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Brahma-related gene 1 (Brg1) epigenetically regulates CAM activation during hypoxic pulmonary hypertension

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Aims Establishment of an inflammatory milieu following elevated leukocyte adhesion to the vascular endothelium, which is mediated by transcriptional activation of cell adhesion molecules (CAMs), contributes to the pathogenesis of chronic hypoxia-induced pulmonary hypertension (HPH). The epigenetic switch that dictates CAM transactivation in response to hypoxia in endothelial cells leading up to HPH is not fully appreciated.

Methods and results We report here that brahma-related gene 1 (Brg1) and brahma (Brm), two catalytic components of the mammalian chromatin remodelling complex, were induced in cultured endothelial cells challenged with hypoxia *in vitro* as well as in pulmonary arteries in an animal model of HPH. Over-expression of Brg1/Brm enhanced, while the depletion of Brg1/Brm attenuated, CAM transactivation and adhesion of leukocytes. Endothelial-specific deletion of Brg1/Brm ameliorated vascular inflammation and HPH in mice. Chromatin immunoprecipitation (ChIP) and re-ChIP assays revealed that hypoxia up-regulated the occupancies of Brg1 and Brm on CAM promoters in a nuclear factor κB (NF-κB) -dependent manner. Finally, Brg1 and Brm activated CAM transcription by altering the chromatin structure surrounding the CAM promoters.

Conclusion Our data suggest that Brg1 provides the crucial epigenetic link to hypoxia-induced CAM induction and leukocyte adhesion that engenders endothelial malfunction and pathogenesis of HPH. As such, targeting Brg1 in endothelial cells may yield promising strategies in the intervention and/or prevention of HPH.

Keywords Hypoxic pulmonary hypertension • Epigenetics • Brg1 • Transcriptional regulation • Adhesion molecules

1. Introduction

Hypoxic pulmonary hypertension (HPH) is a debilitating disease characterized by elevated pulmonary inflammation.[1–3] Elevated inflammation is invariably initiated by the aggregation and adhesion of immune cells on the vessel wall. Indeed, it has been reported that several distinct lineages of immune cells, including lymphocytes, leukocytes, and activated macrophages, accumulate in the lungs following hypoxic challenge.[4–6] These cells, once recruited, contribute to the establishment of a pro-inflammatory microenvironment promoting the pathogenesis of HPH.

The interaction between the vascular endothelium and circulating immune cells is a complicated, multistep process.[7] It starts with the tentative tethering and rolling of immune cells over the endothelial layer, followed by firm adhesion, and then finalized by trans-endothelial migration, leading to the creation of pro-inflammatory niches. Pivotal to this process is the adhesion of leukocytes to the vessel wall facilitated by the up-regulation of cell adhesion molecules (CAMs), including intercellular adhesion molecules (ICAMs), vascular adhesion molecules (VCAMs), and selectins, in vascular endothelial cells.[8] Therefore, accelerated transcription of CAM genes in hypoxic endothelial cells represents a key event that foretells enhanced leukocyte adhesion, perpetuated vascular inflammation, and eventually the development of HPH.

NF-κB/p65 is considered the master regulator of pro-inflammatory transcription in the vasculature.[9] Of note, p65-dependent transcription

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has been found to contribute to vascular endothelial injury under various conditions.[10–12] In particular, p65 is known to exert decompensatory effects on the endothelium inflicted by hypoxic stress.[2] In response to hypoxia, p65 induces and sustains endothelial dysfunction by, at least in part, transactivating a slew of CAM genes.[13–15] The epigenetic underpinnings by which p65 up-regulates CAM gene transcription remain largely undefined.

Unlike the prokaryotic organisms, eukaryotic genes are wrapped by histones into individual nucleosomes to form chromatin that needs to be unfolded in order for the basic transcription machinery to gain access. The process of unwrapping the high-order DNA structure requires a group of highly conserved proteins called chromatin remodelling complex. Initially identified in yeast, this remodelling complex utilizes ATP to mobilize nucleosomes and alter transcription.[16] Brahma-related gene 1 (Brg1) and brahma (Brm) are the catalytic components of the mammalian chromatin remodelling complex playing important roles in maintaining vascular homoeostasis in embryogenesis and promoting a pathogenic agenda under stress conditions. Here, we report that Brg1 is induced by hypoxia and potentiates CAM transactivation in vascular endothelial cells by forming a complex with p65. Brg1 modulates CAM transactivation by influencing histone modifications surrounding the CAM promoters. Importantly, endothelial-specific depletion of Brg1 alleviates HPH in mice. Therefore, targeting Brg1 in endothelial cells may yield promising strategies in the intervention and/or prevention of HPH.

2. Methods

2.1 Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs/EAhy926, ATCC) and human monocytic/macrophage cells (THP-1, ATCC) were maintained in DMEM (Invitrogen) supplemented with 10% foetal bovine serum (Hyclone). Human primary pulmonary arterial endothelial cells (HPECs, Cambrex/Lonza) were maintained in endothelial growth media 2 media with supplements supplied by the vendor; experiments were performed in primary cells between third and sixth passages. The use of human primary cells was in accordance with the principles outlined in the Declaration of Helsinki and approved by the intramural Ethics Committee.

2.2 Plasmids, transient transfection, and luciferase assay

Details for plasmids used in this study can be found in the Supplementary material online. Small interfering RNA (siRNA) sequences were as follows: for human Brg1, AACATGCACCAGATGCACAAG and for human Brm, AAGTCCTGGACCTCCAAGTGT. Transient transfections were performed with Lipofectamine 2000 (Invitrogen). siRNA for p65 has been described before.[17] Luciferase activities were assayed 24–48 h after transfection using a luciferase reporter assay system (Promega). Experiments were routinely performed in triplicate wells and repeated three times.

2.3 Mice

All protocols were approved by the intramural Committee on Ethic Conduct of Animal Studies. Eight-week-old, male C57/BL6 mice were housed in a closed chamber with an ambient air pressure of 405.35 mmHg (~0.53 atm) for 4 weeks to induce pulmonary hypertension. Short-hairpin RNA (shRNA) targeting both Brg1 and Brm (GCUGGAGAAGCAGCAGAAG) were cloned into an endothelium-specific expression vector (Tie2p/eas) and packaged using an endothelium-specific envelope (see Section 2.2) as previously described.[18,19] At Weeks 1 and 3, these mice were injected via sublingual vein purified lentiviral particles. Inhalation anaesthesia was used throughout procedure (1–3% isofluorane supplemented with oxygen). The depth of anaesthesia was assessed by loss of pain and corneal reflexes. Right ventricular systolic pressure and right ventricular hypertrophy were evaluated as described before.[20]

2.4 Protein extraction and Western blot

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100) with a freshly added protease inhibitor tablet (Roche). Western blot analyses were performed with anti-FLAG, anti-β-actin (Sigma), anti-ICAM-1, anti-Brg1, and anti-Brm (Santa Cruz) antibodies. For quantification, the intensity of the blot of the loading control (β-actin) and that of the proteins of interest were measured separately. Then, the intensity of the target protein was divided by the corresponding β-actin intensity. Finally, the ratios of all the other groups were compared with the control group, which is set arbitrarily as 1.

