JMJD8 Regulates Angiogenic Sprouting and Cellular Metabolism by Interacting With Pyruvate Kinase M2 in Endothelial Cells

Jes-Niels Boeckel, Anja Derlet, Simone F. Glaser, Annika Luczak, Tina Lucas, Andreas W. Heumüller, Marcus Krüger, Christoph M. Zehendner, David Kaluza, Anuradha Doddaballapur, Kisho Ohtani, Karine Treguer, Stefanie Dimmeler

Objective—Junmonji C (JmjC) domain–containing proteins modify histone and nonhistone proteins thereby controlling cellular functions. However, the role of JmjC proteins in angiogenesis is largely unknown. Here, we characterize the expression of JmjC domain–containing proteins after inducing endothelial differentiation of murine embryonic stem cells and study the function of JmjC domain–only proteins in endothelial cell (EC) functions.

Approach and Results—We identified a large number of JmjC domain–containing proteins regulated by endothelial differentiation of murine embryonic stem cells. Among the family of JmjC domain–only proteins, Jmjd8 was significantly upregulated on endothelial differentiation. Knockdown of Jmjd8 in ECs significantly decreased in vitro network formation and sprouting in the spheroid assay. JMJD8 is exclusively detectable in the cytoplasm, excluding a function as a histone-modifying enzyme. Mass spectrometry analysis revealed JMJD8-interacting proteins with known functions in cellular metabolism like pyruvate kinase M2. Accordingly, knockdown of pyruvate kinase M2 in human umbilical vein ECs decreased endothelial sprouting in the spheroid assay. Knockdown of JMJD8 caused a reduction of EC metabolism as measured by Seahorse Bioscience extracellular flux analysis. Conversely, overexpression of JMJD8 enhanced cellular oxygen consumption rate of ECs, reflecting an increased mitochondrial respiration.

Conclusions—Jmjd8 is upregulated during endothelial differentiation and regulates endothelial sprouting and metabolism by interacting with pyruvate kinase M2. (Arterioscler Thromb Vasc Biol. 2016;36:1425-1433. DOI: 10.1161/ATVBAHA.116.307695.)

Key Words: cell differentiation ■ cell plasticity ■ chromatin ■ endothelial cells ■ metabolism

Blood vessels are lined with endothelial cells (ECs), which regulate the exchange of nutrients and oxygen within tissues. The outgrowth of new vessels from pre-existing ones, a process named angiogenesis, is essential in organ growth and repair but can also contribute to pathological processes. Angiogenesis is therefore tightly regulated by the demand of the surrounding tissue for oxygen and metabolites.1

A class of proteins characterized by the existence of a Jumonji C (JmjC) domain has been described to function as histone demethylases, which alters chromatin accessibility and thereby regulates gene transcription.2 The enzymatic activity of the protein family depends on the JmjC domain, whereas the presence of additional protein domains leads to their assignment into subgroups.3 For example, C2H2/CSH2 or AT-hook motifs are implicated in the binding and interaction with DNA, whereas plant homeodomain fingers are required for substrate-specific binding.4,5 In addition, some JmjC domain–containing proteins are capable of regulating nonhistone protein functions by hydroxylation or demethylation.6,7 It is already documented that both types of JmjC protein functions are important for the regulation of stem cell plasticity and differentiation.8-11 Recent studies have highlighted the importance of JmjC proteins in the regulation of angiogenesis. Specifically, JHDM1B (KDM2B) mediates in vitro endothelial network formation via FGF2 signaling;12 JMJD6 is required for angiogenic sprouting through controlling the splicing of vascular endothelial growth factor (VEGF) receptor 1,12 whereas JARID1B (KDM5B) epigenetically represses the proangiogenic genes HOXA5 and CCL14, respectively, thereby controlling angiogenesis.13,15 Hypoxia, a condition of reduced oxygen availability, is not only a...
strong stimulus for the outgrowth of new vessels but induces the expression of several JmjC proteins, such as JHDM2A (KDM3A), JMJD2B (KDM4B), JMJD2C (KDM4C), and JMJD2D (KDM4D).16–18 Additionally, the catalytic activity of the JmjC domain depends on the cofactors Fe(II), 2-oxoglutarate, and oxygen.19 Thereby changes in oxygen levels and availability of metabolites profoundly affect the enzymatic activity of JmjC domain proteins and therefore control transcriptional and post-transcriptional downstream effector pathways.20 In this regard, the regulation of transcriptional activity of the transcriptional master regulator under oxygen tension, the hypoxia-inducible factor 1α (HIF1α) by the JmjC domain-containing protein FIH1 (HIF1AN), is most prominent. The transcription factor HIF1α is hydroxylated by FIH1 via an oxygen-consuming reaction and thereby is transcriptionally inhibited under normal oxygen conditions.6,7,21

However, the regulation of JmjC proteins in EC differentiation and the function of the JmjC domain–only proteins namely, No66 (C14orf169), Jmjd4, and Jmjd8 are unknown.

Here, we show that endothelial differentiation of embryonic stem cells (ESC) significantly regulates a variety of JmjC proteins including Jmjd8. Among the JmjC domain–only proteins, JMJD8 was the only family member that controls angiogenesis. Interestingly, JMJD8 binds to PKM2 and regulates angiogenic sprouting and cellular metabolism of ECs.

