically decreased Survivin protein expression (Figure 2C). Conversely, the expression of the cyclin-dependent kinase inhibitor p21 is increased within the first 16 hours after JQ1 treatment (Figure 2D) highlighting the rapid arrest of proliferation triggered by BRD4 inhibition. Thus, BRD4 regulates many oncogenes implicated in PAH physiopathology.
BRD4 Triggers the Proliferation/Apoptosis Imbalance in PAH-PASMCs

Because these oncogenes are implicated in the proliferation/apoptosis imbalance observed in PAH, we then studied the involvement of BRD4 on this pathological phenotype. We confirmed that our PAH-PASMCs were much more proliferative (Ki67 and MTT) and resistant to apoptosis (Annexin V, TUNEL) compared with healthy cells placed in the same conditions (Figure 3; Online Figure III). Upon BRD4 inhibition by JQ1 (1 µM for 48 hours), proliferation levels were knocked down as assessed by Ki67 (Figure 3A). A 50% decrease was also observed in proliferation levels after siBRD4 treatment (20 nmol/L for 48 hours; Ki67, Figure 3A). However, BRD4 also regulates the apoptosis-resistance phenotype of PAH-PASMCs because its inhibition by a pharmacological inhibitor or siRNA restored apoptosis levels assessed by AnnexinV staining (Figure 3B). As shown in Online Figure III, JQ1 inhibits proliferation (Ki67) even at 0.5 µm with a significant effect on apoptosis (TUNEL) at 1 µM, which explains why this concentration was used throughout our study. Furthermore, we confirmed the JQ1 effect using MTT assay and we showed that BRD4 inhibition induced a 35% decrease in cell survival (Figure 3C). Moreover, in PAH-PASMCs, Bcl-2–mediated, and Survivin-mediated apoptosis is linked to mitochondrial membrane hyperpolarization (tetramethylrhodamine methyl ester [TMRM]; center) and spare respiratory capacity (oxygen consumption rates; right; JQ1 treatment 1 µmol/L for 48 hours); n=3 per experiment. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.

Figure 3. Bromodomain-containing protein 4 (BRD4) triggers pulmonary arterial hypertension (PAH)-distal pulmonary arteries (PAs) smooth muscle cells (PASMC) proliferation and resistance to apoptosis. A, Proliferation (Ki67) was studied after treatment of PAH-PASMCs with a BRD4 inhibitor JQ1 or vehicle (dimethyl sulfoxide [DMSO]) (1 µmol/L for 48 hours), siBRD4 or siSCRM (20 nmol/L for 48 hours). Characteristic photomicrographs of cells immunostained for Ki67 in red and colocalizing with nuclei stained in blue with DAPI. Mean data from the images show the percentages of cells with positive nuclear staining; n=3 to 5 cell lines. Scale bar, 10 µm. B, Apoptosis was measured using Annexin V staining in green. Mean data from the images show percentages of positive cells; n=3 to 5 cell lines. Scale bar, 10 µm. C, BRD4 regulates cell survival and health measured by MTT assay (left), mitochondrial membrane hyperpolarization (tetramethylrhodamine methyl ester [TMRM]; center) and spare respiratory capacity (oxygen consumption rates; right; JQ1 treatment 1 µmol/L for 48 hours); n=3 per experiment. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.
stress. We demonstrated that PAH-PASMCs have decreased spare respiratory capacity and that this was restored to levels comparable with control cells when cells were preincubated with JQ1 for 48 hours (Figure 3C), again demonstrating a beneficial effect of JQ1 on PAH-PASMCs.

**BRD4 Inhibition Reverses Sugen/Hypoxia-Induced PAH**

To further confirm the implication of miR-204 downregulation on increased BRD4 expression in vivo, we nebulized miR-204 mimics in Sugen/hypoxia-induced PAH rats. We demonstrated a robust increase in miR-204 in lungs of mimic-treated rats (Online Figure IVA). As previously published by our group in the monocrotaline model, we confirmed that ectopic increase in miR-204 expression improves hemodynamic parameters (RV systolic pressure and mPAP) in the Sugen/hypoxia-induced PAH rat model. In agreement with our in vitro experiments, forced expression of miR-204 decreased BRD4 expression (Figure 4A), supporting the notion that BRD4 is an important downstream effector of the miR-204 pathway.