2.5 RNA isolation and real-time PCR

RNA was extracted with the RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions were performed using a SuperScript First-strand Synthesis System (Invitrogen). Real-time PCR reactions were performed on an ABI Prism 7500 system. Primers and Taqman probes used for real-time reactions to detect ICAM-1, VCAM-1, E-selectin, Brg1, and Brm were purchased from Applied Biosystems.

2.6 Chromatin immunoprecipitation and re-ChIP

Chromatin immunoprecipitation (ChIP) assay was performed as previously described.[21,22] Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-Brg1, anti-Brm, anti-p65, anti-RNA Pol II (Santa Cruz), anti-acetyl histone H3, anti-acetyl H4, anti-dimethyl H3K4, anti-trimethyl H3K4 (Millipore), or pre-immune IgG. For re-ChIP, immune complexes were eluted with the elution buffer (1% SDS, 100 mM $NaCO_3$), diluted with the re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris (pH 8.1)), and subject to immunoprecipitation with a second antibody of interest. Precipitated genomic DNA was amplified by real-time PCR with primers listed in Supplementary material online, *Table S1*.

2.7 Leukocyte adhesion assay

In vitro leukocyte adhesion assay was performed essentially as described before.[23] THP-1 cells were stained with a fluorescent dye (2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein tetrakis (acetoxymethyl) ester) (Sigma) for 30 min at 37°C. After several washes with PBS, THP-1 cells were co-incubated for 30 min with endothelial cells. Unbound leukocytes were removed by washing and the number of adhered cells was visualized by fluorescence microscopy and analysed with Image-Pro Plus (Media Cybernetics).

2.8 Immunofluorescence staining

The plastic-embedded sections were incubated with primary antibodies, anti-CD31 (BD Biosciences), anti-Brg1 (Santa Cruz), anti-Brm (Santa Cruz), anti-CD3 (BD Biosciences), anti-CD45 (BD Biosciences), and anti-F4/80 (BD Biosciences), followed by incubation with donkey secondary antibodies (Jackson ImmunoResearch). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Sigma). For quantification, positively stained cells were counted. The average of at least three slides was included for each mouse.

2.9 Statistical analysis

One-way analysis of variance with *post hoc* Scheffe analyses were performed using an SPSS package. Unless otherwise specified, *P*-values <0.05 were considered statistically significant (asterisk).

3. Results

3.1 Hypoxia activates Brg1 *in vitro* and *in vivo*

To test the hypothesis that Brg1 might be involved in endothelial injury inflicted by hypoxic stress, we first examined the expression levels of Brg1 in cultured human endothelial cells. Exposure to 1% O_2 caused an up-regulation in the message levels of Brg1 and Brm, in both immortalized endothelial cells (HUVECs) and primary pulmonary arterial endothelial cells (HPECs) (*Figure 1A* and *B*). By comparison, BAF47 and BAF155, two non-catalytic components of the mammalian chromatin remodelling complex, were not significantly altered under the same conditions. In agreement, protein levels of Brg1 and Brm were also augmented by hypoxia (*Figure 1C*). To further probe the effect of hypobaric hypoxia on Brg1 and Brm *in vivo*, C57/BL6 mice were housed in a low-oxygen chamber for 4 weeks to induce hypoxic pulmonary hypertension; pulmonary arteries were then isolated to measure the expression of Brg1 and Brm. As shown in *Figure 1D* and *E*, the levels of Brg1 and Brm were significantly elevated in HPH mice as opposed to age- and sex-matched control mice. Together, these data demonstrate that the expression of Brg1 and Brm fluctuates in

