



## Histone Demethylases KDM4A and KDM4C Regulate Differentiation of Embryonic Stem Cells to Endothelial Cells

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### SUMMARY

Understanding epigenetic mechanisms regulating embryonic stem cell (ESC) differentiation to endothelial cells may lead to increased efficiency of generation of vessel wall endothelial cells needed for vascular engineering. Here we demonstrated that the histone demethylases KDM4A and KDM4C played an indispensable but independent role in mediating the expression of fetal liver kinase (*Flk1*) and *VE-cadherin*, respectively, and thereby the transition of mouse ESCs (mESCs) to endothelial cells. KDM4A was shown to bind to histones associated with the *Flk1* promoter and KDM4C to bind to histones associated with the *VE-cadherin* promoter. KDM4A and KDM4C were also both required for capillary tube formation and vasculogenesis in mice. We observed in zebrafish that KDM4A depletion induced more severe vasculogenesis defects than KDM4C depletion, reflecting the early involvement of KDM4A in specifying endothelial cell fate. These findings together demonstrate the essential role of KDM4A and KDM4C in orchestrating mESC differentiation to endothelial cells through the activation of *Flk1* and *VE-cadherin* promoters, respectively.

### INTRODUCTION

Endothelial cells derived from differentiation of embryonic stem cells (ESCs) or induced pluripotent cells (iPSCs) hold great promise for regenerating blood vessels in diseases associated with endothelial denudation (Kourembanas, 2014; Yoder, 2012). Studies have described differentiation of endothelial cells from ESCs as mirroring embryonic vascular development (Descamps and Emanuelli, 2012; Leeper et al., 2010). The growth factors bone morphogenetic protein-4 (BMP-4), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) are required for specifying the transition of ESCs to the mesoderm and then to the endothelial cell fate as defined by the appearance of Flk1, CD31, and VE-cadherin-positive cells (Li et al., 2007; Park et al., 2013).

Epigenetic regulation through histone modifications is a crucial mechanism mediating lineage-specific gene activation of cells undergoing differentiation (Kooistra and Helin, 2012; Kouzarides, 2007; Ohtani et al., 2011). Histone modifications occurring via mono-methylation, di-methylation, and tri-methylation change histone-DNA binding affinities and the interactions of specific transcription factors with the promoters (Barski et al., 2007; Kouzarides, 2007; Wang et al., 2007). Demethylases may regulate activation of genes responsible for the transition of pluripotent cells to endothelial cells (Kohler et al., 2013; Marcelo et al., 2013). Here we addressed the role of mouse ESC (mESC) histone demethylation in endothelial cell specification. We demonstrated that histone demethylases KDM4A and

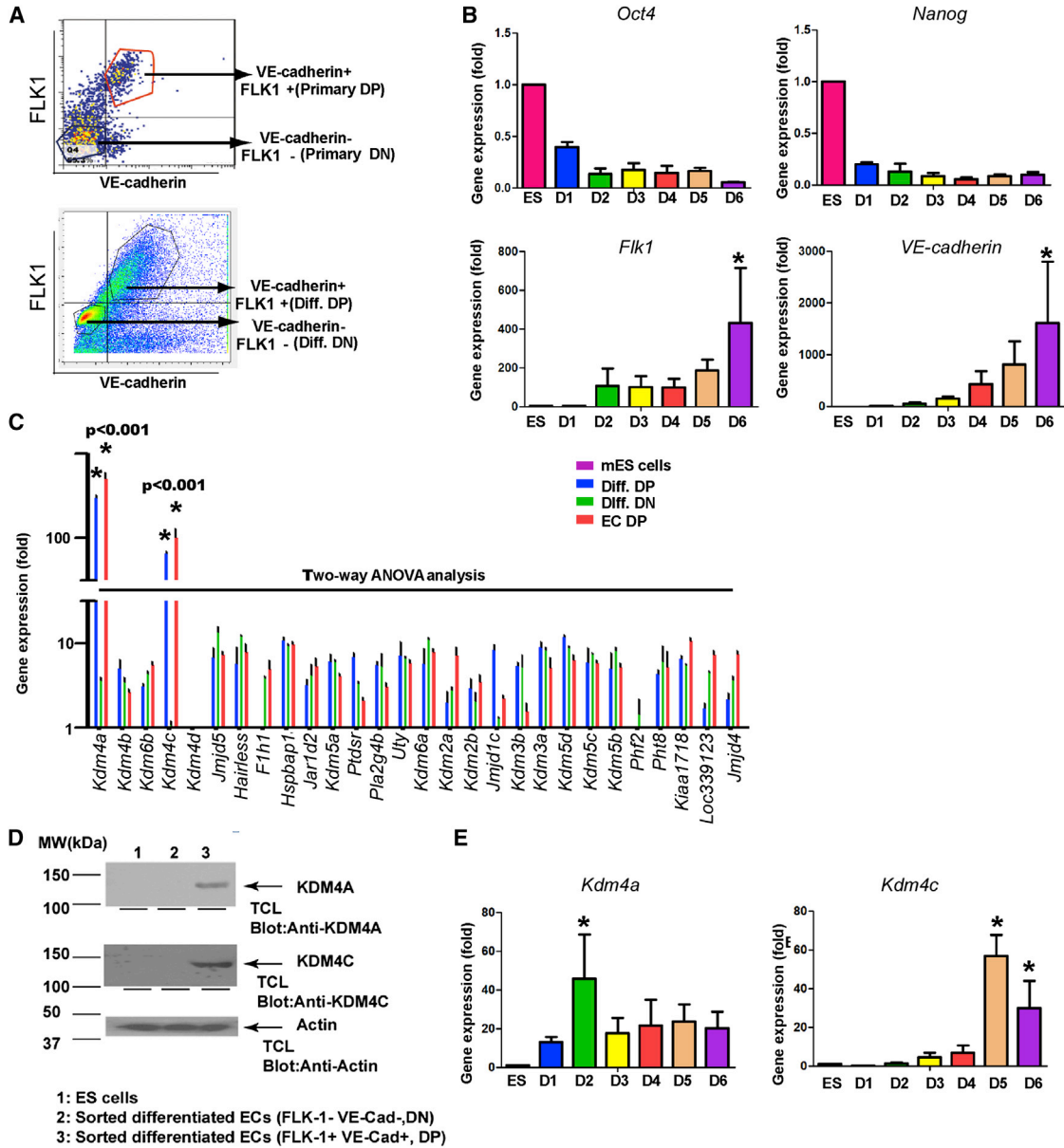
KDM4C independently induced demethylation at histone H3K9 to activate *Flk1* and *VE-cadherin* expression and thus enabled the differentiation of mESCs to endothelial cells. KDM4A targeted the *Flk1* promoter in the early stage of differentiation, whereas KDM4C targeted the *VE-cadherin* promoter later to induce a transition to the endothelial cell lineage. Removal of histone methylation marks on *Flk1* and *VE-cadherin* promoters by KDM4A and KDM4C, respectively, is therefore an essential mechanism of endothelial cell fate specification and vasculogenesis.