To further investigate the potential therapeutic effect of BRD4 inhibition in PAH, we tested whether BRD4 inhibition could reverse PAH in a Sugen/hypoxia-induced PAH rat model. After PAH establishment, rats were treated with either a molecular (siBRD4, 1 nmol/nebulization) or a pharmacological (JQ1, 1 µmol/nebulization) BRD4 inhibitor (experimental timeline presented in Online Figure V A). Before euthanization, a comprehensive pressure-volume evaluation was performed to measure mPAP, RV systolic pressure, CO, and total pulmonary resistance. We demonstrated that either molecular (siBRD4) or pharmacological (JQ1, 1 µmol/nebulization) BRD4 inhibitor significantly improved hemodynamic measurements (Figure 4B). Indeed, BRD4 inhibition by siBRD4 and JQ1 decreased mPAP by 42% and 34%, respectively. Similar effects were also observed with RSVP measurements. BRD4 inhibition also increased CO by over 1.8-fold with both inhibitors, which led to decreased total pulmonary resistance (Figure 4B). These improvements in hemodynamic measurements were associated with a reduction in RV hypertrophy as assessed by echocardiography the day before euthanization.

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**Figure 4. Bromodomain-containing protein 4 (BRD4) inhibition reverses Sugen/hypoxia-induced pulmonary arterial hypertension (PAH).**

A. BRD4 expression was studied by immunoblot in lung tissues of control, Sugen-PAH, Sugen-PAH+negative control mimic (mimic-ctrl), Sugen-pulmonary arterial hypertension (PAH)+mimic-204 (n=5–7 animals/group). Protein expression was normalized to total protein content.

B. Mean pulmonary arterial pressure (mPAP), right ventricle systolic pressure (RVSP), cardiac output (CO), and total pulmonary resistance (TPR) were measured in control, Sugen-PAH, Sugen-PAH+siSCRM, Sugen-PAH+siBRD4 (1 nmol per nebulization), Sugen-PAH+dimethyl sulfoxide (DMSO), Sugen-PAH+JQ1 (1 µmol per nebulization) rats; n=5 to 10 animals/group. C. Vascular remodeling was assessed using hematoxylin and eosin (H&E) stain, by subtracting the lumen area from total pulmonary artery area obtaining vascular wall thickness, which was reported on % over total vessel area. Scale bar, 50 µm; n=5 to 10 animals/group (mean of 10 arteries/rat).

*P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001.
Meloche et al. BRD4 Inhibition Reverses PAH (Online Figure VB). These results strongly support our hypothesis that BRD4 upregulation is pathologically associated with PAH and that its inhibition is a promising therapeutic target.

To determine whether decreased PA wall thickness was accountable for the improvement in mPAP in treated rats, we performed an hematoxylin and eosin staining on harvested lung sections. As expected, BRD4 inhibition by siBRD4 or JQ1 decreased PA wall area by 40% and 25% compared with respective mock-treated rats (Figure 4C), thus supporting our hemodynamic measurements. From a molecular point of view, we measured proliferation and apoptosis within the vascular wall, using Ki67 immunofluorescence staining and TUNEL tagging, respectively. In accordance with our in vitro experiments, JQ1- and siBRD4-treated rats exhibited decreased proliferation and increased apoptosis within the vascular wall (Figure 5), which explains the decreased in PA wall thickness. We also confirmed that our treatment (siBRD4) decreased BRD4 expression by qRT-PCR and immunofluorescence (Online Figure VIA). To determine the specificity of BRD4 overexpression in rats, we measured its expression in other organs, such as kidney and liver, and showed that BRD4 was not overexpressed in these tissues (Online Figure VIB). Moreover, there is no BRD4 protein expression in kidney or liver of any of the studied rats (data not shown). Also, we did not observe any significant differences in weight gain throughout our groups (Online Figure VIC).

Furthermore, we assessed NFATc2 activation in lung sections by immunohistochemistry and showed decreased activation of NFATc2 after siBRD4 and JQ1 treatment (Online Figure VIIA) thus confirming the effects observed in vitro. Moreover, we displayed that JQ1 also plays a key role in the metabolic signature in vivo. The Sugen/hypoxia rats had 50% decreased glucose oxidation/glycolysis ratio as assessed by citrate synthase activity divided by lactate dehydrogenase activity (Online Figure VIB). Our JQ1 treatment reversed this effect in the lungs (Online Figure VIIB). This suggests a beneficial effect of JQ1 for restoring energy metabolism and cell health and further confirms our in vitro observations.