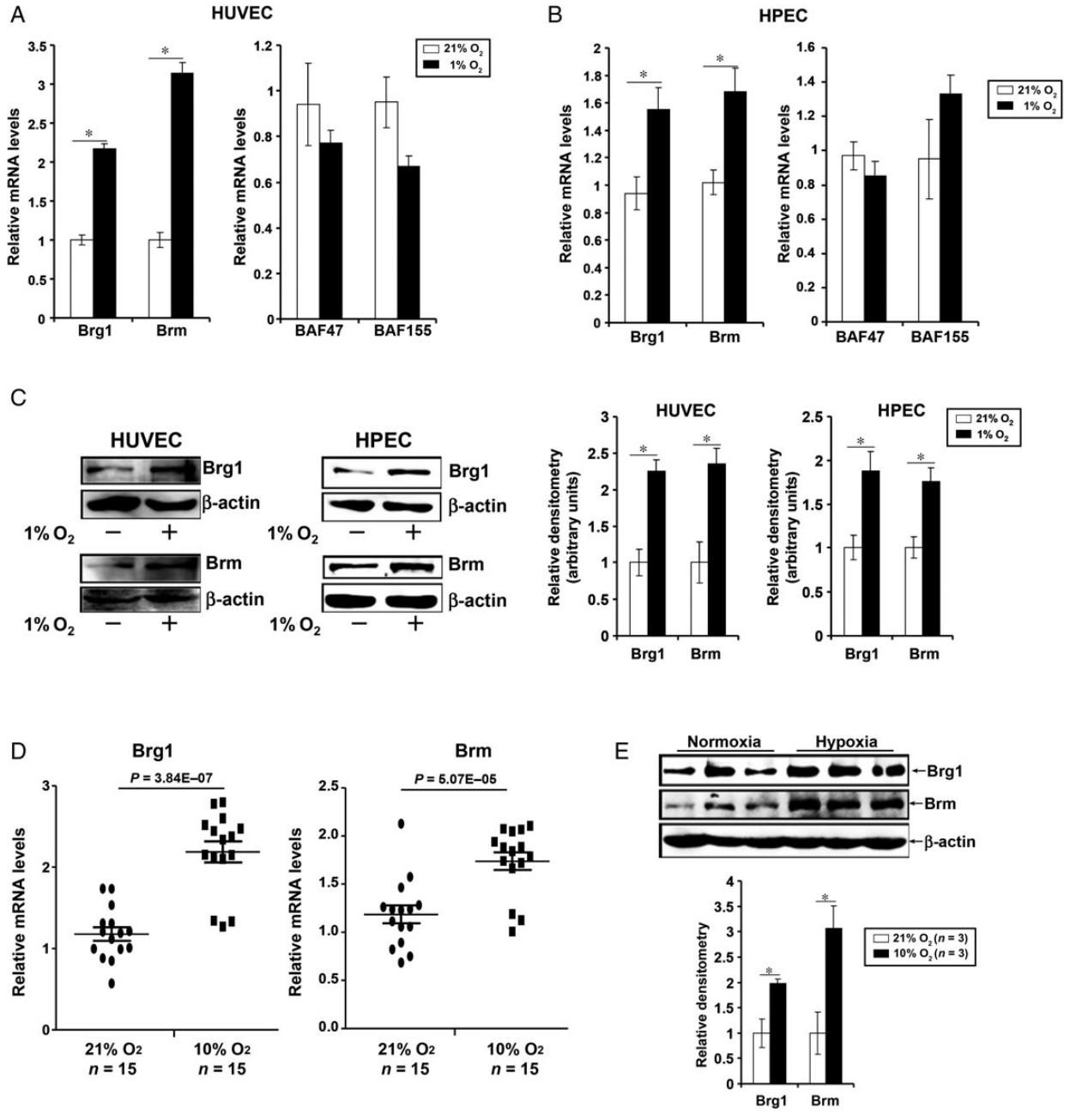

Figure 1 Hypoxia activates the expression of Brg1 and Brm *in vitro* and *in vivo*. (*A*–*C*) HUVECs and HPECs were exposed to 1% O_2 for 24 h. mRNA (*A* and *B*) and protein (*C*) levels of Brg1 and Brm were probed with qPCR and Western blot, respectively. (*D* and *E*) C57/BL6 mice were housed in a low-oxygen chamber for 4 weeks to allow the development of HPH. mRNA (*D*) and protein (*E*) levels of Brg1 and Brm in pulmonary arteries were measured by qPCR and Western blot analyses, respectively.

endothelial cells in response to oxygen tension indicative of a potential role for Brg1/Brm in HPH.

3.2 Brg1 is both sufficient and necessary for hypoxia-induced CAM transactivation

Next, we set out to evaluate the possibility that Brg1 might participate in the regulation of transactivation of CAM genes in endothelial cells in response to hypoxic stress. By themselves, neither Brg1 nor Brm significantly altered the promoter activities of ICAM-1, VCAM-1, and E-selectin genes in reporter assays (*Figure 2A*, Supplementary material online, *Figure S1A* and *B*). Brg1 and Brm were, however, able to enhance the activation of CAM promoters by hypoxia (*Figure 2A*, Supplementary material online, *Figure S1A* and *B*). Enzymatic activity was clearly required for both Brg1 and Brm to potentiate hypoxia-induced CAM transactivation since point mutations (ED, enzyme dead) that interfere with the catalytic domain deprived Brg1 and Brm of their ability to activate CAM promoters under hypoxic conditions (*Figure 2B*, Supplementary material online, *Figure S1C* and *D*). More importantly, over-expression of Brg1 and Brm led to a marked increase in the levels of endogenous adhesion molecules (*Figure 2C* and *D*, Supplementary material online, *Figure S1E* and *F*).

We, then, tackled the question whether depletion of Brg1 or Brm could dampen the induction of adhesion molecules in endothelial cells under hypoxic conditions and thereby alleviate leukocyte adhesion. To this end, we knocked down the expression of endogenous Brg1 and Brm using siRNA. Compared with scrambled (SCR) sequence, specific siRNA significantly down-regulated both mRNA and protein levels of endogenous Brg1 and Brm in endothelial cells (Supplementary material online, *Figure S2A*). In the absence of Brg1 or Brm, induction of CAM promoter activities by hypoxia was severely crippled (*Figure 3A*). Furthermore, the induction of endogenous CAM messages by hypoxia was also limited in both HUVECs (*Figure 3B* and *C*, Supplementary material online, *Figure S2B*), and HPECs (*Figure 3B* and *C*, Supplementary material online, *Figure S2C*). Of note, endogenous expression of all three adhesion molecules seemed to be more sensitive to the loss of Brg1 than Brm. *In vitro* adhesion assay also revealed that the interaction between