### RESULTS

#### Time Course of Expression of KDM4A and KDM4C during mESC Differentiation to Endothelial Cells

Using an established differentiation protocol employing the growth factors BMP-4, bFGF, and VEGF (Blancas et al., 2008), we generated endothelial cells as defined by co-expression of the surface markers FLK1 and VE-cadherin. Fluorescence-activated cell sorting (FACS) analysis showed ~20% FLK1/VE-cadherin double-positive cells on day six (D6) of cell differentiation (Figure 1A). qRT-PCR demonstrated concomitant time-dependent decreases in the expression of the pluripotency regulators *Oct4* and *Nanog* as mESC transitioned into endothelial cells (Figure 1B).

To investigate the role of histone demethylases in mediating the transition to endothelial cells, we first determined expression levels of 28 histone demethylases in the FLK1/VE-cadherin-double positive cells derived from mESCs.



**Figure 1. Expression of KDM4A and KDM4C following mESC Differentiation into Endothelial Cells**

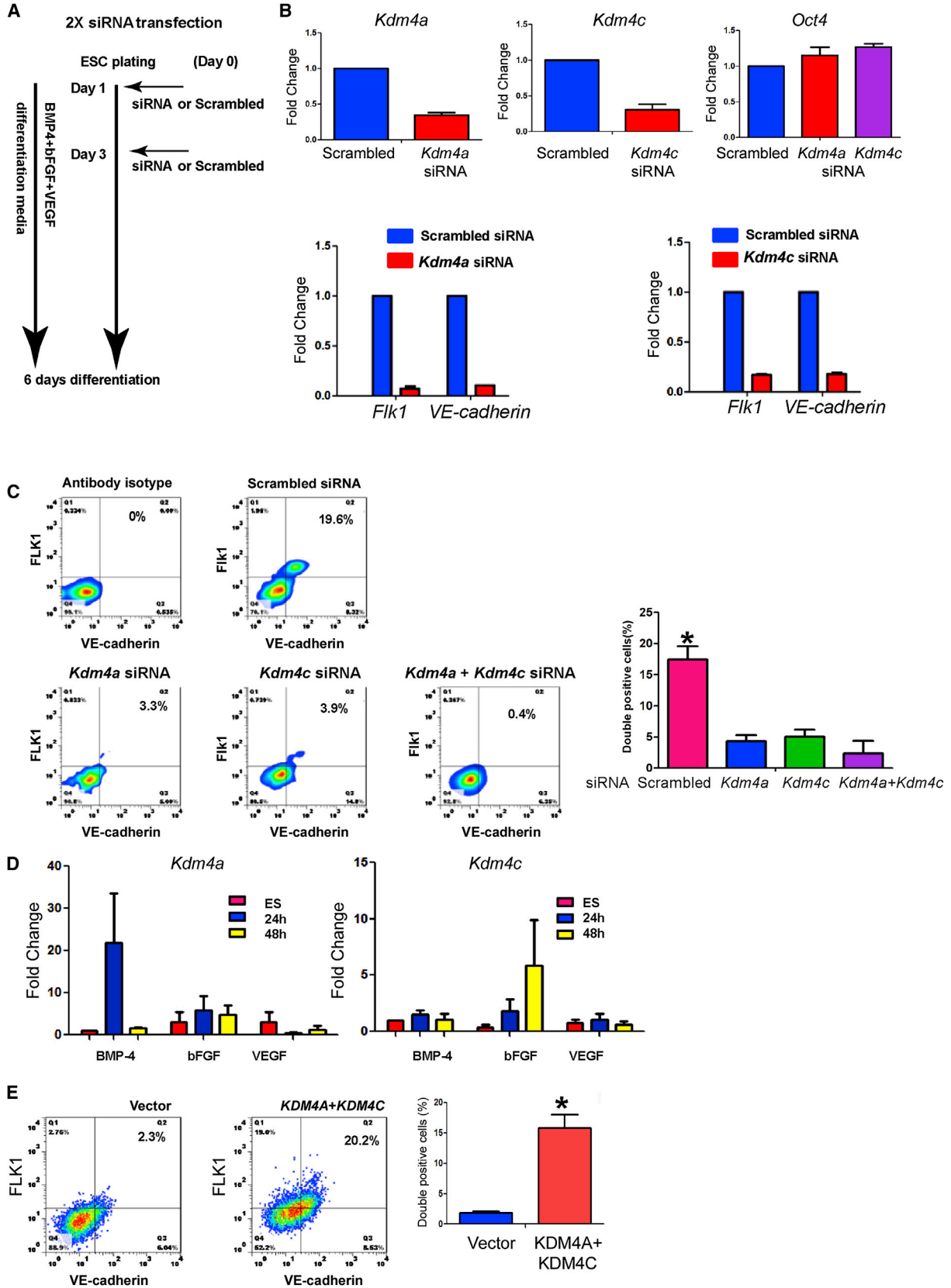
(A) Flow sorting diagram of mESCs differentiated to endothelial cells stained with anti-FLK1 and anti-VE-cadherin antibodies. Cells were separated into FLK1/VE-cadherin-double positive endothelial cells or double-negative control cells.

(B) Time course of gene expression assessed by qRT-PCR for the pluripotency genes *Oct4* and *Nanog* as well as for endothelial genes *Flk1* and *VE-cadherin* during the 6-day differentiation period (D1 to D6; ES are mESCs); error bars represent mean ± SD (n = 3 independent experiments, p < 0.05 by ANOVA).

(C) qRT-PCR screen for 28 known histone demethylases was carried out in mESCs differentiated into FLK1/VE-cadherin-double positive endothelial cells (D6), double-negative control cells (D6), and adult primary mouse endothelial cells. Expression of *KDM4a* and *KDM4c* was markedly increased in mESC-derived and mouse endothelial cells as assessed by two-way ANOVA (all values are normalized to 18S in undifferentiated mESCs). Values are mean ± SD (n = 3 independent experiments, \*p < 0.001 by two-way ANOVA).

(D) Western blotting carried out using anti-KDM4A and anti-KDM4C antibodies in sorted FLK1/VE-cadherin-double-positive cells derived from mESCs at day 6 of differentiation. Representative examples from three independent experiments are shown.

(E) Time course of *Kdm4a* and *Kdm4c* expression determined by qRT-PCR during the day 1 to day 6 period of endothelial cell differentiation from mESCs. Values are mean ± SD (n = 3 independent experiments); gene expression in undifferentiated cells (ES) is set as 1; p < 0.05 by ANOVA.



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We observed that expression of *Kdm4a* and *Kdm4c* was markedly increased in these cells on day 6 of the endothelial differentiation protocol relative to either undifferentiated mESCs or FLK1/VE-cadherin-double negative cells (i.e., non-endothelial cells derived from mESCs) (Figure 1C). Expression of *Kdm4a* and *Kdm4c* was similar to mature adult endothelial cells (Figure 1C). Western blotting confirmed the expression of both KDM4A and KDM4C in the FLK1/VE-cadherin-double-positive but not in the double-negative cells or undifferentiated mESCs (Figure 1D). *Kdm4a* expression increased to the maximal level at day 2 of differentiation and remained elevated for the remainder of the 6-day differentiation period. *KDM4c* expression increased gradually, peaking on day 5, and then declined to an intermediate level on day 6 (Figure 1E).

