Megakaryocytic Leukemia 1 Directs a Histone H3 Lysine 4 Methyltransferase Complex to Regulate Hypoxic Pulmonary Hypertension

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Abstract—Enhanced interaction between vascular endothelial cells and circulating leukocytes, as a result of transcriptional activation of cell adhesion molecules (CAM), helps establish a proinflammatory milieu contributing to the pathogenesis of chronic hypoxia-induced pulmonary hypertension. The molecular switch that dictates CAM transactivation is not clearly defined. Our goal was to determine the involvement of the transcriptional modulator megakaryocytic leukemia 1 (MKL1), also known as myocardin-related transcription factor A (MRTF-A), in CAM transactivation and the underlying mechanism. We report here that compared with wild-type littermates, MKL1/MRTF-A knockout mice were more resistant to the development of hypoxia-induced pulmonary hypertension when exposed to low oxygen pressure. Notably, CAM induction in knockout mice was significantly attenuated with a concomitant reduction of leukocyte adhesion. In cultured vascular endothelial cells, overexpression of MKL1/MRTF-A enhanced, whereas depletion of MKL1/MRTF-A dampened, hypoxia-induced CAM transactivation. In response to hypoxia, MKL1/MRTF-A formed a complex with NF-xB on the CAM promoters. Of interest, MKL1/MRTF-A was responsible for recruiting a histone H3 lysine 4 methyltransferase complex to the CAM promoters. Finally, endothelial-specific silencing of ASH2 and WDR5, 2 key components of the histone H3 lysine 4 methyltransferase complex, ameliorated hypoxia-induced pulmonary hypertension in mice. In conclusion, our data suggest that MKL1/MRTF-A, by coordinating key epigenetic alterations on CAM promoters, provides a critical link to hypoxia-induced endothelial malfunction and contributes to the pathogenesis of hypoxia-induced pulmonary hypertension. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.114.04585.)

Key Words: cell adhesion molecules • epigenetics • hypertension, pulmonary • hypoxia

Vascular endothelium represents the first line of defense against stress stimuli guarding the integrity of the vasculature. As such, disruption of endothelial homeostasis is intimately associated with a host of cardiovascular disorders, including atherosclerosis, cardiac infarction, and hypoxic pulmonary hypertension (HPH).1 Endothelial injury in HPH is reflected by persistent vessel constriction, accumulation of reactive oxygen species, accelerated synthesis and deposition of extracellular matrix proteins, and build-up of proinflammatory milieu. At the transcriptional level, these phenotypic alterations were accompanied by a profound shift in the endothelial transcriptome.2 Expression of adhesion molecules, for instance, is upregulated by chronic hypoxia to sustain a prolonged and stronger interaction between leukocytes and the vessel wall, representing a hallmark event that defines the intensity of proinflammatory response associated with HPH.3 Adhesion molecules (CAM), including intercellular adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAMs), and selectins, are a group of transmembrane proteins that mediate cell–cell interactions. Induction of CAMs by proinflammatory stimuli is observed in multiple cardiovascular disorders in humans; in contrast, CAM blockade has been proven as a promising strategy in preclinical and clinical trials.4–6 CAM transactivation is dictated by several conserved transcription factors, NF-xB being the most prominent activator of CAM transcription. NF-xB is activated by hypoxia...
in endothelial cells to promote the synthesis of ICAM-1, VCAM-1, and E-selectin. Many details regarding NF-xB–dependent CAM transactivation in the context of HPH including the possible involvement of the epigenetic machinery, however, remain to be inked out.

MKL1, variously termed MRTF-A or MAL, is a transcriptional modulator initially discovered as a cofactor for SRF participating in the regulation of smooth muscle cell–specific genes. Several studies have implicated MKL1/MRTF-A in transcriptional regulation in endothelial cells, epithelial cells, platelets, and fibroblasts. One key characteristics of MKL1/MRTF-A is its ability to engage components of the epigenetic machinery, which include histone acetyltransferase, histone demethylase, and chromatin remodeling protein, supporting a role for MKL1/MRTF-A as an epigenetic coordinator bridging sequence-specific transcription factors and the chromatin-altering franchise. Mounting evidence has suggested that MKL1/MRTF-A might act as a stress protein mediating cellular compensatory/decompensatory response to injurious signals. Here, we report that MKL1/MRTF-A deficiency ameliorates HPH in mice paralleling a decrease in CAM expression in the lungs. MKL1/MRTF-A facilitates NF-xB–dependent CAM transactivation in response to hypoxic stress by recruiting a histone H3 lysine 4 (H3K4) methyltransferase complex, the silencing of which is sufficient to stall the pathogenesis of HPH. Therefore, our data portray MKL1/MRTF-A as a key factor in HPH by steering the epigenetic activation of adhesion molecules.

Methods
Methods are available in the online-only Data Supplement.

Results
MKL1 Deficiency Ameliorates HPH in Mice
To verify whether MKL1/MRTF-A, by virtue of activating the transcription of adhesion molecules in vascular endothelial cells, could play a role in the pathogenesis of HPH, we first compared the response of wild-type (WT) and MKL1/MRTF-A–deficient (knockout) mice to the challenge of hypoxic stress. As shown in Figure 1A and 1B, following 3 weeks of exposure to 10% O2, WT mice developed HPH as evidenced by the increase in right ventricular systolic pressure and right ventricular weight. On the contrary, knockout mice exhibited a less severe phenotype. In addition, vascular remodeling was more prominent in WT mice than in knockout mice under hypoxic conditions as evaluated by the thickness of the pulmonary vessel wall and neomuscularization (Figure S1A–S1D in the online-only Data Supplement). Hypoxia resulted in a marked induction of adhesion molecules in the lungs in WT mice, which was significantly diminished in the absence of MKL1/MRTF-A (Figure 1C and 1D). Consequently, there was a decrease in the adhesion of circulating immune cells, including CD3+ T lymphocyte, CD45+ leukocyte, and F4/80+ macrophage, in MKL1/MRTF-A knockout mice when compared with that in WT mice (Figure 1E). We also noticed a decrease in the levels of several proinflammatory mediators, including interleukin-6, interleukin-1, and monocyte chemoattractant protein 1, in the lung homogenates (Figure S1E and S1F) and bronchoalveolar lavage fluid (Figure S1G) in the knockout mice. Flow cytometry also revealed that immune infiltrates were significantly downregulated in the bronchoalveolar lavage fluid in knockout mice when compared with that in WT mice under hypoxic conditions (Figure 1H).