Thus, BRD4 inhibition, by nebulization of either siRNA (siBRD4) or a clinically available inhibitor (JQ1), improved PAH in the Sugen/hypoxia model by providing beneficial effects on the proliferation/apoptosis balance within the vascular wall, suggesting an important role for epigenetic readers in PAH physiopathology, as shown in our schematic representation in Figure 6.

**Discussion**

In this study, we demonstrated for the first time the implication of epigenetic readers, more specifically BRD4, in PAH. We displayed that BRD4 is overexpressed in lungs, distal PAs, and in isolated PASMCs of patients with PAH (Figure 1). Moreover, as predicted by in silico analysis (TargetScan 6.2), decreased miR-204 expression is accountable for BRD4 overexpression in PAH-PASMCs (Figure 1D). As an epigenetic reader, BRD4 has many downstream targets. In our study, we focused on NFAT, Survivin, and Bcl-2 because they play critical roles in the sustainability of this pathological phenotype.
and because they are the predicted targets of BRD4, as well as p21 of its role in cell cycle regulation. We demonstrated that BRD4 is accountable for NFATc2, Survivin, and Bcl-2 overexpression (Figure 2), which is already known to promote the proliferation/apoptosis imbalance observed in PAH-PASMCs.9,10,30,31 As widely described, once activated, NFAT promotes inflammation, proliferation, and also impinges on overall bioenergetic capacity.28 NFAT contributes to the antiapoptotic phenotype by triggering Bcl-2 expression.9 However, Survivin is an inhibitor of apoptosis protein, consequently inhibiting cell death within the vascular wall and promoting vascular remodeling. Thus, by regulating the opening of chromatin by binding to histone tails,14 this epigenetic reader controls their expression (Figure 2) and activation (NFATc2, Online Figure IIC) in PAH. Furthermore, BRD4 also regulates cell cycle progression by altering expression of cell cycle regulators, such as p21 (Figure 2D). This cyclin-dependent kinase inhibitor is involved in cell cycle arrest and is decreased in PAH.32 By regulating these proteins, BRD4 exhibits a crucial role in the propropulsive and antiapoptotic phenotype of PAH-PASMCs (Figure 3; Online Figure III). BRD4 inhibition in PAH-PASMCs also restored a healthy phenotype of PAH-PASMCs (Figure 3B). This was confirmed in vivo by measuring citrate synthase and lactate dehydrogenase activities (Online Figure VIIB). In vivo, both pharmacological (JQ1) and molecular (siRNA) inhibition of BRD4 reversed Sugen/hypoxia-induced apoptosis, and increased proliferation.

PAH by improving pulmonary pressures and right heart functions (Figure 4). A schematic representation of our signaling pathway is presented in Figure 6.

The fact that BRD4 triggers NFATc2 is consistent with a recent study in cancer that demonstrated, by chromatin immunoprecipitation, the presence of BRD4 on several nuclear factor κ-light-chain-enhancer of activated B cells–binding sites on NFAT promoter in osteosarcoma cells.11 In this cancer context, BRD4, activated by receptor activator of nuclear factor-κB ligand, strongly bound to the NFAT promoter at precise nuclear factor κ-light-chain-enhancer of activated B cells consensus sites and acted as a coactivator of nuclear factor κ-light-chain-enhancer of activated B cells. Our group previously demonstrated an miR-204–dependent regulation of NFATc2 in PAH via an Src-homology 2 domain-containing phosphatase 2/Src/signal transducer and activator of transcription 3 (STAT3) axis.39 In this study, we showed that BRD4 is also regulated by miR-204 (Figure 1D) and that BRD4 inhibition decreased NFATc2 expression (Figure 2A), thus decreasing cell proliferation (Figure 3A). However, it is not clear whether this miR-204/BRD4/NFATc2 axis is a part of the STAT3 network or if it is an STAT3-independent pathway. Interestingly, BRD4 was shown to directly interact with the transcription factor STAT3, enhancing transcription of its target genes.32 It is possible to speculate that STAT3 and BRD4, both overexpressed in PAH secondary to miR-204 downregulation, interact together on the NFAT promoter, thus increasing its transcription. Further investigation would be needed to confirm this interaction.