### KDM4A and KDM4C Regulate mESC Transition to Endothelial Cells

We next determined the roles of KDM4A and KDM4C in generating endothelial cells. mESCs were transfected with *Kdm4a* or *Kdm4c* siRNA on days 1 and 3 to achieve optimal knockdown during the 6-day differentiation period (Figure 2A). Depletion of either KDM4A or KDM4C by siRNA treatment induced 60%–80% reduction in mRNA expression in each case (Figure 2B). Compared with scrambled siRNA, siRNA-induced depletion of either *Kdm4a* or *Kdm4c* significantly reduced the expression of both Flk1 and VE-cadherin (Figure 2B).

Next using FACS we found that either depletion of *Kdm4a* or *Kdm4c* significantly reduced the mESC transition to endothelial cells as compared with control siRNA-treated mESCs (Figure 2C). We observed 16% FLK1/VE-cadherin-double-positive cells in the samples treated with scrambled siRNA versus only 4% and 5% in *Kdm4a*- and *Kdm4c*-depleted cells, respectively (Figure 2C). Simultaneous knockdown of both histone demethylases did not

further suppress the generation of double-positive cells (Figure 2C).

We next determined whether KDM4A and KDM4C were downstream targets of BMP4, bFGF, and VEGF, the growth factors used to induce differentiation to endothelial cell. We observed that BMP-4 induced the expression of *Kdm4a* at 24-hr post-stimulation (Figure 2D). In contrast, *Kdm4c* was induced by bFGF with a maximum response seen at 48-hr post-stimulation (Figure 2D). In contrast, VEGF did not appear to significantly induce either *Kdm4a* or *Kdm4c* expression (Figure 2D). We next determined whether overexpressing *Kdm4a* and *Kdm4c* might substitute for BMP-4 and bFGF and hence promote differentiation of mESC to endothelial cells even in the absence of these growth factors. In these experiments, mESC transfected with *Kdm4a* and *Kdm4c* were stimulated with VEGF alone. We found that *Kdm4a* and *Kdm4c* overexpression induced endothelial specification and differentiation in 15% of the cell population (Figure 2E) in the absence of bFGF and BMP-4, a yield similar to what we had observed in the presence of all three growth factors (Figure 1A).

### KDM4A and KDM4C Induce Histone Demethylation at Flk1 and VE-Cadherin Promoters

We determined histone demethylation of *Flk1* and *VE-cadherin* promoters and binding of KDM4A and KDM4C to histones associated with these promoters. In addition, we studied KDM4A and KDM4C binding to the histones associated with the promoter of the transcription factor *Er71* based on its described role in endothelial cell lineage specification (Park et al., 2013). We observed that the *Flk1* promoter was enriched in the KDM4A-histone complex, whereas the *VE-cadherin* promoter was primarily found in KDM4C-histone complex (Figure 3A). There was no detectable *Er71* promoter interaction with either KDM4A

## Figure 2. KDM4A and KDM4C Mediate mESC Differentiation to Endothelial Cells

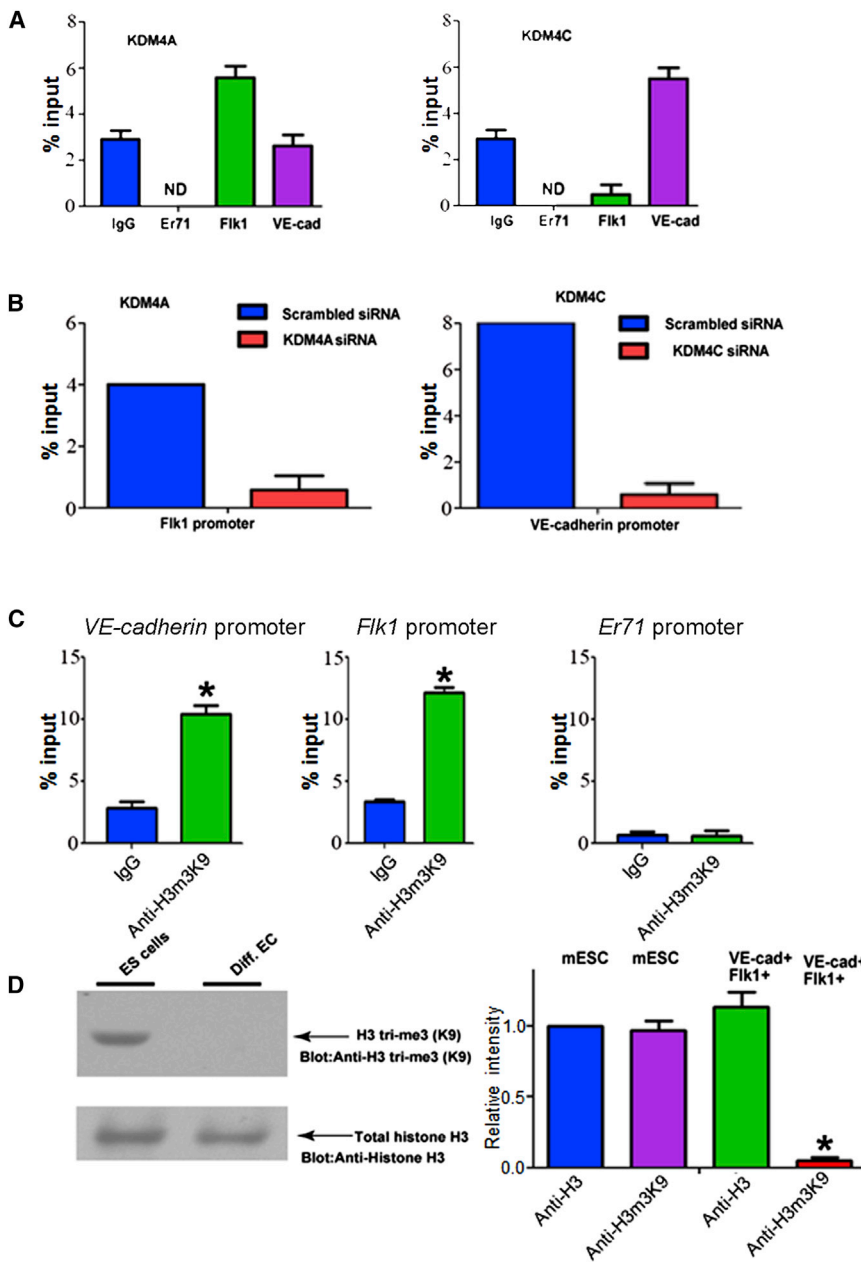
(A) Diagram showing experimental protocol for siRNA treatment. mESC were transfected on days 1 and 3 to achieve maximum knockdown of protein. The cells were differentiated in medium containing BMP-4, bFGF, and VEGF for 6 days.