To tackle the question whether mice with endothelial-specific deletion of MKL1/MRTF-A could phenocopy mice with germ-line MKL1/MRTF-A deletion in terms of hypoxic pulmonary hypertension, we used a recently validated lentiviral system carrying MKL1/MRTF-A targeting short hairpin RNA (Endo-shMKL1) driven by the Tse2 gene promoter to specifically knockdown MKL1/MRTF-A in the endothelium. Of interest, endothelial MKL1/MRTF-A depletion led to a decrease in RSVP (Figure S2A) and right ventricular weight (Figure S2B), which accompanied a simultaneous downregulation of adhesion molecules in the lungs under hypoxic conditions (Figure S2C). Similarly, levels of proinflammatory mediators were reduced in the lungs (Figure S2D and S2E) and in the bronchoalveolar lavage fluid (Figure S2F), so were the immune infiltrates in the bronchoalveolar lavage fluid (Figure S2G). Therefore, MKL1/MRTF-A deficiency, likely MKL1/MRTF-A in the endothelium, ameliorates HPH in mice in conjunction with reduced CAM expression and pulmonary inflammation.

MKL1 Contributes to Hypoxia-Induced CAM Transactivation
Having observed that the suppression of HPH in mice by MKL1/MRTF-A ablation was accompanied by decelerated synthesis of CAMs in the lungs, we hypothesized that MKL1/MRTF-A might mediate hypoxia-induced CAM transactivation. Indeed, MKL1/MRTF-A activated promoter activities of ICAM-1, VCAM-1, and E-selectin genes in endothelial cells in a dose-dependent manner (Figure S3A). Overexpression of MKL1/MRTF-A potentiated transcriptional activation of adhesion molecules by hypoxia (Figure 2A–2C). In addition, there was a clear synergy between MKL1/MRTF-A overexpression and hypoxia in the activation of endogenous levels of adhesion molecules (Figure 2D and 2E). As a result, adhesion of leukocytes to endothelial cells was markedly enhanced (Figure S3B).

On the contrary, interfering with MKL1/MRTF-A activity with either dominant negative mutation (Figure S3C and S3E) or short hairpin RNA–mediated knockdown attenuated CAM activation in response to hypoxia (Figure S3D). In addition, induction of endogenous message levels of adhesion molecules by hypoxia was markedly alleviated when cells were depleted of MKL1/MRTF-A with different pairs of small interfering RNA (Figure 2F and 2G; Figure S3F–S3H). Consequently, adhesion of leukocyte to endothelial cells was attenuated (Figure 2H).

Because previous investigations suggest that the small G-protein RhoA is required for MKL1/MRTF-A activation, we probed the role of RhoA in hypoxia-induced CAM transactivation. Knockdown of RhoA (Figure S4A for efficiency) attenuated promoter activities (Figure S4B), message levels (Figure S4C), and protein levels (Figure S4D) of adhesion molecules in response to hypoxia. Cumulatively, these
led to a reduction in leukocyte adhesion to endothelial cells in vitro (Figure S4E). Taken together, these data suggest that MKL1/MRTF-A is a key modulator of CAM gene upregulation in endothelial cells under hypoxic stress possibly acting downstream of RhoA.

**Hypoxia Stimulates the Recruitment of MKL1 to CAM Promoters**

We have previously shown that MKL1/MRTF-A is recruited to the ICAM-1 promoter by NF-κB/p65 in response to increased intracellular levels of oxidized low-density lipoprotein.12
Figure 2. Megakaryocytic leukemia 1 (MKL1) potentiates the induction of adhesion molecules by hypoxia. A–C, Promoter-luciferase fusion constructs for intercellular adhesion molecule (ICAM-1; A), vascular cell adhesion molecule (VCAM; B), or E-selectin (C) were transfected into EAhy92.6/human umbilical vein endothelial cells (HUVECs) with or without wild-type (WT) MKL1 followed by exposure to 1% O₂. Data are expressed as relative luciferase unit (RLU). MKL1 overexpression enhanced cell adhesion molecules (CAM) promoter activities. D and E, EAhy92.6/HUVECs were transfected with MKL1 or an empty vector (EV) followed by exposure to 1% O₂. mRNA (D) and protein (E) levels of adhesion molecules were probed by quantitative polymerase chain reaction (qPCR) and Western, respectively. MKL1 overexpression augmented endogenous CAM expression. F and H, EAhy92.6/HUVECs and human primary pulmonary endothelial cells (HPECs) were transfected with small interfering RNA targeting MKL1 (siMKL1) or scrambled RNA (SCR) followed by exposure to 1% O₂. mRNA (F) and protein (G) levels of adhesion molecules were measured by qPCR and Western, respectively. MKL1 depletion repressed CAM expression. H, In vitro adhesion assay was performed with EAhy92.6/HUVECs as described under in Methods in the online-only Data Supplement. MKL1 depletion suppressed leukocyte adhesion in vitro.
Therefore, we probed whether hypoxia could promote the occupancy of MKL1/MRTF-A on CAM promoters. As shown in Figure 3A, there was more MKL1/MRTF-A binding to the same region where p65 binds on all 3 CAM promoters in cells challenged with 1% O2. In contrast, little binding was detected in the coding regions of these promoters (data not shown). Moreover, MKL1/MRTF-A binding on the CAM promoters was enhanced in the lungs of HPH mice when compared with the control mice (Figure 3B). More important, hypoxia stimulated the interaction between MKL1/MRTF-A and p65 on the CAM promoters (Figure 3C). Knockdown of endogenous p65 by siRNA markedly reduced the binding of MKL1/MRTF-A on all 3 promoters (Figure 3D). Together, these data suggest that consistent with its role as a potent transactivator of CAM genes, MKL1/MRTF-A occupancy on the CAM promoters was enhanced by hypoxia in a p65-dependent manner. We also found that RhoA silencing suppressed MKL1/MRTF-A binding (Figure S5A) and disrupted MKL1-p65 interaction (Figure S5B) on CAM promoters under hypoxic conditions, consistent with the fact that RhoA is required for CAM induction by hypoxia.