In addition to the oncogenes NFAT, Bcl-2, and Survivin, we showed that BRD4 regulates p21 expression. In this study, we found that BRD4 inhibition rapidly (within the first 16 hours) increased p21 expression (Figure 2D). In cancer, p21 is a known target of BRD4, as many studies show increased p21 expression on BRD4 inhibition.15,17,33 In these studies, they suggest that the BRD4 effect on p21 is through the transcription factor Myc. Nevertheless, we did not find a clear evidence of Myc regulation by BRD4 in PAH (data not shown), although it is known that Myc expression is increased in PAH.34 This suggests that BRD4-dependent regulation of p21 in PAH may be Myc independent. It was suggested in the literature that peroxisome proliferator–activated receptor γ, another key factor in PAH physiopathology,35,36 could regulate p21 expression.37,38 Thus, p21 expression could also be triggered through regulation of this signaling pathway. Nonetheless, further studies would be needed to the specific regulation of p21 in PAH. A possible implication of other BET proteins, such as BRD2 could also be implicated in this peroxisome proliferator–activated receptor γ pathway because BRD2 corepressed peroxisome proliferator–activated receptor γ.39

Our proliferation/apoptosis experiments revealed a greater JQ1 effect compared with specific BRD4 siRNA (Figure 3A and 3B). This may not be surprising because JQ1 is a pan-BET selective bromodomain inhibitor and other BRD proteins are overexpressed in distal PAs of patients with PAH (Online Figure IA). Nevertheless, JQ1 has a higher affinity for the bromodomain 1 of BRD4.40 The differences that we found between JQ1 and siBRD4 results in vitro suggest that other members of the BET family, in addition to BRD4, may play a
role in PAH physiopathology and that inhibition of their activity may have synergic and beneficial effects for the reversal of PAH-PASMCs phenotype. Just like BRD4, BRD2 and BRD3 bind to acetylated histones and are found on genes promoters associated with chromatin remodelers and transcription factors.16,24–40 BRD2 and BRD4 are often associated to cell cycle control and inflammation in multiple inflammatory diseases and cancers, although the role of BRD3 in the cell is less understood.16,40–43 Further investigations are required to determine a possible role for BRD2 and BRD3 in PAH.

As mentioned above, BRD4 is associated, in addition to its proliferative and antiapoptotic implications, with inflammatory processes in multiple diseases, such as rheumatoid arthritis,42 atherosclerosis,44 and chronic obstructive pulmonary disease.41 This paves the way for the use of BRD4 inhibitors to modulate the autoimmune arm of PAH pathobiology. Indeed, autoimmune diseases, such as connective tissue diseases, make a favorable seedbed for associated PAH occurrence and autoantibodies are implicated in the development of both idiopathic PAH and PAH associated with connective tissue diseases.44 Interestingly, although it is currently widely recognized that the T-helper 17 cells are crucial effectors of the autoimmune diseases, a T-helper 17 cell polarization response and autoantibodies are implicated in the development of both idiopathic PAH and PAH associated with connective tissue diseases.44 Interestingly, although it is currently widely recognized that the T-helper 17 cells are crucial effectors of the autoimmune diseases, a T-helper 17 cell polarization has been recently demonstrated in PAH.46 Because BET bromodomain inhibition has been shown to suppress T-helper 17–mediated pathology, inhibition of autoimmune-mediated vascular injuries in PAH may be a point of therapeutic intervention by JQ1.

Furthermore, BRD4 was also shown to regulate proliferation, migration, and interleukin 6 (IL-6) release by fibroblasts in lungs of patients with idiopathic pulmonary fibrosis,46 suggesting that BRD4 might have a role in physiopathology of pulmonary hypertension associated with pulmonary fibrosis. BRD4 and BRD2 sustain inflammation by activating nuclear factor κ-light-chain-enhancer of activated B cells and by regulating its target genes.45 Chromatin immunoprecipitation analyses showed that BRD4 directly binds to promoter of the proinflammatory cytokines IL-6 and tumor necrosis factor-α, increasing their expression in activated macrophages in chronic inflammatory diseases.40 Interestingly, these proinflammatory cytokines are upregulated in PAH and activate STAT3 signaling pathway.25,28 Furthermore, our group previously demonstrated the role of these proinflammatory cytokines to cause DNA damage in PASMCs, resulting in a poly(ADP-ribose) polymerase 1 increased expression and activation and subsequent miR-204 downregulation.8 Thus, BRD4 might not only play a role in the onset of PAH but also in the sustainability of this disease by maintaining this inflammatory state.