(B) The effect of *Kdm4a* and *Kdm4c* knockdown on expression of endothelial-specific markers. Efficiency of *Kdm4a* and *Kdm4c* knockdown was 60%–80%. Oct4 expression was not suppressed by either *Kdm4a* or *Kdm4c* knockdown ( $n = 4$  per group). Both *Kdm4a* and *Kdm4c* knockdown resulted in 90% and 80%, respectively, reduction in *Flk1* and *VE-cadherin* expression on day 6 ( $n = 3$  independent experiments).

(C) Flow cytometry assessment of cell surface expression of Flk1 and VE-cadherin on day 6 of differentiation following treatment with scrambled, *Kdm4a*, *Kdm4c*, or *Kdm4a* and *Kdm4c* siRNAs. Quantification in the bar graph shows that the percentage of FLK1 and VE-cadherin double positive cells was reduced from 17% to less than 5%; FACS profile is representative ( $n = 3$  independent experiments). Quantification values are mean  $\pm$  SD;  $p < 0.05$ . Combining *Kdm4a* with *Kdm4c* siRNAs did not further suppress differentiation.

(D) Treatment of mESCs with BMP-4 alone (2 ng/ml) is sufficient to induced *Kdm4a* expression at 24-hr post-stimulation. Treatment with bFGF alone (10 ng/ml) induced *Kdm4c* expression at 48-hr post-stimulation. Error bars represent mean  $\pm$  SD ( $n = 3$  independent experiments). VEGF stimulation did not induce either KDM4A or KDM4C expression during the 48-hr period.

(E) *Kdm4a* and *Kdm4c* overexpression substitutes for BMP-4 and bFGF. mESCs were transfected with *Kdm4a* and *Kdm4c* and cultured in medium containing only VEGF. FACS analysis on day 6 demonstrated that the fraction of double-positive cells was 20%. Bar graph summarizes data from ( $n = 3$ ) three independent experiments.



**Figure 3. KDM4A and KDM4C Bind to Histones Associated with *Flk1* and *VE-cadherin* Promoters, Respectively**

(A) Differentiated cells were subjected ChIP-qPCR analysis using control IgG, anti-KDM4A, and anti-KDM4C antibodies (see [Experimental Procedures](#)). The ChIPs were subjected to qPCR using oligonucleotides to amplify the promoter regions of *VE-cadherin*, *Flk1*, and the transcription factor *Er71*. Chromatin binding to IgG-ChIP was considered background. *Flk1* promoter was enriched in KDM4A ChIP, whereas *VE-cadherin* promoter was enriched in KDM4C ChIP. *Er71*-promoter was not detectable (ND) in either ChIP. Values are mean  $\pm$  SD ( $n = 3$  independent experiments).

(B) *Kdm4a* knockdown prevented enrichment of *Flk1*-promoter in the ChIP assay, whereas *Kdm4c* knockdown prevented enrichment of the *VE-cadherin* promoter. Values are mean  $\pm$  SD ( $n = 3$  independent experiments).

(C) ChIP assay was performed using an anti-histone H3 tri-methylation at lysine (K9) antibody to assess methylation of histones at the promoters of *VE-cadherin*, *Flk1*, and *Er71* in undifferentiated mESCs; values are mean  $\pm$  SD ( $n = 3$  independent experiments).

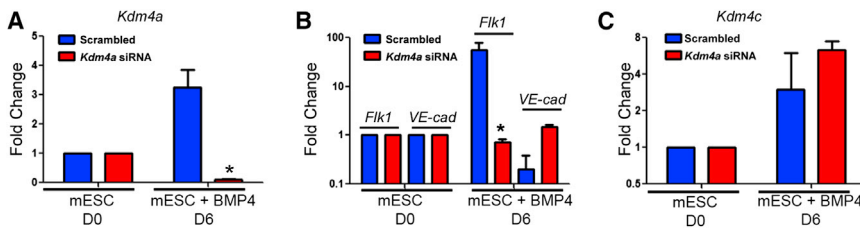
(D) Histone 3 tri-methylation at lysine 9 (H3K9) was assessed by western blotting in mESCs and FLK1/*VE-cadherin*-double-positive cells. An antibody against total histone H3 was used as loading control. The blot is representative of three independent experiments. Bar graph (mean  $\pm$  SD) shows significant reduction of histone H3 tri-methylation (K9) in endothelial cells as compared to undifferentiated ESCs ( $p < 0.05$  by Student's *t* test).

or KDM4C (Figure 3A). In control experiments, siRNA-induced knockdown of *Kdm4a* significantly decreased the detection of the *Flk1* promoter, whereas downregulation of KDM4C reduced detection of the *VE-cadherin* promoter (Figure 3B).

We also determined whether KDM4A and KDM4C functioned by modifying histone 3 methylation at lysine 9 (H3K9) in mESC since H3K9 is the preferred target of both demethylases (Berry and Janknecht, 2013; Cloos et al., 2006, 2008; Couture et al., 2007; Klose et al., 2006; Ng et al., 2007). Both *VE-cadherin* and *Flk1* promoters were enriched in H3K9 immunoprecipitates as compared

with control IgG (Figure 3C). However, there was no detectable enrichment of the *Er-71* promoter in H3K9 immunoprecipitates (Figure 3C). These results show that histones associated with *VE-cadherin* and *Flk1* promoters were methylated in the undifferentiated state. In contrast to the undifferentiated cells, histone H3K9 tri-methylation was barely detectable in the emergent endothelial cells (Figure 3D).

We then addressed whether BMP-4 induction of *Flk1* was mediated by KDM4A since KDM4A expression was upregulated by BMP-4 (Figure 2A). To avoid confounding the effects of VEGF and bFGF that are routinely added to the



**Figure 4. KDM4A Regulates *Flk1* Expression in mESCs Stimulated with BMP-4**

(A) mESCs were transfected with either scrambled or *Kdm4a* siRNA and stimulated with BMP-4 to induce mesodermal lineage commitment. qRT-PCR confirmed greater than 90% knockdown efficiency in cells harvested on day 6; values are mean  $\pm$  SD (n = 3 independent experiments,  $p < 0.05$  by Student's t test).

(B) *Flk1* and *VE-cadherin* expression evaluated by qRT-PCR in either scrambled or *Kdm4a* siRNA-transfected cells after 6 days differentiation confirmed significant reduction in *Flk1* expression following *Kdm4a* knockdown. Values are mean  $\pm$  SD (n = 3 independent experiments;  $p < 0.05$  by Student's t test).

(C) *Kdm4c* expression was determined by qRT-PCR demonstrated that downregulation of *Kdm4a* had no effect on *Kdm4c* gene expression. Values are mean  $\pm$  SD (n = 3 independent experiments).

endothelial differentiation medium, only BMP-4 was used in these experiments. Using siRNA to suppress KDM4A expression (Figure 4A), we observed that *Kdm4a* knockdown significantly decreased *Flk1* expression at day 6 after BMP-4 stimulation (Figure 4B), suggesting that *Flk1* is a crucial downstream target of KDM4A. Importantly, depletion of *Kdm4a* had no significant effect in modifying *Kdm4c* expression as compared with control siRNA-treated cells (Figure 4C), suggesting that these demethylases functioned independently.