MKL1 Coordinates Epigenetic Alterations on Target Promoters by Recruiting an H3K4 Methyltransferase Complex

Hypoxia-induced transactivation is associated with enhanced assembly of the basic transcriptional machinery and dynamic histone modifications surrounding the promoter region of genes.22 We sought to determine how MKL1/MRTF-A would contribute to this process. There was a significant increase in the occupancy of RNA polymerase II on the CAM promoters after hypoxic stress accompanied by elevated levels of acetylated histones H3 and H4, as well as dimethylated and trimethylated H3K4, all of which were blocked by siRNA-mediated silencing of MKL1/MRTF-A (Figure 4A–4C). In contrast, monomethylated H3K4 was not altered. Neither were global levels of modified histones affected (Figure S6C). More importantly, acetylated histone H3 (Figure S6A), acetylated histone H4 (Figure S6B), dimethylated H3K4 (Figure 4D), and trimethylated H3K4 (Figure 4E) levels were significantly decreased in the lungs of knockout mice as opposed to WT mice, indicating that MKL1/MRTF-A might be wired to the epigenetic circuit by orchestrating histone modifications. RhoA depletion, on the contrary, prevented the deposition of active histone marks on the CAM promoters (Figure S7A–S7D).

The observation that MKL1/MRTF-A loss-of-function caused the erasure of H3K4 methylation on CAM promoters raised an intriguing possibility that MKL1/MRTF-A might be interacting with and recruiting an H3K4 methyltransferase complex. Through proteomic screening, we have identified ASH2, a key component of the mammalian complex of proteins associated with SET (COMPASS)/COMPASS-like H3K4 methyltransferase complex, as a binding partner for MKL1/MRTF-A (manuscript under consideration elsewhere). The interaction between ASH2 and MKL1/MRTF-A was confirmed by coimmunoprecipitation (Figure 5A). We also observed an interaction between MKL1/MRTF-A and WDR5,23 which is required for the function of the H3K4 methyltransferase complex similar to ASH2 (Figure S8A). Occupancy of ASH2 (Figure 5B) and WDR5 (Figure S8C) on the CAM promoters was increased by hypoxia paralleling the recruitment of and SET1, the catalytic unit of COMPASS.24
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Chromatin immunoprecipitation assays using lung lysates also confirmed the recruitment of ASH2 to the CAM promoters in HPH mice (Figure 5C). Furthermore, although hypoxia did not affect global ASH2–MKL1 interaction (Figure S8B), it enhanced the interaction between MKL1/MRTF-A and ASH2 on the CAM promoters (Figure 5D), suggesting that hypoxia probably modulates the interaction between MKL1/MRTF-A and ASH2 in a gene context-specific manner. Silencing of MKL1/MRTF-A completely abrogated the binding of COMPASS proteins (Figures 5E; Figure S8C and S8D).

Figure 4. Megakaryocytic leukemia 1 (MKL1) coordinates epigenetic alterations on target promoters. A–C, EAhy92.6/human umbilical vein endothelial cells (HUVECs) were transfected with siMKL1 or scrambled RNA (SCR) followed by exposure to 1% O2. Chromatin immunoprecipitation (ChIP) assays were performed with indicated antibodies. Precipitated genomic DNA was amplified with primers spanning cell adhesion molecules (CAM) promoters. MKL1 depletion rendered the chromatin structure surrounding CAM promoters more repressive in vitro. D and E, Wild-type (WT) or MKL1-deficient (knockout [KO]) mice were induced to develop HPH as described in Methods in the online-only Data Supplement. ChIP assays were performed using lung lysates with anti-dimethylated histone H3 lysine 4 (H3K4Me2; D) or anti-trimethylated histone H3 lysine 4 (H3K4Me3; E). MKL1 deficiency erased methylated histone H3K4 on CAM promoters in the lungs. ICAMs indicates intercellular adhesion molecules; and VCAMs, vascular cell adhesion molecules.
Meanwhile, occupancies of COMPASS proteins on the CAM promoters were also downregulated by RhoA knockdown (Figure S9). Thus, MKL1/MRTF-A orchestrates epigenetic regulation of CAM transactivation in response to hypoxia by helping assemble an H3K4 methyltransferase complex on the CAM promoters.