In regard to our in vivo experiments, siBRD4 treatment surprisingly seems more efficient in restoring hemodynamic measurements than JQ1 treatments conversely to what we observed in vitro. This may be explained by the short half-life of JQ1. Nevertheless, we think that BRD4 is a new therapeutic target for PAH because of its role in multiple oncogenic pathways implicated in the disease. Also, the BRD4 inhibitor JQ1 is currently in clinical trials and no toxicity has yet been reported, except for a small weight loss, which was not observed in our animal model (Online Figure VIC) probably because of the route of administration. Indeed, in our study, we administrated both JQ1 and siBRD4 by nebulization, creating a localized site of administration. The strength of administrating our therapeutics this way is to inhibit the target locally to induce the desired effect only where it is needed and to prevent possible side effects elsewhere in the body. This is worth taking into consideration because JQ1 treatment by intraperitoneal injection reduced cardiac hypertrophy in mice models with transverse aortic constriction.49 Nevertheless, cardiac hypertrophy (compensated RV) is needed for PAH patient’s survival.50,51 Interestingly, we observed BRD4 overexpression in RV of patients with PAH, as well as trends for increased levels of BRD2 and BRD3 (Online Figure IA and IC). This might suggest a role for BRD4 and other BET proteins in PAH RV hypertrophy and also in triggering the switch from a compensated RV to RV failure. This aspect will be explored in future studies.

In conclusion, this is the first study to provide evidence for the implication of the epigenetic reader BRD4 in PAH physiopathology. By acting as a coactivator to promote genes transcription resulting in cell cycle progress, BRD4 accounts for many PAH features, including NFATc2, Bcl-2 and Survivin upregulation, p21 repression, cell proliferation, mitochondrial dysfunction, and resistance to apoptosis. Thus, our study does not only demonstrate the implication of epigenetic readers in PAH but also supports the translational potential of existing BRD4 inhibitors as new therapies for PAH.

Acknowledgments
We thank the Respiratory Health Network tissue bank (Institut Universitaire de Cardiologie et de Pneumologie de Québec [IUCPQ] site) and the Pulmonary Hypertension Research Group for their help and advice throughout the project. We also thank Dr Ferraro from the Centre Hospitalier de L’Université de Montréal for the help in obtaining tissues for PAH patients’ ongoing lung transplantation. We would also acknowledge the help of Serge Simard, biostatistician from the CRIUCPQ (Centre de Recherche de l’Institut Universitaire de Cardiologie et de Pneumologie de Québec), in reviewing this study.

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Disclosures
None.

References


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**Novelty and Significance**

**What Is Known?**

- BRD4 inhibitors (such as JQ1) are being investigated in clinical trials.
- BRD4 regulates expression of several oncogenes in cancer, which are also implicated in pulmonary arterial hypertension (PAH) pathogenesis.

**What New Information Does This Article Contribute?**

- We show that bromodomain-containing protein 4 (BRD4) is overexpressed in the lung tissue and pulmonary vasculature from human patients with PAH.
- Treatment with a BRD4 inhibitor imparts beneficial effects in a rat model of PAH.
- The study provides evidence for the involvement of the epigenetic reader BRD4 in PAH physiopathology.
Bromodomain-Containing Protein 4: The Epigenetic Origin of Pulmonary Arterial Hypertension

Jolyane Meloche, François Potus, Mylène Vaillancourt, Alice Bourgeois, Ian Johnson, Laure Deschamps, Sophie Chabot, Grégoire Ruffenach, Sarah Henry, Sandra Breuils-Bonnet, Ève Tremblay, Valérie Nadeau, Caroline Lambert, Renée Paradis, Steeve Provencher and Sébastien Bonnet