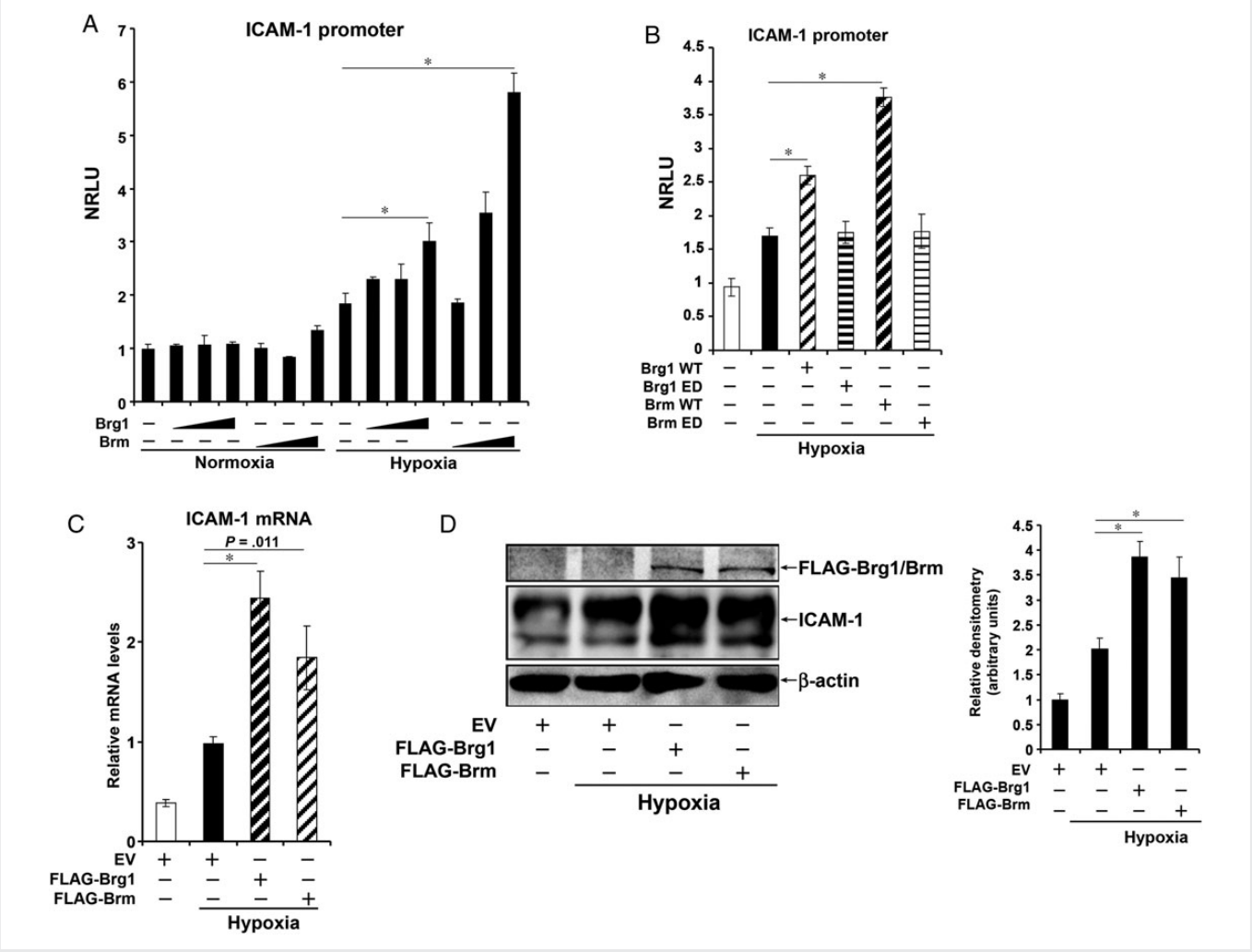

Figure 2 Brg1 and Brm potentiate hypoxia-induced ICAM-1 transcription. (*A*) An ICAM-1 promoter construct was transfected into HUVECs with increasing doses of Brg1 or Brm and then exposed to 21 or 1% O_2 for 24 h. Luciferase activities were expressed as normalized relative luciferase units (NRLU). (*B*) An ICAM-1 promoter construct was transfected into HUVECs with indicated expression constructs and then exposed to 21 or 1% O_2 for 24 h. Luciferase activities were expressed as NRLU. (*C* and *D*) HUVECs were transfected with expression constructs for Brg1 or Brm as indicated and then exposed to 21 or 1% O_2 for 24 h. mRNA (*C*) and protein (*D*) levels of ICAM-1 were measured by qPCR and Western blot analyses, respectively.

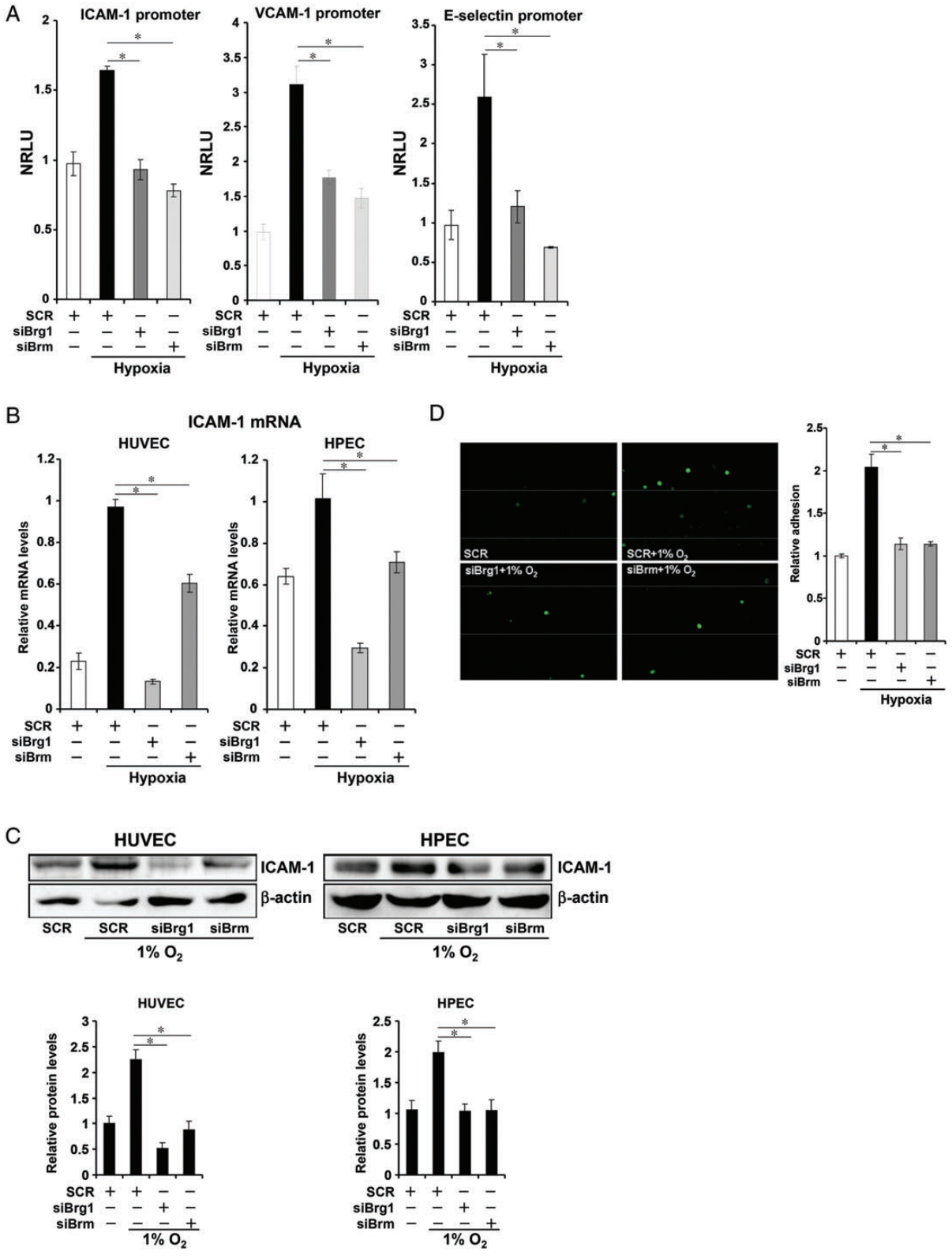

Figure 3 Depletion of Brg1 and Brm down-regulate ICAM-1 induction by hypoxia. (*A*) CAM promoter constructs were transfected into HUVECs with scrambled (SCR) or specific siRNA and then exposed to 21 or 1% O_2 for 24 h. Luciferase activities were expressed as NRLU. (*B* and *C*) HUVECs or HPECs were transfected with SCR or specific siRNA as indicated and then exposed to 21 or 1% O_2 for 24 h. mRNA (*B*) and protein (*C*) levels of ICAM-1 were measured by qPCR and Western blot analyses, respectively. (*D*) HUVECs were transfected with SCR or specific siRNA as indicated and then exposed to 21 or 1% O_2 for 24 h. An *in vitro* adhesion assay was performed as described in Section 2. Data were expressed as relative adhesion compared with the control group.