COMPASS Proteins Participate in CAM Transactivation by Hypoxia

Next, we assessed the role of ASH2, WDR5, and SET1 in CAM transactivation-mediated by hypoxia/MKL1. Coexpression of ASH2, WDR5, or SET1 with MKL1/MRTF-A enhanced the promoter activities of ICAM-1 (Figure 6A), VCAM-1 (Figure 6B),
and E-selectin (Figure 6C) genes under normoxic conditions. Meanwhile, ASH2, WDR5, and SET1 also potentiated the promoter activities under hypoxic conditions (Figure 6D). On the contrary, knockdown of individual COMPASS components (Figure S10A for validation) normalized endogenous CAM expression in hypoxia-treated endothelial cells (Figure 6E and Figure 6. Complex of proteins associated with SET (COMPASS) proteins mediate hypoxia-induced cell adhesion molecules (CAM) activation in vitro. A–C, Different CAM promoter-luciferase plasmids were transfected into EAhy92.6/human umbilical vein endothelial cells (HUVECs) along with indicated expression constructs. Overexpression of COMPASS proteins synergized with megakaryocytic leukemia 1 (MKL1) to activate CAM promoters. D, intercellular adhesion molecule (ICAM)-1 promoter-luciferase construct was transfected into EAhy92.6/HUVECs along with indicated expression constructs followed by exposure to 1% O$_2$. Overexpression of COMPASS proteins synergized with hypoxia to activate CAM promoters. E–G, EAhy92.6/HUVECs and human primary pulmonary endothelial cells (HPECs) were transfected with indicated siRNAs followed by exposure to 1% O$_2$. mRNA (E) and protein (F) levels of adhesion molecules were assessed by quantitative polymerase chain reaction and Western. Depletion of individual COMPASS proteins attenuated endogenous CAM expression. G, Endothelial–leukocyte interaction was evaluated by in vitro adhesion assay. Depletion of individual COMPASS proteins attenuated leukocyte adhesion in vitro. SCR indicates scrambled RNA; and VCAM, vascular cell adhesion molecules.
Figure 7. Endothelial-specific depletion of ASH2 and WDR5 attenuates hypoxic pulmonary hypertension (HPH) in mice. C57/BL6 mice were injected via tail vein lentiviral particles carrying endothelial-specific shRNA targeting ASH2 or WDR5. Right ventricular (RV) hypertrophy was evaluated by RV systolic pressure (SP; A) and RV/left ventricle (LV)+S (B). Endothelial-specific silencing of ASH2/WDR5 suppressed RVSP and RV/LV+S in mice. C and D. Expression of cell adhesion molecule (CAM) in the lungs were measured by quantitative polymerase chain reaction (C) and Western (D). n=3 for each group. Endothelial-specific silencing of ASH2/WDR5 repressed CAM expression in the lungs in mice. Protein quantifications were performed with Image Pro based on 3 independent experiments. n=3 to 5 mice per group. E and F, Chromatin immunoprecipitation (ChIP) assays were performed using lung lysates from indicated groups with anti-dimethylated histone H3 lysine 4 (H3K4Me2; E) or anti-trimethylated histone H3 lysine 4 (H3K4Me3; F). Endothelial-specific silencing of ASH2/WDR5 erased H3K4 methylation on CAM promoters in the lungs in mice. G, Representative images of immunofluorescence staining of pulmonary vessels (50–100 μm) with indicated antibodies; scale bar, 20 μm. Arrows show immune cells adhered to the endothelium. Quantifications were performed using Image Pro, and data are expressed as relative adhesion. n=3 to 5 mice per group. H, A model depicting the mode of action for MKL1 in the pathogenesis of HPH. ICAMs indicates intercellular adhesion molecules; SCR, scrambled RNA; and VCAM, vascular cell adhesion molecules.
As a result, endothelial–leukocyte interaction was significantly diminished (Figure 6G). Together, these data suggest that COMPASS proteins play essential roles in hypoxia-induced, MKL1/MRTF-A–dependent CAM transactivation.

**Targeted Depletion of ASH2 and WDR5 in the Endothelium Averts HPH in Mice**

Finally, we asked whether endothelial-specific silencing of ASH2 or WDR5, by blocking the induction of CAMs, could affect the pathogenesis of HPH in mice. To achieve this goal, we harnessed an endothelium-targeting lentiviral delivery system in which the short hairpin RNA sequence was under the control of the Tie2 promoter/enhancer, whereas the viral envelope protein engineered to specifically recognize vascular endothelium. Immunofluorescence staining revealed that envelope protein engineered to specifically recognize vascular endothelium. Immunofluorescence staining revealed that mice injected with Endo-shAsh2 or Endo-shWdr5 showed specific knockdown of ASH2 or WDR5 in the endothelium (Figure S10B). In contrast, neither fibroblast cells (delimited by FSP1; Figure S10C) nor epithelial cells (labeled by SP-C; Figure S10D) showed significant loss of ASH2/WDR5 expression. Of note, endothelial-specific targeting of either ASH2 or WDR5 dampened the elevation of right ventricular systolic pressure (Figure 7A) and right ventricular hypertrophy (Figure 7B) in hypoxia-challenged mice. Meanwhile, medial layer expansion and neomuscularization (Figure S11A–S11C) were less prominent in mice with endothelium depleted of ASH2 or WDR5.

In agreement of normalized HPH phenotype in mice, ASH2/WDR5 knockdown in the endothelium also suppressed the induction of ICAM-1, VCAM-1, and E-selectin at the mRNA level (Figure 7C) and the protein level (Figure 7D). Downregulation of CAM transactivation mirrored decreases in H3K4 dimethylation (Figure 7E) and trimethylation (Figure 7F) on the promoters. In accordance, adhesion of leukocytes to the pulmonary vessels was blocked (Figure 7G). Collectively, these data provide a functional link between ASH2/WDR5-mediated H3K4 methylation, CAM transactivation, and the pathogenesis of HPH in vivo.

**Discussion**

Persistent pulmonary inflammation, in which endothelium-mediated recruitment of leukocytes plays a key role via the expression of adhesion molecules, has been observed in patients with HPH. Yet, there has been no evidence that targeting any individual adhesion molecule could alleviate and stall the pathogenesis of HPH, suggesting that it might be the combinatory effect of a group of adhesion molecules that contributes to the progression of HPH. The current study delineates a novel epigenetic circuit underscoring the transcriptional activation of adhesion molecules in endothelial cells in response to hypoxic stress both in vitro and in vivo (Figure 7F), providing clues to the development of novel strategies against HPH.