### KDM4A and KDM4C Are Required for Capillary Tube Formation and Vasculogenesis In Vivo

mESCs were transfected with either scrambled, *Kdm4a*, or *Kdm4c* siRNA on days 1 and 3 of the differentiation protocol. Cells harvested on day 6 were plated onto Matrigel-coated plates for 16 hr. Control siRNA-treated cells formed tubes, whereas KDM4A- and KDM4C-depleted cells did not form tubes (Figure 5A). Experiments were also made in which unsorted day 6 cells were mixed with Matrigel and implanted subcutaneously into nude mice. At day 7 post-implantation, Matrigel plugs were retrieved, section, and stained for PECAM1 and VE-cadherin. We observed PECAM1 and VE-cadherin-positive blood vessels in the control group pre-treated with scrambled siRNA, whereas there was a virtual absence of blood vessels when implanted cells were pre-treated with siRNAs targeting *Kdm4a*, or *Kdm4c*, or both (Figure 5B).

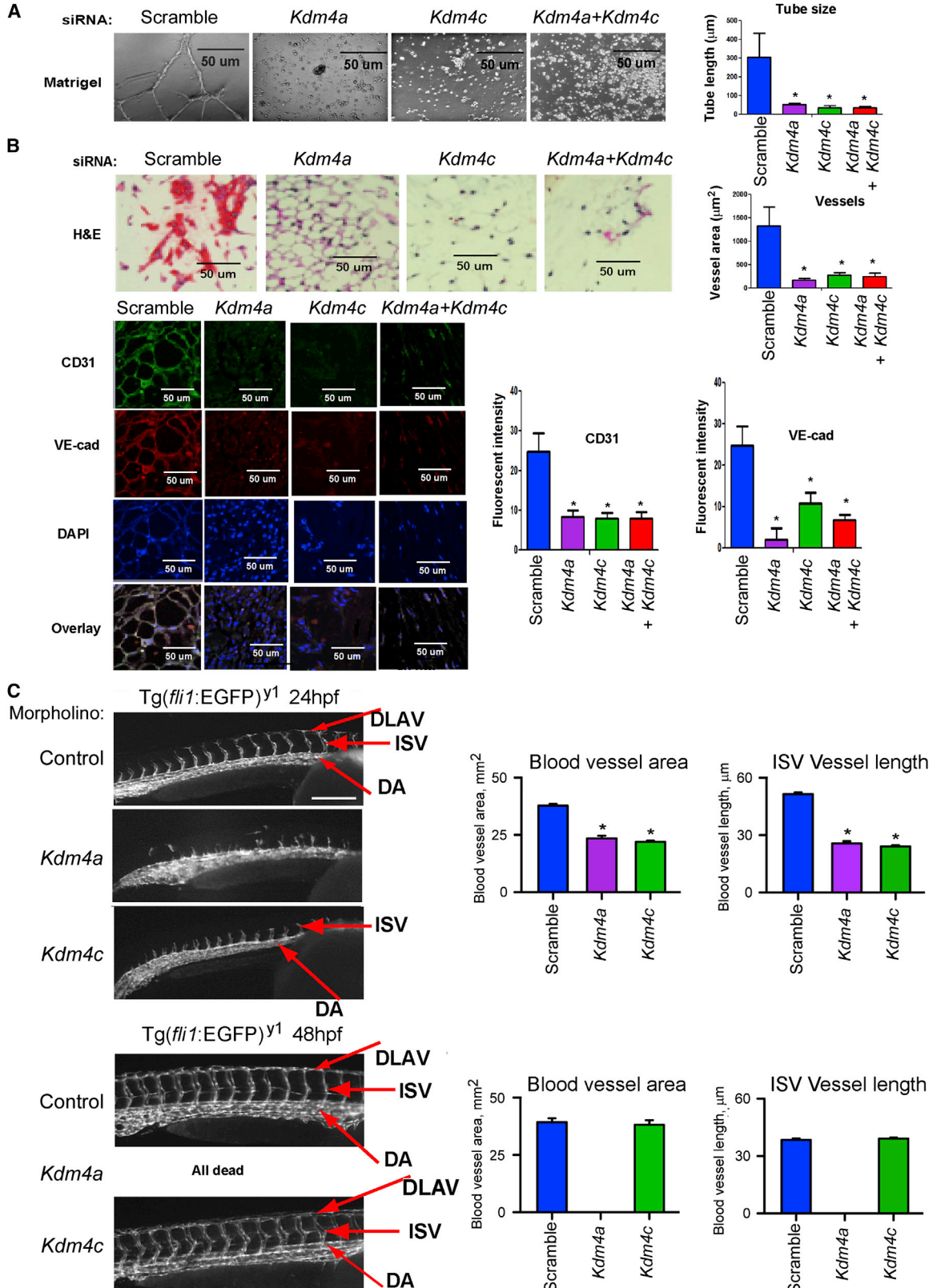
We also used the zebrafish model to address the role of KDM4A and KDM4C in regulating vascular development in vivo. Zebrafish embryos were either injected with control morpholino (MO), or *Kdm4a* MO, or *Kdm4c* MO, and observed for up to 48-hr postfertilization (hpf). In the control group, endothelial cells grew out from the dorsal aorta (DA) adjacent to intersomitic boundaries in a stereotypical manner at 24 hpf (Figure 5C). Bipolar sprouts grew toward the dorsal side until they passed the horizontal myoseptum, where they extended in anterior and poste-

rior directions into two branches that fused with branches of neighboring sprouts to form the characteristic dorsal longitudinal anastomotic vessels (DLAVs; Figure 5C). Downregulation of either *KDM4a* or *KDM4c* caused defects in vasculogenesis and angiogenesis. At a low MO dose (2 ng), most of the embryos of the *Kdm4a*-MO or *Kdm4c*-MO groups demonstrated profoundly defective intersegmental vessels and complete absence of DLAV at 24 hpf. These defects were more severe in the *Kdm4a*-MO group as compared with the *Kdm4c*-MO group (Figure 5C). None of the *Kdm4a*-MO-injected zebrafish survived by 48 hpf, whereas the *Kdm4c*-MO-injected group survived and showed relatively small impairment of vascular development at this time (Figure 5C). These results are consistent with the mouse results showing that KDM4A participated in the early phase of mESC differentiation to endothelial cells and KDM4C mediated subsequent differentiation to mature endothelial cells. The results also suggest that compensatory mechanisms may be activated in vivo following *Kdm4c* knockdown, which only partially restored vascular development in 48-hpf embryos.