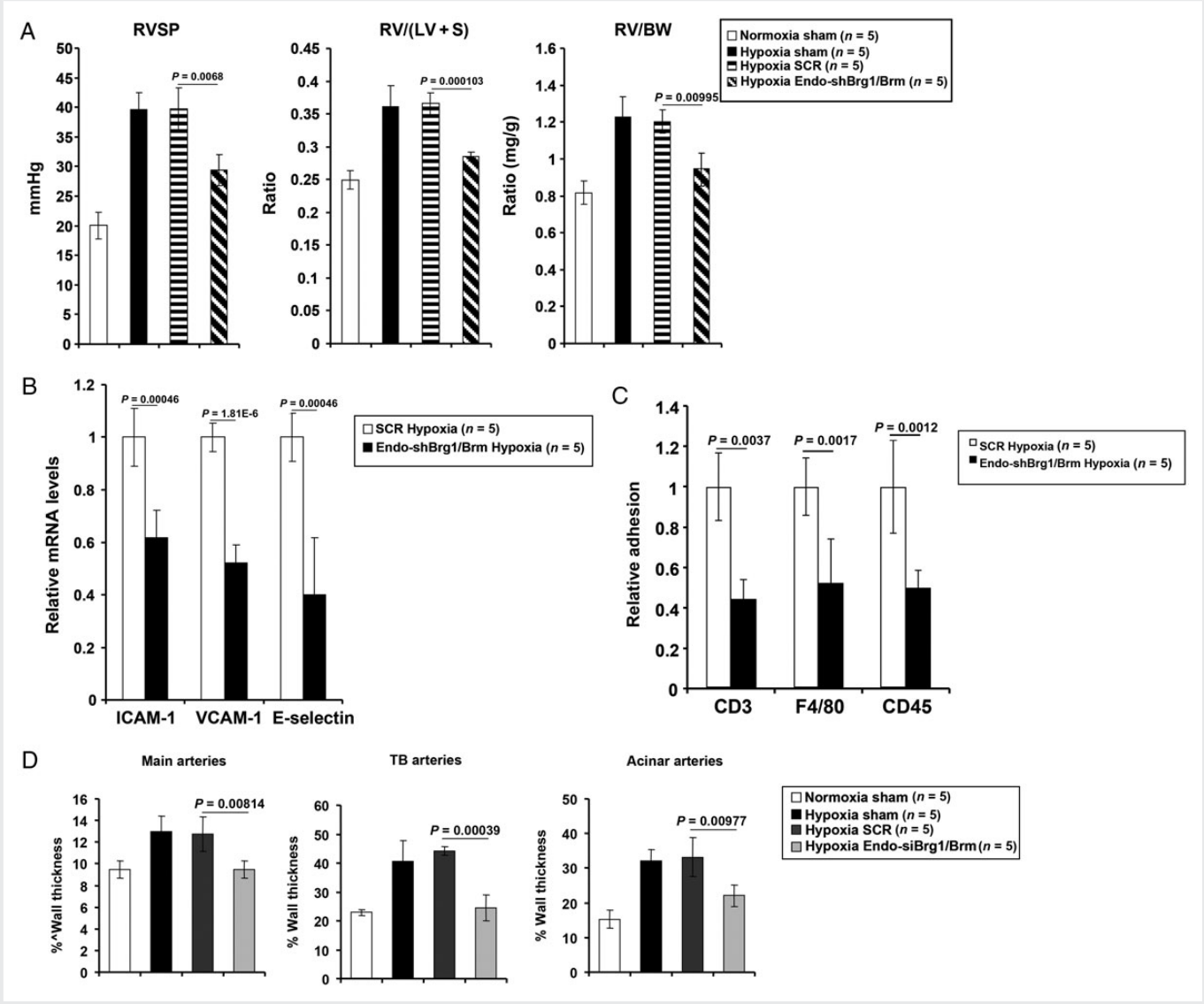

Figure 4 Endothelial-specific knockdown of Brg1/Brm ameliorates HPH in mice. (*A*) Right ventricular hypertrophy was evaluated by right ventricular systolic pressure (RVSP), dividing right ventricular weight (RV) with combined weight of LV and ventricular septum (S), and right ventricular weight over body mass. $n = 5$ for each group. (*B*) Expression levels of CAM genes in aortic arteries were assessed by qPCR. $n = 5$ each group. (*C*) Immunofluorescence staining was performed as described in Section 2. Adhered immune cells were counted and data are expressed as relative adhesion. $n = 5$ for each group. (*D*) Pulmonary vascular remodelling was assessed by the percentage of medial wall thickness for vessels of different sizes as indicated. $n = 5$ for each group.

endothelial cells and leukocytes was weakened by the elimination of Brg1 and Brm (*Figure 3D*). Collectively, these data suggest that Brg1 and Brm are both sufficient and necessary for hypoxia-induced CAM transactivation and leukocyte adhesion *in vitro*.

3.3 Depletion of Brg1/Brm suppresses CAM induction and ameliorates HPH in mice

To verify whether our observation that Brg1/Brm enhanced hypoxia CAM transactivation and leukocyte adhesion in cultured endothelial cells could be extrapolated to *in vivo* settings, we harnessed an animal model of HPH in which C57/BL6 mice were housed in a low oxygen chamber for 4 weeks. In the meantime, these mice were injected via sublingual vein lentiviral particles carrying an endothelial-specific vector[18] that encodes shRNA targeting both Brg1 and Brm. Immunofluorescence staining revealed that the expression of Brg1 and Brm was effectively reduced in the pulmonary endothelium, as delineated by CD31, by these viral particles (Supplementary material online, *Figure S3A* and *B*). As a result, elevation of right ventricular pressure (RVSP) was significantly relieved by endothelial-specific Brg1/Brm silencing, as was hypertrophy of the right ventricle as evaluated by the ratio of weight of the right ventricle (RV) to the weight of the left ventricle (LV) plus ventricular septal (VS) and RV weight over body weight (*Figure 4A*). On the other hand, maximum and minimum d*p*/d*t* (Supplementary material online, *Figure S3C*) and heart rate (Supplementary material online, *Figure S3D*) were not significantly altered. Correction of HPH by Brg1/Brm depletion was accompanied by the down-regulation of all three CAM molecules in pulmonary arteries (*Figure 4B*, Supplementary material online,

Figure S3E). As a result, there was decreased recruitment of immune cells to the vessels, including T lymphocytes (CD3-positive), leukocytes (CD45-positive), activated macrophages (F4/80-positive), dendritic cells (H2-IE/A-positive), and neutrophils (CD11b-positive) (*Figure 4C*, Supplementary material online, *Figure S3F–K*). Furthermore, remodelling of pulmonary vasculature, as measured by vessel wall thickness, was suppressed in mice injected with endo-siBrg1/siBrm compared with mice injected with control shRNA (*Figure 4D*, Supplementary material online, *Figure S3L*). In aggregate, these data clearly support the notion that silencing of Brg1 and Brm stalled development of HPH, likely owing to the normalization of endothelial function *in vivo*.