Consistent with our previous finding that MKL1/MRTF-A is indispensable for oxidized low-density lipoprotein–induced CAM activation in cultured endothelial cells, our data as summarized here clearly portray MKL1/MRTF-A as a critical factor for vascular inflammation associated with HPH by virtue of connecting the epigenetic machinery to hypoxia-induced transactivation of adhesion molecules. The phenotype described here is consistent with a previously published rat model in which MKL1/MRTF-A was silenced by lentivirus-mediated delivery of short hairpin RNA. More importantly, endothelial-specific depletion of MKL1/MRTF-A suppressed pulmonary inflammation and attenuated HPH in mice (Figure S2), in agreement with our recent finding that endothelial MKL1/MRTF-A mediates angiotensin II–induced cardiac hypertrophy. In light of our previous report that MKL1/MRTF-A is responsible for increased synthesis of endothelin in hypoxia-challenged cells, these data collectively support the role of endothelial dysfunction, mediated by MKL1/MRTF-A, in the pathogenesis of HPH. MKL1/MRTF-A deficiency also significantly relieved hypoxia-induced elevation of left ventricular pressure and vascular remodeling. The current data set, however, should be interpreted with caution in defining the cell autonomous role for MKL1/MRTF-A as MKL1/MRTF-A is expressed not only in the vascular endothelium but also in other types of cells, including smooth muscle cells, fibroblast cells, and epithelial cells in the lungs in mice (Figure S12). First, cytokines/chemokines secreted by leukocytes fine-tune the proliferation and migration of smooth muscle cells. Thus, diminished vascular remodeling in MKL1/MRTF-A knockout mice could presumably result from dampened leukocyte adhesion. Second, MKL1/MRTF-A is known to directly participate in the phenotypic modulation of smooth muscle cells, a process key to vascular remodeling in HPH. In addition, correction of right ventricular hypertrophy could be explained by normalized pulmonary circulation and particularly restored endothelial function in the absence of MKL1/MRTF-A. An equally plausible explanation could be that MKL1/MRTF-A promotes vascular hypertrophy by responding to pressure overload in the cardiomyocyte. Third, MKL1/MRTF-A is known to regulate fibroblast to myofibroblast transition, a key process in HPH pathogenesis. The possibility that MKL1/MRTF-A deficiency in fibroblast may stall fibroblast to myofibroblast transition and consequently HPH progression cannot be ruled out. Finally, the observation that MKL1/MRTF-A interacts with NF-κB/p65 suggests that MKL1/MRTF-A could also play a potential role in regulating the phenotype of circulating hematopoietic cells such macrophages and platelets in response to hypoxic stimulation. Indeed, our recent finding that MKL1/MRTF-A recruits the COMPASS complex to drive the expression proinflammatory mediators (eg, interleukin-1β) induced by lipopolysaccharide in macrophages seems to lend this model some support. Given the universal expression pattern of MKL1/MRTF-A, these lingering ambiguities will have to be addressed using a tissue-specific MKL1/MRTF-A knockout model.

MKL1/MRTF-A has been demonstrated to interact with multiple epigenetic factors, including chromatin remodeling proteins, histone acetyltransferase, and histone demethylase. Downregulation of adhesion molecules as a result of MKL1/MRTF-A silencing was accompanied by the disappearance of acetylated histones H3 and H4, as well dimethylated and trimethylated H3K4 on the promoters of the corresponding genes. Of key importance, we have identified a histone H3K4 methyltransferase complex as part of the epigenetic
machinery enlisted by MKL1/MRTF-A to activate adhesion molecules and to advance the pathogenic agenda imposed by hypoxic stress in mice. Germ-line deletion of either ASH2 or WDR5 results in developmental arrest and embryonic lethality. Our data indicate that endothelial-specific depletion of ASH2 or WDR5 in adult mice protected the mice from HPH. Hang et al have reported that conditional knockout of Brg1, a chromatin remodeling protein necessary for vessel formation, in adult mice abrogates pressure overload–induced cardiac hypertrophy, a finding in line with our observation and echoing the assertion that inadvertent activation of developmentally critical factors may herald detrimental effects in postembryonic life.

Recently, several independent investigations have implicated H3K4 methylation in endothelial injury. In a string of publications, El-Osta and co-workers have reported that H3K4 mono-, but not di-, or trimethylation, mediated by SET7, was responsible for p65 upregulation by hyperglycemic stress. On the contrary, Takizawa et al suggest that induction of plasminogen activator inhibitor 1 in the endothelium in mice with type I diabetes mellitus was predominantly controlled by H3K4 trimethylation. Still, Zhong and Kowluru note that repression of SOD2 expression by high glucose was synonymous with a reduction in H3K4 monomethylation and dimethylation. These seemingly conflicting data nonetheless argue for a role of H3K4 methylation in shaping the endothelial phenotype under stress conditions. Whereas hypoxia can modulate both global- and gene-specific H3K4 methylation in cancer cells, little is known about its effect on H3K4 methylation in the setting of plasminogen activator inhibitor 1 in the endothelium and especially in the settings of HPH. Because single-gene–based epigenetic analysis tends to give limited and sometimes biased information toward the understanding of such complex disease states as HPH, a genome-wide survey of H3K4 methylation status before and after stress using chromatin immunoprecipitation–sequencing would greatly further our knowledge on the pathogenesis of HPH. In addition, it would be interesting to determine how MKL1/MRTF-A contributes to the makeup of H3K4 methylation in endothelial cells and more importantly how this process translates to a specific pathobiological process.

We have previously shown that hypoxia increases MKL1/MRTF-A expression and nuclear localization in cultured endothelial cells and in the lungs in mice. We show here that hypoxia promotes the recruitment of MKL1/MRTF-A to the CAM promoters, which is dependent on RhoA (Figure S4, S7, and S9). The RhoA-MKL1/MRTF-A axis plays important roles in cell and tissue injury. Inhibition of the RhoA-ROCK signaling has been demonstrated to attenuate pulmonary hypertension in several different animal models although the underlying mechanism is ill-defined. Our data as presented here indicate that RhoA is indispensable for hypoxia-induced CAM induction in endothelial cells (Figure S4). More intriguingly, RhoA silencing affects the disposition of modified histones on the CAM promoters by influencing MKL1/MRTF-A–dependent COMPASS recruitment (Figures S7 and S9). These observations are consistent with a string of recent findings that seem to argue for a connection between cytoskeletal reorganization and epigenetic gene regulation. Clearly, a thorough examination of RhoA-dependent epigenetic alterations across the genome would further our understanding of HPH pathogenesis and benefit a more rationalized exploitation of RhoA inhibitors in HPH intervention. A long held view is that the hypoxic response is intimately related to the inflammatory response in vivo although there lacks unequivocal evidence whether a protein or a group of proteins can bridge these 2 responses. Our report here that MKL1/MRTF-A regulates pulmonary inflammation under hypoxic conditions seems to suggest that MKL1/MRTF-A might just be such as a moderator. In light of our previous reports that highlight the role of MKL1/MRTF-A in mediating the effects of oxidized low-density lipoprotein, lipopolysaccharide, and angiotensin II and the fact that MKL1/MRTF-A is universally expressed in the vasculature, this noncanonical pathway probably holds a key position during inflammatory situations within the vasculature.