## DISCUSSION

Here we describe the role for histone demethylases KDM4A and KDM4C in the epigenetic regulation of mESC differentiation to endothelial cells. We showed that KDM4A and KDM4C targeted the tri-methyl histone 3 lysine 9 (H3K9me3), a known repressive marker of gene regulation (Black et al., 2012; Berry and Janknecht 2013) at *Flk1* and *VE-cadherin* promoters. Demethylation of H3K9me3 enabled de-repression at the promoters and thereby induced expression of *Flk1* and *VE-cadherin* genes, the lineage markers of endothelial cells (Kohler et al., 2013; Park et al., 2013).

Differentiation of ESCs into the mesodermal progenitors and then into endothelial cells involves the orderly



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expression of developmental transcription factors such as Er71 (Kataoka et al., 2011; Lee et al., 2008), upregulation of the VEGF receptor Flk1 (Kohler et al., 2013; Park et al., 2013), and expression of endothelial cell-specific proteins such as VE-cadherin, which allow the cells to form stable adherens junctions and functional vascular networks (Park et al., 2013). In the present study, we demonstrated that KDM4A and KDM4C were essential epigenetic regulators of mESC differentiation to endothelial cells in mice and also contributed to vascular development in the zebrafish. KDM4A and KDM4C functioned independently through histone demethylation at the *Flk1* and *VE-cadherin* promoters, respectively, to induce the endothelial cell lineage. Interestingly, we found that *Kdm4c* knockdown also suppressed *Flk1* expression despite there being no interaction between KDM4C and histones at the *Flk1* promoter, therefore suggesting that KDM4C targets additional genes regulating *Flk1* expression.

Our study examined the role histone demethylases in the endothelial differentiation of ESCs. Using a screen for 28 histone demethylases in FLK1/VE-cad-double-positive endothelial cells, we observed that mRNA and protein expression of *Kdm4a* and *Kdm4c* were markedly increased during endothelial differentiation. Importantly, the levels of KDM4A and KDM4C increased at day 6 of differentiation to the same extent seen in adult endothelial cells, suggesting that ESC-derived endothelial cells resembled mature endothelial cells in their histone demethylation signature. Although we did not examine human ESC differentiation to endothelial cells, their emergence may similarly involve KDM4A and KDM4C since histone demethylases are evolutionarily conserved (Berry and Janknecht, 2013; Black et al., 2012; Hou and Yu, 2010; Ye et al., 2012).

To buttress the evidence that binding of KDM4A and KDM4C to *Flk1* and *VE-cadherin* promoters reduced trimethylation of H3K9me3, we compared enrichment of H3K9me3 at the *Flk1* and *VE-cadherin* promoters in mESCs

relative to the differentiated endothelial cells. In these studies, we sorted FLK1/VE-cadherin-double-positive cells and determined H3K9me3 methylation. The results clearly demonstrated that methylation was markedly reduced in differentiated cells as compared with undifferentiated cells. In addition, we knocked down *Kdm4a* or *Kdm4c* and determined enrichment of H3K9me3 at the *Flk1* and *VE-cadherin* promoters and observed persistent methylation of the *Flk1* promoter following *Kdm4a* knockdown but not following *Kdm4c* knockdown (data not shown). These results together show the critical role of KDM4A and KDM4C in regulating demethylation at level of *Flk1* and *VE-cadherin* promoters and point to these demethylases as central to differentiation of ESCs to endothelial cells.

ESC differentiation into endothelial cells requires the presence of the growth factors BMP-4, bFGF, and VEGF (Blancas et al., 2008), but it is unknown whether endothelial differentiation induced in this manner requires epigenetic regulation. Our data showed that BMP-4 induced the expression of KDM4A within 24 hr, consistent with the central role of KDM4A upregulation during the critical early phase of endothelial lineage commitment. We also observed that KDM4A bound to histones on Flk1 promoter and that *Flk1* expression increased 24 hr after the induction of KDM4A. Since Flk1 enables mesodermal progenitors to commit to endothelial lineage fate (Park et al., 2013; Yamashita et al., 2000), our results suggest that an orchestrated differentiation program initiated by BMP-4 is responsible for mediating the upregulation of KDM4A. Interestingly, KDM4C expression was not dependent on KDM4A, as evident by the finding that KDM4A knockdown did not affect the expression of KDM4C. We also observed that KDM4C expression was maximal at 72 hr and induced the expression of VE-cadherin on day 5. Thus, it appears that KDM4A initiated the earlier mesodermal differentiation, whereas KDM4C participates in specifying differentiation of cells toward endothelial cells. A model suggested

### Figure 5. KDM4A and KDM4C Are Required for Capillary Tube Formation and Vasculogenesis In Vivo

(A) mESCs were transfected with *scrambled*, *Kdm4a*, or *Kdm4c* siRNA and underwent differentiation protocol. Unsorted differentiated cells were plated on Matrigel, and tube formation was evaluated by phase-contrast microscopy. Knockdown of either *Kdm4a* or *Kdm4c* prevented tube formation. Values are mean  $\pm$  SD ( $n = 3$  independent experiments). Bar graphs of tube lengths in indicated groups ( $p < 0.05$  by Student's  $t$  test).

(B) Unsorted differentiated cells treated as in (A) were suspended in Matrigel containing VEGF and injected subcutaneous into nude mice. Matrigel plugs were harvested on day 7 and assessed by H&E staining. Bar graphs show vessel area. Immunostaining was performed using anti-CD31 and anti-VE-cadherin antibodies. Values are mean  $\pm$  SD ( $n = 3$  independent experiments). Bar graphs show CD31 and VE-cadherin signal intensity ( $p < 0.05$  by Student's  $t$  test).

(C) Targeting either *Kdm4a* or *Kdm4c* with MOs induced significant defects in vasculogenesis in zebrafish embryos, with defects being more prominent in the *Kdm4a*-MO group. In each case, fertilized zebrafish eggs were injected with 2-ng Control-MO, *Kdm4a*-MO, or *Kdm4c*-MO, and the embryos were observed at 24 and 48 hpf. *Kdm4a* morphants displayed only residual inter-somite vessels (ISV) derived from DA and complete lack of DLAV, whereas *Kdm4c*-MO showed a less severe phenotype with reduced number of DLAV at 24 hpf. *Kdm4a* morphants did not survive beyond 36 hpf. In contrast, although the response was delayed in *Kdm4c* morphants, they developed an apparently normal vasculature at 48 hpf similar to control embryos. Bar graphs show blood vessel area and the length of inter-somite vessels at 24 and 48 hpf ( $p < 0.05$  by ANOVA).





by our observations is that once FLK1 is expressed through KDM4A, the newly generated FLK1-positive cells in response to VEGF signaling induce expression of KDM4C to mediate VE-cadherin expression and thereby the endothelial lineage commitment.

Importantly, endothelial cells generated by KDM4A- and KDM4C-induced histone demethylation formed capillaries and vascular networks when implanted in mice. We also observed in the zebrafish model that downregulation of KDM4A and KDM4C orthologs impaired vascular development with the effect of KDM4A being markedly greater than KDM4C, consistent with the role of KDM4A in regulating the early transition to mesoderm and KDM4C in specifying endothelial cell fate. By 48 hr, the zebrafish vasculature also appeared normal in KDM4C knockdown animals, suggesting that in contrast to absence of KDM4A, compensatory mechanisms were activated in the absence of KDM4C capable of generating endothelial cells and restoring vascular development.