3.4 Hypoxia promotes the recruitment of Brg1 and Brm to the CAM promoters

Having established a role for Brg1/Brm in the pathogenesis of HPH *in vitro* and *in vivo*, we sought to determine the mechanism by which Brg1/Brm potentiate the transactivation of CAM genes in response to hypoxia in endothelial cells. NF-κB/p65 is known to be responsible for the activation of all three CAM promoters.[24] Therefore, we hypothesized that, in response to hypoxic stress, NF-κB could recruit Brg1 and Brm to the CAM promoters to activate transcription. Indeed, ChIP assay indicates there was a dramatic increase in the occupancies of Brg1 and Brm on the promoter region of the ICAM-1 gene spanning the NF-κB site under hypoxic conditions as early as 24 h following exposure; the increase was sustained at 48 h (*Figure 5A*). In contrast, there was little change in the binding of either Brg1 or Brm on the intronic region of the ICAM-1 gene where there is no NF-κB response element (Supplementary material online, *Figure S4A*). More importantly, hypoxia recruited more Brg1 and Brm to the ICAM-1 promoter *in vivo* in pulmonary arteries isolated from HPH mice when compared with the control mice (*Figure 5B*). Similarly, we also observed that hypoxia elevated the recruitment of Brg1 and Brm to the VCAM-1 promoter (Supplementary material online, *Figure S4B*) and E-selectin promoter (Supplementary material online, *Figure S4C*). Consistent with these observations, re-ChIP assays demonstrate that hypoxia promoted the interaction between Brg1/Brm and NF-κB/p65 on the ICAM-1 promoter (*Figure 5C*).

To further explore the interplay between p65 and Brg1/Brm in hypoxia-induced transactivation of CAM genes, we knocked down the expression of p65 with siRNA in endothelial cells. As shown in *Figure 5D*, siRNA targeting p65 blunted the occupancy of p65 on the ICAM-1 promoter. Consequently, neither Brg1 nor Brm was able to bind to the ICAM-1 promoter without p65. Similar scenarios took place on the VCAM-1 (Supplementary material online, *Figure S4D*) and E-selectin (Supplementary material online, *Figure S4E*) promoters. Reciprocally, depletion of Brg1 or Brm also negatively affected the binding of p65 on all three CAM promoters (*Figure 5E*, Supplementary material online, *Figure S4F* and *G*). Combined, these data support a model wherein a Brg1/Brm/p65 complex forms on the CAM promoters in response to hypoxia to activate transcription in endothelial cells.

3.5 Brg1 and Brm differentially influence the chromatin structure of the CAM promoters

Histone modifications, or the 'histone code', have been tabbed as a core component of the epigenetic machinery that plays an essential role in transcriptional regulation.[25] Therefore, we asked whether Brg1 and Brm could modulate CAM transcription by influencing histone modifications surrounding the CAM promoters. Hypoxia induces an accumulation of acetylated histones H3 (AcH3) and H4 (AcH4), dimethylated H3K4 (H3K4Me2), and trimethylated H3K4 (H3K4Me3), all of which herald transcriptional activation, on the CAM promoter regions (*Figure 6A*, Supplementary material online, *Figure S5A* and *B*). Ablation of Brg1 greatly reduced the levels of AcH3, H3K4Me2, and H3K4Me3, but failed to impact AcH4 (*Figure 6A*, Supplementary material online, *Figure S5A* and *B*). The depletion of Brm, on the other hand, decreased the levels of AcH3 and H3K4Me3, albeit not as potently as that of Brg1, but did not alter either AcH4 or H3K4Me2. As a result, the recruitment of RNA polymerase II (Pol II) was hampered probably leading to a decelerated transcription rate. In conclusion, these data suggest that Brg1 and, to a lesser extent, Brm can form a crosstalk with the histone modification machinery to influence hypoxia-induced CAM transactivation.

4. Discussion

Under physiological conditions, the pulmonary endothelium is refractory to the adhesion and extravasation of circulating immune cells, keeping the inflammatory response in check. Low oxygen tension invokes a change in the endothelial transcriptome that includes transactivation of multiple adhesion molecules. The ensuing aggregation and adhesion of immune cells to the vessel wall guarantee a strong pro-inflammatory response, ultimately leading to the development of hypoxic pulmonary hypertension. Here, we provide evidence that the chromatin remodelling proteins Brg1 and Brm link epigenetic transactivation of CAM genes in hypoxic endothelial cells to the pathogenesis of HPH.

Numerous investigations have implicated Brg1 and Brm in the regulation of cellular response to stress cues that range from ischaemia,[26] to pressure overload,[27] to excessive nutrition.[17] Specifically, Brg1 and/or Brm seems to precipitate the damages rendered by stress stimuli and pivot the cells and organs that are under challenges to a decompensated phenotype. Hang *et al.*[27] have demonstrated that conditioned deactivation of Brg1 in the myocardium prevents the deterioration of pathological cardiac hypertrophy. A recent report by Tian *et al.*[17] suggests that silencing of Brg1/Brm alleviates high-fat diet-induced steatohepatitis. We show here that the levels of Brg1 and Brm increased in response to hypoxic stress *in vitro* and *in vivo* (*Figure 1*). More importantly, endothelial-specific deletion of Brg1/Brm abrogated CAM induction and normalized pulmonary structure and function in an animal model of HPH (*Figure 4*). Thus, our data lend additional support to the notion that post-embryonic activation of the chromatin remodelling complex may be associated with an aggravated pathology in adults.