Perspective

Our data lend support to a model wherein the transcriptional modulator MKL1/MRTF-A, through recruiting a histone H3K4 methyltransferase complex, acts as a switch turning on specific histone modifications to induce NF-kB–dependent CAM expression in endothelial cells. Future investigations building on these results will provide potential therapeutic solutions to the treatment of HPH.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is New?**

• We show here that (1) germ-line ablation and endothelial-specific knock-down of megakaryocytic leukemia 1 (MKL1)/myocardin-related transcription factor A (MRTF-A) attenuates hypoxic pulmonary hypertension (HPH) in mice; (2) MKL1/MRTF-A regulates pulmonary inflammation by potentiating transactivation of adhesion molecules (CAM) under hypoxic conditions; (3) MKL1 activates CAM transcription by recruiting a histone H3 lysine 4 methyltransferase complex (COMPASS); and (4) endothelial-specific depletion of COMPASS proteins attenuates HPH in mice.

**What Is Relevant?**

• Elevated CAM in the vascular endothelium contributes to recruitment and adhesion of leukocytes and pulmonary inflammation, leading to the pathogenesis of HPH. Our data link MKL1/MRTF-A-dependent, epigenetic activation of CAM expression to endothelial dysfunction, pulmonary inflammation, and HPH. We expect that our findings will be of significant interest to a broad readership in the basic, clinical, and pharmaceutical communities.

**Summary**

Enhanced interaction between vascular endothelial cells and circulating leukocytes, as a result of transcriptional activation of cell adhesion molecules, helps establish a proinflammatory milieu contributing to the pathogenesis of chronic HPH. We report here that the transcriptional modulator MKL1/MRTF-A is responsible for recruiting a histone H3 lysine 4 methyltransferase complex to the CAM promoters to promote pulmonary inflammation and HPH pathogenesis. Endothelial-specific depletion of MKL1/MRTF-A or components of the COMPASS complex ameliorated HPH in mice. In conclusion, our data suggest that MKL1/MRTF-A, by coordinating key epigenetic alterations on CAM promoters, provides a critical link to hypoxia-induced endothelial malfunction and contributes to the pathogenesis of HPH.
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Megakaryocytic leukemia 1 (MKL1) directs an H3K4 methyltransferase complex to regulate hypoxic pulmonary hypertension

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Running title: MKL1 epigenetically activates CAM

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Methods

Cell culture

Human umbilical vein endothelial cells (HUVEC/EAhy926, ATCC) and human monocytic/macrophage cells (THP-1, ATCC) were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone). Human primary pulmonary arterial endothelial cells (HPEC, Invitrogen) were maintained in M200 media with supplements supplied by the vendor; experiments were performed in HPECs between 3<sup>rd</sup> and 6<sup>th</sup> passages. Three separate batches of cells were used in this study. Where indicated, hypoxia (1% O<sub>2</sub>) was achieved by a mixture of ultra-high purity gases (5% CO<sub>2</sub>, 10% H<sub>2</sub>, 85% N<sub>2</sub>) in a 37°C incubator (Thermo Fisher).

Plasmids, Transient Transfection, and Luciferase Assay

Expression constructs for MKL1 (WT and DN), ASH2, WDR5, SET1, and short hairpin RNA (shRNA) plasmid targeting MKL1 were gifts from Dr. Prywes<sup>1, 2</sup>, Dr. Min Wu<sup>3</sup>, and Dr. Parmacek<sup>4</sup>. Promoter luciferase fusion constructs for ICAM-1, VCAM-1, and E-selectin were provided by Dr. Stratowa<sup>5</sup>, Dr. Redondo<sup>6</sup>, and Dr. Sachais<sup>7</sup>. Small interfering RNA were synthesized using the following sequences: for MKL1 #1, GUGUCUUGGUGUGUUA and #2, CUGCGUGCAUAUCAAGAACA; for ASH2, GCCUGGUAAUUUGAAAUCAdTdT; for WDR5, GTGGAAGAGTGACTGCTAA; for SET1, CAGCGTATTATGAAAGCTGGA; for RhoA, GACAUGCUUGCUCAUAGUCTT. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) essentially as previously described<sup>8</sup>. For expression plasmids, an empty vector was included as a control. For shRNA plasmids, a non-target plasmid was included as a control. A small amount of GFP plasmid (30ng/well) was always co-transfected with indicated constructs for monitoring and normalization of transfection efficiency. Luciferase activities were assayed 24-48 hours after transfection using a luciferase reporter assay system (Promega). Data were normalized by both protein concentration and GFP fluorescence and were expressed as arbitrary relative luciferase unit (RLU) compared with the control group.

Protein Extraction, Immunoprecipitation, and Western Blot

Whole cell lysates were obtained by re-suspending cell pellets in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor tablet (Roche). Specific antibodies or pre-immune IgGs (P.I.I.) were added to and incubated with cell lysates overnight before being absorbed by Protein A/G-plus Agarose beads (Santa Cruz). Precipitated immune complex was released by boiling with 1X SDS electrophoresis sample buffer. Western blot analyses were performed with anti-β-actin (Sigma), anti-ICAM-1 (Abgent, AJ1386a), anti-VCAM-1 (Santa Cruz, sc-8304), and anti-E-selectin (Santa Cruz, sc-14011) antibodies.

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 were purchased from Applied Biosystems.

**Mice**

All protocols were approved by the intramural Committee on Ethic Conduct of Animal Studies. MKL1 knockout (KO) mice were obtained from Steve Morris's laboratory as previously described. Female MKL1 KO mice have mammary epithelial deficiency rendering them unable to feed the cubs but are otherwise indistinguishable from wild type littermates under physiological conditions. 8-week old, male mice were housed in a low oxygen chamber (10% O₂) for three weeks to induce HPH. shRNA targeting, ASH2 (CGAGTCTTTGTCGACCCATCAT), or WDR5 (GCCGTTCCATTTCAACCGTGAT) were cloned into an endothelium-specific expression vector (Tie2p/eas) and packaged using an endothelium-specific envelope (2.2) as previously described. At week 1 and week 3, these mice were injected with lentivirus via tail vein. A similar endothelial-specific knockdown system targeting MKL1 has been described and validated recently. Measurement of RVSP and heart weight was performed essentially as previously described. Bronchoalveolar lavage (BAL) was collected from anesthetized mice using two 1-ml aliquots of PBS. Cells were centrifuged and resuspended in 1 ml PBS. Cells were used for flow cytometry and the BAL fluid (BALF) was used to analyze pro-inflammatory mediators by ELISA.