Growth factors such as BMP-4 initiate the commitment of pluripotent stem cells to the mesodermal lineage (Winnier et al., 1995). It is not known how a single growth factor such as BMP-4 activates the extensive transcriptional program required for mesodermal commitment. Our finding that overexpression of *Kdm4A* and *Kdm4C* can supplant BMP-4 and bFGF stimulation, respectively, suggests that histone demethylation driven by KDM4A and KDM4C upregulates the gene expression of critical mesodermal and vascular lineage markers, including to FLK1 and VE-cadherin. We did not study whether KDM4A and KDM4C upregulation concomitantly suppresses the expression of pluripotency regulators and pathways mediating commitment to non-mesodermal lineages, but this is an important question that needs to be addressed in future studies. By identifying the epigenetic switches acting downstream of growth factors driving vascular differentiation, the present findings pave the way for novel epigenetic approaches to ensure highly efficient endothelial differentiation while minimizing the need for exogenous recombinant growth factors.

In contrast to the distinct roles of KDM4A and KDM4C in mediating the expression of *Flk1* and *VE-cadherin*, we observed that neither histone demethylase was involved in mediating the expression of the transcription factor Er71. The epigenetic regulation of Er71 expression is not known, but it may involve other epigenetic mechanisms such as histone de-acetylation or DNA methylation (Park et al., 2013).

One limitation of our study is that we cannot exclude additional targets of KDM4A and KDM4C besides FLK1 and VE-cadherin or other epigenetic regulatory mechanisms that may also contribute to endothelial lineage specification. For example, the fact that suppression of KDM4C also affected the levels of the comparatively early differentiation marker Flk1 suggests that KDM4C may

have additional targets beyond VE-cadherin that regulate mesodermal or vascular commitment. Another important limitation is the focus on Flk1 and VE-cadherin. However, we chose to focus on these two markers because Flk1 indicates early mesodermal commitment and VE-cadherin is a highly specific marker of mature endothelial cells, but future studies may be needed to address whether KDM4A and KDM4C also regulate the differentiation into subsets of endothelial cells that do not express VE-cadherin.

In summary, our results elucidate the role of KDM4A and KDM4C in regulating the differentiation of mESCs to endothelial cells. KDM4A initiated differentiation by targeting the *Flk1* promoter, whereas KDM4C specified endothelial cell fate by targeting the *VE-cadherin* promoter. Vasculogenesis in mice and zebrafish was also dependent on KDM4A and KDM4C. As histone demethylation in *Flk1* and *VE-cadherin* promoters by KDM4A and KDM4C acted sequentially to induce the transition of ESCs to endothelial cells, our findings raise the intriguing possibility of activating these histone demethylases to induce vascular endothelial differentiation and de novo vasculogenesis.

## EXPERIMENTAL PROCEDURES

### mESC Culture and Reagents

mESCs (D3) were maintained on gelatin-coated plates in DMEM supplemented with leukemia inhibitory factor (LIF; Chemicon), 15% defined fetal bovine serum (Hyclone), nonessential amino acids (GIBCO), 50- $\mu$ M  $\beta$ -mercaptoethanol (GIBCO), 2 mM L-glutamine (Mediatech), 0.1- $\mu$ g/ml penicillin (Sigma-Aldrich), 0.2- $\mu$ g/ml streptomycin (Sigma-Aldrich), and monothioglycerol (MTG), and ascorbic acid (Sigma-Aldrich).

### mESC Differentiation into Endothelial Cells and siRNA Transfection

mESCs were co-cultured and maintained with fibroblast feeder cells. For differentiation, cells were cultured feeder free for at least two passages and seeded on collagen-coated plates 1 day before initiation of the ESC differentiation. ESC medium was replaced with endothelial cell differentiation medium containing BMP-4 (2 ng/ml), bFGF (10 ng/ml), and VEGF (50 ng/ml), which was changed daily (Kohler et al., 2013). *KDM4a* and *KDM4c* siRNAs (Table S1) and scrambled negative control siRNA were transfected using RNAiMAX Transfection reagent (Invitrogen) following manufacturer's protocol. Cells were transfected twice to achieve the best transfection efficiency.

### Flow Cytometry

Flow cytometric analysis and sorting were performed on a Beckman Coulter CyAn II (Beckman Coulter), as previously described (Kohler et al., 2013). Antibodies purchased from eBiosciences were specific anti-Flk-1 (anti-mouse CD309 [Flk1]-APC, Clone Avas12a1, catalog number 17-5821-81), anti-VE-cadherin (anti-human CD144- PE, Clone 16B1, catalog number 12-1449-82),



and IgG isotype controls. Data were analyzed using the manufacturer's software.

### Protein Separation for Western Blotting

The cultured cells were washed twice with PBS and then lysed with HEPES containing buffer (60 mM KCl, 15 mM NaCl, 15 mM HEPES [pH 7.9], 4 mM MgCl<sub>2</sub>, 0.5-M DTT, 1% Triton X-100, supplemented with Proteinase inhibitor). The sample was spun down at 13,000 rpm for 5 min at 4°C, and the supernatant was collected. Laemml loading buffer was added into samples, and samples were heated to 95°C for 5 min. Proteins were separated on 9% polyacrylamide gels and transferred to polyvinyl difluoride (PVDF) membranes. Non-specific binding sites were blocked with 5% milk in TBS-T buffer. Polyclonal anti-KDM4A (catalog number ab105953, Abcam) and KDM4C (catalog number ab93694, Abcam) antibodies and rabbit anti-H3K9me3 antibody (catalog number ab8898, Abcam) were used at 1:1,000 dilution. The membrane was incubated with the primary antibody for 2 hr at room temperature or overnight in a cold room. Goat anti-rabbit or rabbit anti-mouse secondary antibody from BioRad was used at 1:10,000 dilution and incubated with the membrane for 1 hr at room temperature. Enhanced chemiluminescence (ECL) was used for signal detection (GE Healthcare).

### qRT-PCR

Total RNA was isolated from cells using the RNeasy plus extraction kit (QIAGEN) and 1-μg total RNA was used for reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed on a 7500 Fast Real Time PCR System (Applied Biosystems) using SYBR Green PCR mix (Applied Biosystems). Gene specific (Table S2) and promoter-enhancer specific oligonucleotides were synthesized by Integrated DNA Technologies.