In the mouse model of HPH, Brg1/Brm silencing led to a remarkable decrease in the adhesion of several different lineages of immune cells, consistent with the reduction of ICAM-1, VCAM-1, and E-selectin. In addition, pulmonary remodelling as evaluated by the vessel wall thickness was suppressed (*Figure 4D*). Thickening of the vessel wall in the lungs challenged with hypobaric hypoxia is believed to be mediated by proliferation and migration of vascular smooth muscle cells (VSMCs).[28] Several humoral factors, derived primarily from immune cells attracted to the endothelium, are known to modulate VSMC proliferation and migration. Activated macrophages, for example, can secrete interleukin 6 and macrophage migration inhibitory factor to promote VSMC proliferation and migration.[4,29] On the other hand, T lymphocytes produce CCL5/RANTES to influence VSMC behaviour.[30] It is likely that the observed correction of vascular structure in Brg1/Brm-depleted HPH mice is a reflection of a normalized profile of local humoral factors following reduced adhesion of immune cells and inflammation. Further studies employing animal models with selective deprivation of a subset of immune cells will help clarify this issue.[31]

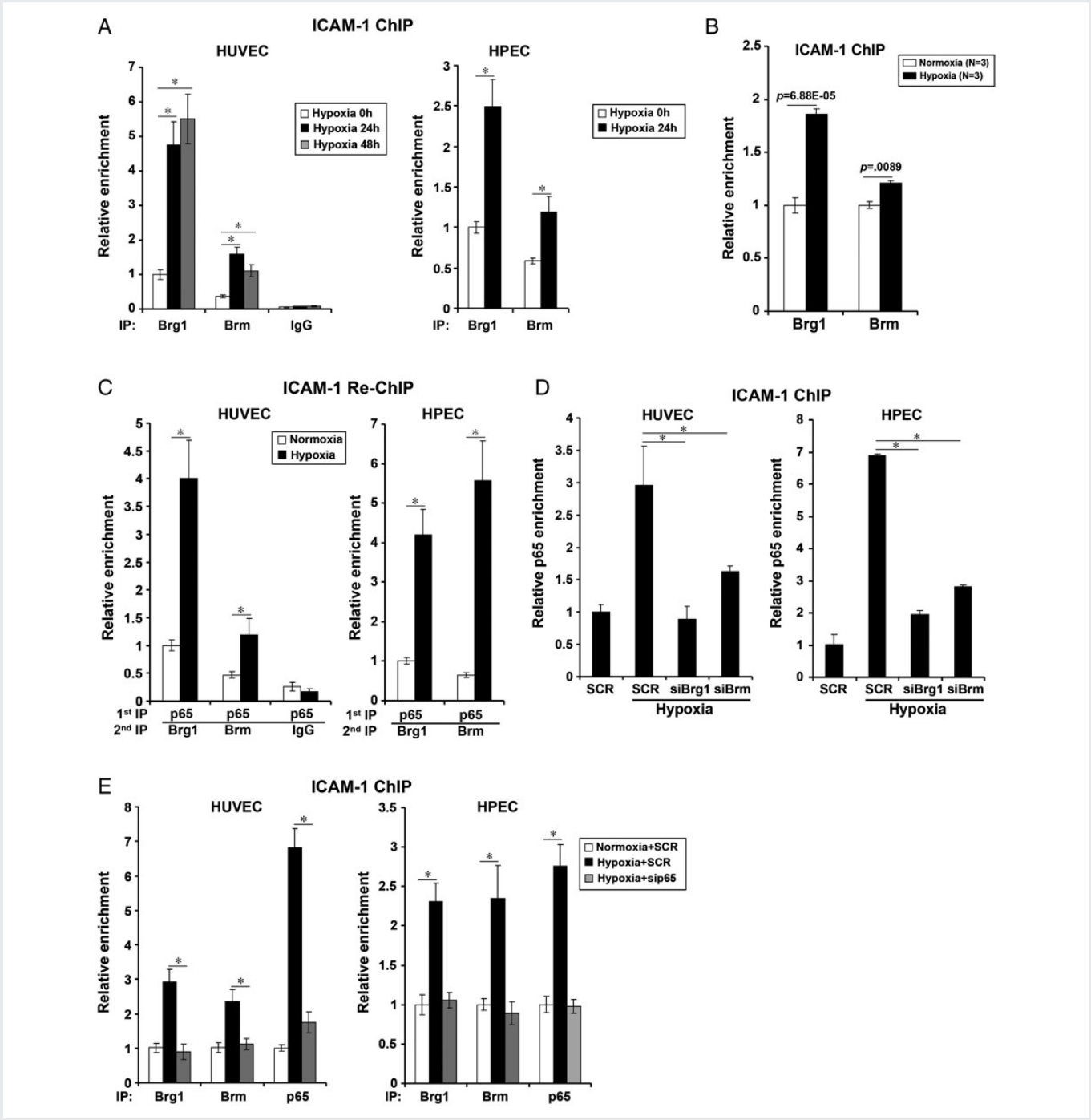

Figure 5 Hypoxia promotes the interplay between Brg1/Brm and NF-κB/p65 on the ICAM-1 promoter. (*A*) HUVECs or HPECs were exposed to 1% O_2 and harvested at indicated time points. ChIP assays were performed with indicated antibodies. (*B*) C57/BL6 mice were induced to develop HPH as described in Section 2. ChIP assays were performed using pulmonary arteries from the HPH mice or control mice. (*C*) HUVECs or HPECs were exposed to 1% O_2 for 24 h. Re-ChIP assays were performed with indicated antibodies. (*D*) HUVECs or HPECs were transfected with SCR or specific siRNA as indicated and then exposed to 21 or 1% O_2 for 24 h. ChIP assays were performed with indicated antibodies. (*E*) HUVECs or HPECs were transfected with SCR or specific siRNA as indicated and then exposed to 21 or 1% O_2 for 24 h. ChIP assays were performed with anti-p65.

One major finding of the current study is the identification of an NF-κB/Brg1 complex on the CAM promoters in response to hypoxia. Previously, a seminal study by Smale *et al.* had implicated Brg1/Brm in NF-κB-dependent transcription by demonstrating that lack of Brg1/Brm crippled the synthesis and secretion of several pro-inflammatory mediators in macrophages.[32] This observation has been corroborated by a couple of follow-up investigations that collectively suggest a role of Brg1 in determining the dynamics of the NF-κB controlled transcriptome.[17,26] We provide clear evidence here that NF-κB interacts with both Brg1 and Brm on the ICAM-1 promoter