**Histology**

Immunohistochemistry was performed as previously described. Briefly, parallel sections were blocked with 10% normal goat serum for 1 hour at room temperature and then incubated with anti-α-SMA (Sigma; 1:100). Staining was visualized by incubation with an appropriate biotinylated secondary antibody and developed with a streptavidin-horseradish peroxidase kit (Pierce) for 20min. Sections were counterstained with hematoxylin. Pictures were taken using an Olympus IX-70 microscope. Vessel muscularity was determined using α-SMA–stained lung sections, with ~10 fields counted per animal. Vessels ≤ 50mm that were completely surrounded with α-SMA were considered vascularized. Data are expressed as the percentage of total vessels.

**Immunofluorescence staining**

The plastic-embedded sections were incubated with primary antibodies, anti-CD31 (BD Biosciences), anti-ASH2 (Santa Cruz, sc-81184), anti-WDR5 (Santa Cruz, sc-135245), anti-CD3 (BD Biosciences, 554829), anti-CD45 (BD Biosciences, 610266), or anti-F4/80 (BD Biosciences, 552958), followed by incubation with donkey secondary antibodies (Jackson ImmunoResearch). The nuclei were counterstained with DAPI (Sigma).

**Leukocyte Adhesion Assay**
THP-1 cells were stained with a fluorescent die (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 analyzed with Image-Pro Plus (Media Cybernetics).

**Chromatin Immunoprecipitation (ChIP)**

Chromatin was cross-linked with 1% formaldehyde. Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with protease inhibitor tablet. DNA was fragmented into ~500 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-MKL1 (Santa Cruz, sc-32909), anti-p65 (Santa Cruz, sc-372), anti-RNA Pol II (Santa Cruz, sc-9001), anti-acetyl histone H3 (Millipore, 17-615), anti-acetyl histone H4 (Millipore, 06-598), anti-dimethyl H3K4 (Millipore, 07-030), anti-trimethyl H3K4 (Millipore, 07-473), anti-ASH2 (Bethyl Laboratories, A300-489A), anti-WDR5 (Bethyl Laboratories, A300-429A), anti-SET1A (Bethyl Laboratories, A300-289A), or pre-immune IgG. Precipitated genomic DNA was amplified by real-time PCR with using the following primers: for human ICAM-1 gene, 5′-CCTGCCACCGCCGCCC-3’ and 5′-AGGGGCGGTGCTGTTCCTCC-3’; for human VCAM-1 gene, 5′-AAATCAATTCCATGCGGATA-3’ and 5′-AAGGCTTTGTGTGCCAGG-3’;; for human E-selectin gene, 5’-GGCCTCAGCCCGAGTAGTGC-3’ and 5’-CTGCTCGCTCGCTCGCAG-3’; for mouse ICAM-1 gene, 5′-AGGGGACTAGGCGGCTGAGCCATCAG-3’ and 5′-GAACGAGGGCTTCGGTTT3’; for mouse VCAM-1 gene, 5′-GAACGAGGGCTTCGGTTT3’ and 5′-TAATTGGCTTCTTGCAGGCCGCTTCTGCAG-3’; for mouse E-selectin gene, 5′-ATACAAATATAAGAGC-3’ and 5′-ATCCGTTCTTCGCAGCTTGC-3’.

**Enzyme-linked immune absorbance assay (ELISA)**

ELISA was performed using mouse lung homogenates and BALF to measure IL-1, IL-6, and MCP-1/CCL2 (Pierce).

**Flow cytometry**

Flow cytometry was performed as previously described using FACStar (Becton-Dickinson) with the following antibodies (eBioscience): FITC-labeled rabbit anti-mouse CD45, PE-labeled rat anti-mouse CD3, FITC-labeled rat anti-mouse F4/80.

**Statistical analysis**

Data are presented as mean±SD. For experiments concerning multiple groups, one-way ANOVA with post-hoc Scheffé analyses were performed to evaluate the
differences. The differences between two (control and experimental) groups were determined by two-sided, unpaired Student’s t-test. $p$ values smaller than .05 are considered significant. For in vivo experiments, all $p$ values are spelled out. Sample size for all animal experiments was between 3 to 5 per group while all in vitro experiments were repeated at least three times with a power analysis showing $\beta>80$. 
References