### Chromatin Immunoprecipitation-qPCR Assay

To analyze the interaction of KDM4A, KDM4C, and histone H3 (tri methyl K9) with *Flk1* and *VE-cadherin* promoter segments, we used chromatin immunoprecipitation (ChIP) assay. The assays were performed according to the manufacturer's protocol (Abcam). ChIP entailed the use of specific antibodies and controls such as IgGs and beads alone. To minimize background binding, samples were diluted. In brief, cells were cross-linked with 0.75% formaldehyde (as the links it forms are reversible) at room temperature for 10 min, stopped by glycine (0.125 M) at room temperature for 5 min, and washed twice with PBS solution. The cells were lysed with FA buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, protease Inhibitors) and then subjected to sonication (20 s for ten times) on ice. Polyclonal anti-KDM4A, anti-KDM4C, anti-histone H3 (tri methyl K9) antibodies were purchased from Abcam. Chromatin binding to IgG-ChIP was considered background. The primers used in ChIP-qPCR were as follows: *Er71* promoter, forward, 5'-AGCTTCAAACAAAACCTCTTC-3' and reverse, 5'-TCTTAAGGGGGGAATTTATG-3'; *Er71\_10kb*, forward, 5'-CTGG TACCTGTCCTGCTTC-3' and reverse, 5'-AATGCAAACCTTACAA GTACA-3'; *Flk1* promoter, forward, 5'-CTGTGTTTCCTTAGATCG CG-3' and reverse, 5'-CCTGCACCTCGCGCTGGGCA-3'; *Flk1\_10kb*,

forward, 5'-AGGACTTTTAAATGAATGGA-3' and reverse, 5'-TCCAG GCTGTGACTCACAGG-3'; *VE-cadherin* promoter, forward, 5'-GGA CATCAGTATTCCAGGTA-3' and reverse, 5'-GGAACGAGTGGCTCA CTCTA-3'; and *VE-cadherin\_10kb*, forward, 5'-TGACCTGCTTGAG TTCCAGT-3' and reverse, 5'-TTTGCATCTACATTCATGAG-3'.

For ChIP-qPCR, we used 5% of starting chromatin as input, and the raw Ct value were recorded. Since the starting input was 5%, dilution factor (DF) of 20 or 4.321 cycles (i.e., log<sub>2</sub> of 20) was subtracted from the raw Ct value (i.e., adjusted to 100% input). ChIP-qPCR was run in triplicates and Ct values recorded. Data relative to input were normalized for both background levels and input chromatin used for ChIP. Thus, percentage input (y axis) was calculated from 20\*2<sup>-(adjusted input - Ct)</sup> and plotted as bar diagram for IgG and anti-HDM4A and anti-KDM4C antibodies (x axis).

### Tube Formation and Matrigel Plug Assay

Six-well plates were coated with growth factor-reduced Matrigel (BD Biosciences) according manufacturer's instructions. After Matrigel polymerization, 2-million differentiated cells were seeded; tube-like structures were assessed after 16 hr using phase contrast microscopy (Olympus).

All mouse experiments were carried out according to protocols approved by the Animal Care and Use Committee (University of Illinois at Chicago). Two-million mESCs were transfected with siRNAs targeting either *KDM4a*, *KDM4c*, or both and underwent the differentiation protocol for 6 days. The differentiated cells (2 million) for each condition were mixed with Matrigel containing VEGF and injected into the abdominal subcutaneous skin of anesthetized nude mice for additional 7 days. All Matrigel plugs were carefully harvested, fixed with formalin solution, paraffin embedded, sectioned, and subjected to H&E or immunostaining staining using anti-CD31 and anti-VE-cadherin antibodies. The differentiated cells used in the Matrigel studies were not sorted for endothelial markers.

### Zebrafish Experiments

Zebrafish experiments were carried out according to protocols approved by Animal Care and Use Committee (University of Illinois at Chicago). The breeding colony of zebrafish (*Danio rerio*, *Sheer* strain, Dr. H. Tomasiewicz, University of Wisconsin) was maintained at standard conditions (28.5°C [pH 7.5]) and staged according to morphology (Kimmel et al., 1995). The transgenic line Tg(*flil*:EGFP)<sup>y1</sup> (Zebrafish International Resource Center) was sequentially outcrossed to the *Sheer* fish to obtain optically transparent fish harboring *flil*:EGFP transgene. Homozygous *Sheer* fish harboring *flil*:EGFP were used to obtain stronger intensity of the fluorescent signal and a better acquisition of long time-lapse series. *Kdm4A* and *Kdm4C* orthologs are well conserved in vertebrates. The sequences of zebrafish and human proteins shared 47% and 50% identity, respectively. For *Kdm4a* and *Kdm4c* knockdown, the following custom translation-blocking MOs were designed and purchased, along with standard control MOs, from Gene Tools: 5'-GGCTGACCGAGTCCCTTAACACTGAC-3' (KDMab\_ZF MO), 5'-CACTTGCCCCAACACCTGCCATCCT-3' (KDMC\_ZF MO), as well as a random control oligo 25-N and a GFP-positive control. MOs reconstituted in nuclease-free water were dissolved in nuclease-free water, and their concentrations were determined with NanoDrop.



Fertilized eggs were collected after timed mating of adult zebrafish and injected at one- to four-cell stage with the indicated amounts of MO into the yolk immediately below the blastomeres using a Pneumatic PicoPump (World Precision Instruments). MOs were injected using 0.1% phenol red in 0.5- to 2-nl Danieau buffer, and the volume of the injected drop was estimated with a micrometer scale. The efficacy of a translation-blocking MO was first assessed by targeting GFP in a strain expressing the GFP gene under the control of the *fli1* promoter. The efficacy of MOs was assessed by RT-PCR with superscript one-step RT-PCR system (Invitrogen) and using as template total RNA from either control, *Kdm4A* or *Kdm4A* MO injected embryos. RNA was extracted with Trizol from 24- and 48-hr pooled embryos and treated with DNase. Embryos were injected with 2-, 5-, or 10-ng MOs in a 1- to 2-nl volume. Injection of control MOs (Random control oligo and GFP positive control at 10 ng) verified that injections at this concentration did not induce MO-mediated toxicity, and the same or lower MO concentrations were utilized in all experiments. For imaging zebrafish vessels, live embryos were dechorionated manually with a micro tweezers, anesthetized using 0.016% tricaine (Sigma), and mounted in 1% low melting agarose. Zebrafish embryos were visualized with stereo zoom microscope (Discovery V8, Zeiss). For fluorescent microscopy, a GFP filter was used. For visualization of vasculature formation, zebrafish embryos (24–48 hpf) were captured by AxioCam-HR Zeiss Digital Camera. ImageJ (<http://rsb.info.nih.gov/ij/>) was used for image processing. Images were analyzed with Metamorph software (Molecular Devices).

### Statistical Analysis

The statistical data were analyzed using the GraphPad Prism software. Results are shown as means  $\pm$  SD unless specified otherwise. Comparisons between any two groups were performed using the two-tailed unpaired Student's *t* test. Comparisons of multiple values among more than two groups were performed using two-way ANOVA with post hoc Bonferroni's correction. Differences were considered statistically significant when  $p < 0.05$ .

### SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2015.05.016>.

### AUTHOR CONTRIBUTIONS

A.B.M., J.R., L.W., and Y.A.K. designed the experiments. L.W. and S.R. carried out the experiments. L.W., K.K.W., S.R., K.T., Y.A.K., J.R., and A.B.M. analyzed the data and wrote the manuscript.

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