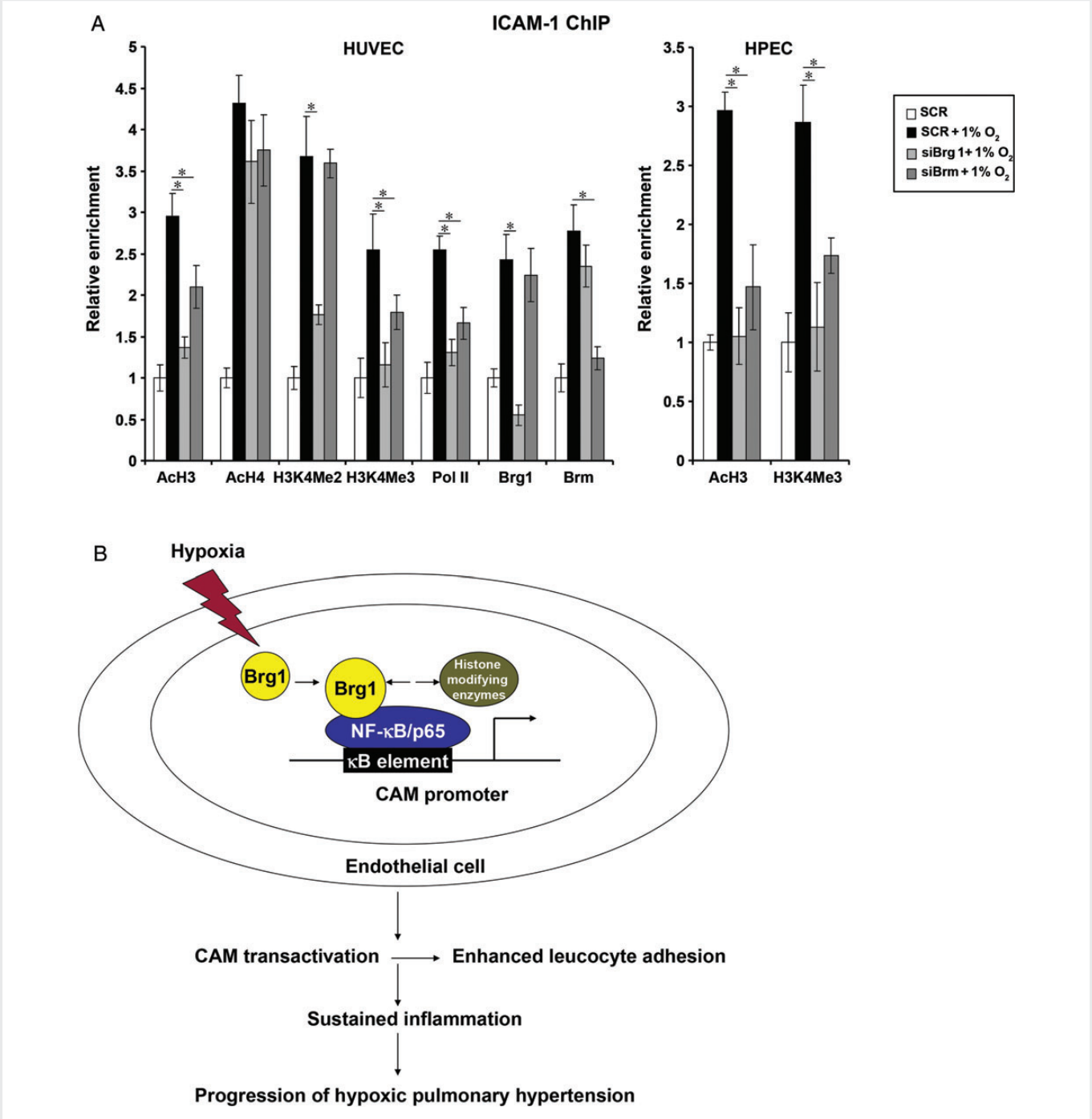

Figure 6 Brg1 and Brm differentially influence the chromatin structure of the ICAM-1 promoter in response to hypoxia. (*A*) HUVECs or HPECs were transfected with SCR or specific siRNA as indicated and then exposed to 21 or 1% O_2 for 24 h. ChIP assays were performed with indicated antibodies. (*B*) A model depicting the mode of action for Brg1 in hypoxia-induced CAM transactivation in endothelial cells and the pathogenesis of HPH.

under hypoxic conditions (*Figure 5C*). Intriguingly, silencing of Brg1 or Brm weakened the binding of NF-κB to target promoters, indicative of the reciprocal nature of the NF-κB-Brg1/Brm interplay. NF-κB binding to its cognate element is sensitive to a specific chromatin conformation.[33] Therefore, a plausible explanation would be that altered chromatin structure as a result of Brg1/Brm elimination negatively affects the affinity of NF-κB for its targets (*Figure 6A*). We would like to propose that Brg1/Brm may demarcate the chromatin structure for NF-κB-specific transcription. Extensive ChIP-seq analyses will shed more light on the interaction between NF-κB and Brg1/Brm on a genome-wide scale.

A few caveats should be considered before the conclusion drawn here can be further extrapolated. First, lung-specific deletion of Brg1 has been reported to promote carcinogenesis in mice.[34] Therefore, any attempt

of targeting Brg1 to alleviate HPH should be weighed against the risk of cancer. Secondly, the current lentiviral targeting system used in this study to specifically eliminate Brg1 from the endothelium is not immune from a possible leakage. In this regard, the role of Brg1 in the pathogenesis should be evaluated more cautiously. For instance, Brg1 has been reported to modulate transforming growth factor (TGF-β) signalling, a key growth factor involved in HPH.[35] It is likely that the attenuated vascular remodelling in the absence of Brg1 could be a result of a skewed TGF-β effect independent of Brg1-mediated CAM activation. Alternatively, Brg1 might fine-tune the transcription of other NF-κB target genes (e.g. inducible NO synthase/iNOS) that contribute to HPH. Finally, hypobaric hypoxia used on animals might have some effects different from that of the normobaric condition used on cells.[36] Clearly, further investigation is warranted before a more definitive role could be assigned to Brg1 in the pathogenesis of HPH.

A somewhat counterintuitive, though not completely unprecedented, finding is that d*p*/d*t* max values were up-regulated in mice challenged with hypoxic stress and that the correction of RVSP by Brg1/Brm silencing in the endothelium did not alter d*p*/d*t* max (Supplementary material online, *Figure S3E*). Mizuno *et al.*[37] have shown that p53 deficiency exacerbates hypoxia-induced elevation of RSVP in mice without much alteration in either heart rate or d*p*/d*t* max compared with wild-type littermates. A couple of other studies have also demonstrated that increased RVSP in hypoxic mice is accompanied by an up-regulation (~10%) in d*p*/d*t* max instead of a decrease.[38,39] One plausible explanation is that, under the current experimental conditions (4 weeks following 10% O_2 challenge), the heart was still in a compensated state with increased d*p*/d*t* max and that improvement of endothelial function as a result of Brg1/Brm knockdown was sufficient to reverse RVSP (a vascular response) but not enough to override the effect of hypoxia on cardiomyocytes (directly responsible for d*p*/d*t* max values). A likely solution to this conundrum would be to simultaneously deplete Brg1/Brm from the vascular endothelium and the myocardium.

In summary, our finding identifies Brg1 as a key regulator of CAM transactivation in endothelial cells by engaging the histone modification enzymes to alter chromatin structure. Since the catalytic domain of Brg1 is clearly necessary for its activity (*Figure 2*), the development of pharmaceutical inhibitors specific for Brg1 may prove beneficial in the intervention and/or prevention of hypoxic pulmonary hypertension.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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