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SUPPLEMENTAL MATERIAL

Detailed Methods

Cell culture and treatments
PAH-PASMCs were isolated from pulmonary arteries with a diameter smaller than 1000 μm from 6 PAH patients (2 idiopatic PAH, 1 associated PAH and 3 heritable PAH), all with confirmed diagnostic defined by a mean PA pressure over 25 mmHg. The smooth muscle cell phenotype was confirmed using α-smooth muscle actin (Sigma). Control PASMCs (n=6 cell lines) were purchased from Cell Application (San Diego, CA). PASMCs were grown in high-glucose DMEM supplemented with 10% FBS (Sigma) and 1% antibiotic/antimotic (Life Technologies) and used from fourth to sixth passage. BRD4 was inhibited with a clinically relevant dose of (+)-JQ1 (JQ1) (1 μM for 48h, Selleckchem), diluted in Dimethyl sulfoxide (DMSO). Lipofectamine 2000 (Life Technologies) was used to transfect PASMCs with specific BRD4 siRNA (siBRD4) (20nM for 48h; Life Technologies) or its negative control (siRNA negative control #2, siSCRM) (Life Technologies). The implication of miR-204 was assessed using miRIDIAN miR-204 mimic (200nM for 48h) (Thermo Fisher Scientific) and AntagomiR-204 (hairpin inhibitor, 200nM for 48h) (Thermo Fisher Scientific). For each experiment, we used a proper control (mimics or hairpin inhibitor negative control from Thermo Fisher Scientific).

Proliferation and apoptosis measurements
To study the effect of BRD4 on PASMC proliferation and apoptosis in vitro, cultured human PAH-PASMCs were exposed to 10% FBS (proliferation) or 0.1% FBS (starvation, which is known to promote apoptosis). Proliferation was measured by immunofluorescence staining using Ki67 antibody (Millipore, 1:250). Apoptosis was measured by immunofluorescence using ApoAlert Annexin V-FITC Apoptosis kit (Clontech) and Apoptag apoptosis detection kit (Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), Millipore). Percent of positive PASMCs was determined and divided by the total amount of cells, calculated with DAPI (total nuclei). MTT assay (Sigma) was also used to measure proliferation according to the manufacturer’s instructions. The absorbance at 575nm and 690nm were measured using a microplate reader (Synergy H1, BioTek). PASMC mitochondrial membrane potential (ΔΨm) was assessed using tetramethylrhodamine methyl ester perchlorate (TMRM, Invitrogen) after JQ1 treatment to evaluate the effect of inhibiting BRD4 on ΔΨm, since a hyperpolarized mitochondria reflects resistance to apoptosis. Briefly, live cells were treated with TMRM 10μM and Hoechst 100μM (Life Technologies) during 30 minutes at 37°C. Fluorescence was measured using AxioVision software on 20 to 50 cells/experiment in 3 experiments. Real time measurements of oxygen consumption rate were performed using Seahorse XF24 extracellular flux analyzer in order to assess cell spare respiratory capacity, using the XF Mito Stress Test Kit (Seahorse Bioscience) according to the manufacturer’s instructions. Briefly, the spare respiratory capacity is the difference between maximal uncontrolled oxygen consumption rates compared to baseline measurements. The maximal oxygen consumption rate is observed after addition of an electron transport chain accelerator: FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone). FCCP is an uncoupling agent, which disrupts mitochondrial membrane potential and ATP synthesis, leading to a rapid oxygen and energy consumption (without ATP production). The spare respiratory capacity reflects the viability and survival of cells as well as their capacity to respond to stress. All experiments were at least performed in triplicate.

Quantitative RT-PCR and immunobLOTS
To measure BRD4, NFATc2 and Bcl-2 mRNA expression, total mRNA was extracted from PASMCs using a trizol protocol. Quantitative RT-PCR (qRT-PCR) was calculated with the β2-microglobulin (β2M) or 18S as housekeeping genes (Taqman Gene Expression Assay, Applied Biosystem), as previously described. NFATc2 mRNA levels were normalized to β2M, because of its lower expression and all other genes were normalized to 18S. Protein expression of BRD4 (Abcam, 1:5000), NFATc2 (Abcam, 1:1000), Survivin (Cell Signaling, 1:1000) and p21 (Cell Signaling, 1:1000) were quantified using the
Chemidoc (Bio-Rad) and normalized to total protein content (calculated with amidoblack or stainfree). Briefly, using the Image Lab 5.0 Software (Bio-Rad), we calculated the total intensity of protein per lane and did a ratio of the protein of interest intensity on the intensity of total amount protein in that lane, although only one band of the amidoblack is shown in main Figures. Total protein stainings are presented in Online Figures (whole membrane stained with amidoblack). The arrow represents the band shown in main Figure. Results were expressed in fold of control. All experiments were at least performed in triplicate.