**A**

WT normoxia  |  WT hypoxia  |  KO normoxia  |  KO hypoxia

**B**

WT normoxia  |  WT hypoxia  |  KO normoxia  |  KO hypoxia

0–50μm

50–100μm

**C**

% wall thickness

0–50μm  |  50–100μm

WT  |  WT  |  KO  |  KO

**D**

% total vessels

Normoxia  |  Hypoxia

WT  |  WT  |  KO  |  KO

**E**

Lung homogenates

IL-6  |  IL-1  |  MCP-1

WT  |  KO  |  WT  |  KO  |  WT  |  KO

**F**

Lung homogenates

IL-6  |  IL-1  |  MCP-1

WT  |  KO  |  WT  |  KO  |  WT  |  KO
Fig.S1: MKL1 deficient (KO) and age/sex-matched control C57/Bl mice were allowed develop HPH as described under Methods. (A) Representative H&E staining photographs of lung sections. (B-D) Representative α-SMA staining photographs of lung sections are shown (B). Wall thickness (C) was expressed as the medial wall thickness divided by the diameter of the vessel. N=3 for each group. Vascularization (D) was determined as described under Methods. N=3 for each group. (E, F) Levels of pro-inflammatory mediators in the lungs were measured by qPCR (E) and ELISA (F). N=3~5 mice for each group. (G, H) BAL was collected and processed as described under Methods. Supernatant was used to examine pro-inflammatory mediators by ELISA (G) and cell pellets were used to examine immune infiltrates by flow cytometry (H). N=3~5 mice for each group.
Fig.S2: C57/BL6 mice were induced to develop HPH and endothelial MKL1 was silenced by a lentivirus-based system as described under Methods. (A, B) Right ventricular hypertrophy was evaluated by RVSP (A) and RV/LV+S (B). (C) Expression levels of adhesion molecules in the lungs were examined by qPCR. (D, E) Levels of pro-inflammatory mediators in the lungs were measured by qPCR (D) and ELISA (E). (F, G) BAL was collected and processed as described under Methods. Supernatant was used to examine pro-inflammatory mediators by ELISA (F) and cell
pellets were used to examine immune infiltrates by flow cytometry (G). N=3~5 mice for each group.
Fig.S3: (A) Different CAM promoter luciferase plasmids were transfected into EAhy92.6/HUVECs along with increasing concentrations of MKL1 expression construct. Data are expressed as RLU. (B) MKL1 expression construct or an empty vector (EV) was transfected into EAhy92.6/HUVECs followed by exposure to 1% O2. Endothelial-leukocyte interaction was evaluated by in vitro adhesion assay. (C) Promoter-luciferase fusion constructs for ICAM-1, VCAM-1, or E-selectin were transfected into EAhy92.6/HUVECs with or without dominant negative (DN) MKL1 followed by exposure to 1% O2. Data are expressed as RLU. (D) Promoter-luciferase fusion constructs for ICAM-1, VCAM-1, or E-selectin were transfected into EAhy92.6/HUVECs with or without shRNA plasmid targeting MKL1 (shMKL1) followed by exposure to 1% O2. Data are expressed as RLU. (E) MKL1 DN or EV was transfected into EAhy92.6/HUVECs followed by exposure to 1% O2. mRNA levels of adhesion molecules were examined by qPCR. (F) EAhy92.6/HUVECs were transfected with indicated siRNA. mRNA and protein levels of MKL1 were examined by qPCR and Western. (G, H) EAhy92.6/HUVECs and HPECs were transfected with small interfering RNA targeting MKL1 (siMKL1#2) or scrambled RNA (SCR) followed by exposure to 1% O2. mRNA (G) and protein (H) levels of adhesion molecules were measured by qPCR and Western, respectively.
Fig S4: (A) EAhy92.6/HUVECs were transfected with SCR or siRhoA as indicated. Knockdown efficiency was examined by qPCR and Western. (B) Promoter-luciferase fusion constructs for ICAM-1, VCAM-1, or E-selectin were transfected into EAhy92.6/HUVECs with SCR or siRhoA followed by exposure to 1% O2. Data are expressed as RLU. (C-E) EAhy92.6/HUVECs were transfected with SCR or siRhoA followed by exposure to 1% O2. mRNA (C) and protein (D) levels of adhesion molecules were measured by qPCR and Western, respectively. (E) In vitro adhesion assay was performed with EAhy92.6/HUVECs as described under Methods.
Fig.S5: (A) EAhy92.6/HUVECs were transfected with SCR or siRhoA followed by exposure to 1% O₂. ChIP assays were performed with anti-MKL1. (B) EAhy92.6/HUVECs were transfected with SCR or siRhoA followed by exposure to 1% O₂. Re-ChIP assays were performed with indicated antibodies.
Fig. S6: (A, B) Wild type (WT) or MKL1 deficient (KO) mice were induced to develop HPH as described under Methods. ChIP assays were performed using lung lysates with anti-AcH3 (A) or anti-AcH4 (B). (C) EAhy92.6/HUVECs were transfected with SCR or siMKL1 followed by exposure to 1% O2. Global levels of modified histones were examined by Western.
**Fig. S7:** EAhy92.6/HUVECs were transfected with SCR or siRhoA followed by exposure to 1% O₂. ChIP assays were performed with anti-acetyl H3 (A), anti-acetyl H4 (B), anti-dimethylated H3K4 (C), or anti-trimethylated H3K4 (D).
Fig. S8: (A) Whole cell lysates extracted from EAhy92.6/HUVECs were immunoprecipitated with indicated antibodies. (B) EAhy92.6/HUVECs were exposed to hypoxia for 24 hours. Whole cell lysates were immunoprecipitated with indicated antibodies. (C) EAhy92.6/HUVECs were exposed to 1% O₂ and harvested at indicated time points. ChIP assays were performed with indicated antibodies. (D, E) EAhy92.6/HUVECs were transfected with siMKL1 or SCR followed by exposure to 1% O₂. ChIP assays were performed with anti-WDR5 (D) or anti-SET1 (E).
Fig.S9: EAhY92.6/HUVECs were transfected with SCR or siRhoA followed by exposure to 1% O2. ChIP assays were performed with anti-ASH2 (A), anti-WDR5 (B), or anti-trimethylated SET1 (C).
Fig S10: (A) EAhy92.6/HUVECs were transfected with indicated siRNAs. mRNA expression was measured by qPCR and Western. (B-D) C57/BL6 mice were injected via tail vein lentiviral particles carrying endothelial-specific shRNA targeting ASH2 or WDR5. (B) Immunostaining was performed with anti-ASH2 or anti-WDR5 (Red) and anti-CD31 (green) followed by counter-staining with DAPI. Arrows, typical endothelial cells with ASH2/WDR5 expression. (C) Immunostaining was performed with anti-ASH2 or anti-WDR5 (Red) and anti-FSP1 (green) followed by counter-staining with DAPI. Arrows, typical fibroblast cells with ASH2/WDR5 expression. (D) Immunostaining was performed with anti-ASH2 or anti-WDR5 (Red)
and anti-SP-C (green) followed by counter-staining with DAPI. Arrows, typical epithelial cells with ASH2/WDR5 expression.
Fig.S11: C57/BL6 mice were injected via tail vein lentiviral particles carrying endothelial-specific shRNA targeting ASH2 or WDR5. (A) Representative H&E staining photographs of lung sections. (B) Representative α-SMA staining photographs of lung sections. Wall thickness (C) was expressed as the medial wall thickness divided by the diameter of the vessel. Vascularization (D) was determined as described under Methods.
Fig. S12: (A-D) Immunofluorescence stainings were performed on paraffin embedded lung sections with anti-MRTF-A (red) and anti-CD31 (green, A), or anti-α-SMA (green, B), or anti-SP-C (green, C), or anti-FSP (green, D) followed by counter-staining with DAPI. Arrows, cells with MRTF-A expression. Scale bar, 50μm.