Animal models
Male Sprague-Dawley rats (250-300g) (strain 400, Charles River) were randomly distributed between groups (5 to 10 animals/group). All hemodynamic measurements were performed blinded to the condition. A subcutaneous injection of 20mg/kg SU5416 (Tocris Bioscience) was performed. Rats were then placed in normobaric hypoxic chambers (10% O₂ and 90% N₂) for 3 weeks. Once PAH was established (after 5 weeks), animals were treated by nebulization every four days for 2 weeks with either the BET inhibitor JQ1 (1μmol, Selleckchem), vehicle (DMSO), siBRD4 (1nmol, Life Technologies) or siRNA Negative Control # 2 (1nmol, Life Technologies). PAH progression was monitored every week by non-invasive 2D-Doppler echocardiography. Before sacrifice, all rats underwent a comprehensive pressure-volume evaluation by closed chest right heart catheterization (SciSence catheters). Briefly, rats will be anesthetized with 3- 4% isoflurane induction and maintained with 2% during procedures. A high-fidelity catheter (1.9F PV- 6.0mm Elec Spacing (#FTH-1912B-6018), Transonic Science Instruments Inc.) allowing measurement of instantaneous RV pressure and volume will be advanced through the jugular vein in close-chest animals and measurements will be recorded continuously using LabScribe2 software (iWorks System Inc). We measured measure mean pulmonary arterial pressure (mPAP), RV systolic pressure (RVSP) and cardiac output (CO), as previously described. Total pulmonary resistance (TPR) was calculated by dividing mPAP with the CO. Histology measurements were performed as previously described. Briefly, pulmonary artery wall thickness was measured in 10 arteries/animal using Hematoxylin and Eosin (H&E) staining on lung sections. PA wall thickness was calculated by the percentage of the PA wall area on total artery area.

Cofocal microscopy, immunofluorescence and immunohistochemistry
Immunofluorescence stainings were performed on 5μm paraffin-embedded lung section and only distal PAs (<75μm) were investigated. BRD4 (Abcam, 1:250), Ki67 (Millipore, 1:250) and α-smooth muscle actin (Sigma, 1:400) were used as primary antibodies and Alexa Fluor 488 and 594 (Life Technologies, 1:1000) were used as secondary antibodies. PASMC apoptosis within the arteries were measured using Apoptag apoptosis detection kit (TUNEL, Millipore). Colocalization analyses (with DAPI) were performed using the Zen system from Zeiss. To assess NFATc2 activity (nuclear translocation), lung sections were stained with NFATc2 primary antibody (Abcam; 1:250) followed by immunohistochemistry detection with secondary biotinylated-anti-mouse antibody and the Vectastain HRP ABC Reagent (Vector Laboratories, Burlingame, CA) was used for detection. The sections were counterstained with Hematoxylin. In all cases, percent of positive cells was assessed by dividing the amount of positive nuclei on total nuclei/artery in 10 arteries/rat for at least 5 rats/group.
Supplemental Figures

Online Figure I. Total membranes stained with amidoblack, which were used for total protein content normalization. The arrow represents the band shown in main figures.
Online Figure II. Other BET proteins are also increased in PAH. A, BRD4 expression was measured in RV of controls and PAH patients. n=5 controls and 4 PAH, *p<0.05. B, BRD2 and BRD3 protein levels were studied by immunoblot in distal PAs of control donors as well as patients with PAH. n=9 control and 6 PAH, **p<0.01. C, Although not significantly, but other BET proteins also have increased expression in RV of PAH patients. n=5 controls and 4 PAH.
Online Figure III. Total membranes stained with amidoblack, which were used for total protein content normalization. The arrow represents the band shown in main figures.
**Online Figure IV. BRD4 regulates NFATc2 activation.**

**A,** BRD4 protein expression after JQ1 treatment (1 µm for 48 hours) is slightly increased. n=3. **B,** BRD4 siRNA efficiency was confirmed using qRT-PCR (n=3) and western blot. **C,** NFATc2 activity was assessed by nuclear translocation assay. Representative photomicrographs of cells immunostained for the detection of NFATc2 in red and colocalizing with nuclei stained in blue with DAPI. Scale bar represents 20 µm. Mean data from the images show the percentages of cells with positive nuclear staining. n=3 to 5 cell lines. *p<0.05, ***p<0.001 and ****p<0.0001
Online Figure V. Total membranes stained with amidoblack, which were used for total protein content normalization. The arrow represents the band shown in main figures.
Online Figure VI. BRD4 triggers PAH-PASMC proliferation and resistance to apoptosis in a dose-dependent manner. A, Representative photomicrographs of cells after different doses of JQ1 for 48 hours immunostained for the detection of Ki67 (proliferation) in red and colocalizing with nuclei stained in blue with DAPI. Scale bar represents 20μm. Mean data from the images show the percentages of cells with positive nuclear staining. n=3. B, Representative photomicrographs of cells after different doses of JQ1 for 48 hours immunostained for apoptosis (TUNEL) in red and colocalizing with DAPI (blue). Scale bar represents 10μm. Mean data from the images show the percentages of cells with positive nuclear staining. n=3. C, Representative photomicrographs of cells immunostained with Tetramethylrhodamine methyl ester (TMRM) in orange and Hoechst in blue. Scale bar represents 20μm. *p<0.05, **p<0.01 and ***p<0.001.
Online Figure VII. Profile of Sugen rats after inducing miR-204 and inhibition BRD4. A, miR-204 expression was measured using qRT-PCR on n=5 to 7 rats per group. B, Ectopic increase in miR-204 levels decreased hemodynamic measurements in Sugen/hypoxia rats. Right ventricle (RV) systolic pressure (RVSP) and mean pulmonary arterial pressure (mPAP) were assessed by right heart catheterization. n=5 to 7 animals/group. *p<0.05, ***p<0.001 and ****p<0.0001. C, Total membrane (in Figure 4A) stained with amidoblock, which was used for total protein content normalization. The arrow represents the band shown in Figure 4A.
Online Figure VIII. Experimental design of the Sugen/hypoxia experiment. A, Rats received a subcutaneous injection of Sugen (SU5416) 20mg/kg on day 0 and put in hypoxia for 3 weeks. We waited 3 weeks after the hypoxia period before starting our treatment. Rats were treated by intra-tracheal nebulization every 4 days for 2 weeks (3 nebulizations total). B, Right ventricular (RV) hypertrophy was assessed by echocardiography the day before sacrifice. n=5 to 7 animals/group. *p<0.05 and ***p<0.001.
Online Figure IX. BRD4 is specifically overexpressed in the lungs. A, BRD4 expression was assessed by qRT-PCR in siBRD4-treated rats compared to their mock control. n=5 to 8 rats per group. ***p<0.001. Representative photomicrographs of pulmonary arteries immunostained for BRD4 (in red) to assess the effectiveness of BRD4 siRNA administration. Vascular smooth muscle cells were identified using a smooth muscle actin staining (in green) and nuclei are stained in blue with DAPI. Mean data from the images show the percentages of cells with a positive nuclear staining. Scale bars represent 40 μm. n=mean of 10 arteries/rat in 5 rats/group, ****p<0.0001. B, BRD4 mRNA level was measured in rat liver and kidney. n=5 animals per group. C, Change in body weight during the 2 weeks treatment period was measured. Data are expressed as the weight gained (in grams).
Online Figure X. BRD4 inhibition in vivo decreases NFATc2 activation and restores energy metabolism. A, NFATc2 activation (nuclear translocation assay) was assessed by immunohistochemistry in 5 rats per group. NFATc2 positive staining is brown and colocalizes with nuclei in blue (as shown by the arrows). Mean data from the images show the percentages of cells with a positive nuclear staining for NFATc2 in 10 arteries per rat. Scale bars represent 30 μm. B, The energy metabolism of lungs of Sugen/hypoxia rats with or without JQ1 treatment was assessed using citrate synthase (CS) activity (glucose oxydation) and lactate dehydrogenase (LDH) activity (glycolysis). The ratio of glucose oxydation over glycolysis was expressed on fold from control lungs (A.U.: Arbitrary Units). n=6 per group. *p<0.05, **p<0.01 and ***p<0.001.
Supplemental Table

Online Table I. Detailed tissue characteristics used in this study

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Supplemental References