**Materials and Methods**

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

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**Nonstandard Abbreviations and Acronyms**

- aa: amino acids
- EC: endothelial cell
- ESC: embryonic stem cell
- JmjC: Jumonji C
- JMJD8: JumonjiC domain–containing protein 8
- PKM2: pyruvate kinase M2

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**Figure 1.** Screening for Jumonji C (JmjC) domain–containing protein expression in endothelial differentiation of mouse embryonic stem cells (ESCs). **A**, Mouse ESCs were cultured in suspension. On day 2 of differentiation, 30 μg/mL vascular endothelial growth factor was added to the medium. Endothelial differentiation was stopped at days 4 and 7, and total RNA was isolated. Expression of the stemness gene Oct4 during endothelial differentiation of murine ESCs. Expression of endothelial markers **(B)** Pecam and **(C)** VE-cadherin in endothelial differentiation of murine ESCs. **D**, Expression of 30 JmjC domain–containing proteins was assessed using quantitative reverse transcription polymerase chain reaction. mRNA expression was normalized to Gapdh expression using the 2−ΔΔCt method. Inset: overview of the JmjC family subgroups. Reprinted from Klose et al3 with permission of the publisher. Copyright ©2006, Nature Publishing Group. Data are shown as mean±SEM, fold change day 0. n=3. Statistical significance was determined using Student t test; *P<0.05.
Results

Regulation of JmjC Domain–Containing Protein Expression by Endothelial Differentiation of ESCs

Endothelial differentiation was induced as previously described and validated by the increased expression of endothelial marker genes and concomitant downregulation of pluripotency markers such as Oct4 (Pou5f1; Figure 1A–1C). Among the 30 annotated JmjC domain–containing proteins, 11 proteins were significantly upregulated, whereas only 2 JmjC domain proteins were significantly decreased at day 7 of endothelial differentiation (Figure 1D). To determine, whether these JmjC proteins are specific for the endothelial lineage, we induced cardiac differentiation of ESC. We found a significant increase of 8 JmjC proteins, whereas 8 JmjC proteins were downregulated at day 8 of cardiac differentiation (Figure I A–IC in the online-only Data Supplement). At day 7 of endothelial differentiation, expression of Jmjd5 (Kdm8), Fih1 (Hif1an), Jmjd4, Jmjd6 (Ptdsr), and Jmjd8 were significantly higher compared with day 0, whereas Hspbap1 was the only JmjC domain–only protein whose expression was significantly decreased (Figure 1D). In contrast, at day 8 of cardiac differentiation, Mina53 (Mina), Jmjd7, and Hspbap1 were significantly decreased, and only Jmjd8 showed a significantly higher expression level when compared with day 0 (Figure 1A in the online-only Data Supplement).

Jmjd8 Acts as a Proangiogenic JmjC Protein In Vitro and In Vivo

To date, only the function of JMJD6 and FIH1 were evaluated in ECs, but a previous study identified JMJD8, NO66 (C14orf169), and JMJD6 the 3 highest expressed JmjC domain–only proteins in human umbilical vein ECs. Therefore, 3 JmjC domain–only proteins Jmjd8, NO66, and Jmjd4 were selected for further functional analysis. To investigate the function of JMJD4, JMJD8, and NO66 in ECs, we used RNA interference (siRNA)–mediated knockdown (Figure 2A). Only the knockdown of JMJD8, but not JMJD4

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Figure 2. Function of selected JmjC domain–only proteins in endothelial cells. A, siRNA-mediated knockdown of NO66, JMJD4, and JMJD8 was confirmed using quantitative polymerase chain reaction (qPCR; n=3). Values normalized to RPLP0 (ribosomal protein, large, P0) mRNA. B, Network formation on matrigel after siNO66, siJMJD4, and siJMJD8 treatment (n=3). Representative pictures are shown in top. *P<0.05 vs Scr-1; #P<0.05 vs Scr-2. C, Human umbilical vein endothelial cells (HUVECs) were either cultured on plastic dishes or on matrigel. Incubated for 24 h followed by analysis of mRNA expression of NO66, JMJD4, and JMJD8 using qPCR (n=3). Data are shown as fold incubation on plastic dish. D, Expression of vascular endothelial growth factor (VEGF) and VEGFR1 mRNA after culturing HUVECs on plastic dishes and on matrigel (n=3). Data is shown as fold incubation on plastic dish. E, Representative pictures of HUVECs cultured on plastic dishes or on matrigel for 24 h. Data are mean±SEM. Statistical significance was determined with Student t test; *P<0.05.
or NO66, significantly reduced endothelial network formation in an in vitro Matrigel assay (Figure 2B). Moreover, we tested the response of ECs in 2D matrigel culture, which increased vascular endothelial growth factor and vascular endothelial growth factor receptor 1 expression (Figure 2C–2E). Cultivation of ECs on Matrigel induced a significant upregulation of JMJD8 and downregulation of NO66 expression, whereas JMJD4 expression was not changed (Figure 2C).

To confirm the angiogenic phenotype of JMJD8 knockdown in ECs, we used 2 additional siRNAs targeting JMJD8 (Figure 3A). Knockdown by all 3 siRNAs targeting JMJD8 significantly reduced network formation in a Matrigel assay (Figure 3B) and endothelial sprouting in a spheroid assay (Figure 3C). On the contrary, survival, cell cycle, and transmigration capacity of ECs were not affected by JMJD8 knockdown (Figure IIA–IIC in the online-only Data Supplement). Because JMJD8 was increased during endothelial differentiation, we further investigated whether JMJD8 is involved in regulation of endothelial marker gene expression during ESC differentiation. Indeed, silencing of JMJD8 in ESC prevented vascular endothelial growth factor–induced EC differentiation as indicated by significantly reduced expression of the endothelial marker gene VE-cadherin (Figure 3D).

Having demonstrated that silencing of JMJD8 reduced angiogenic sprouting and EC differentiation in vitro, we examined the in vivo function of JMJD8 by generating JMJD8−/− mice (Figure IIIA and IIIB in the online-only Data Supplement). JMJD8−/− showed no obvious phenotype, suggesting that embryonic vascular development was not affected. However, the number of capillaries in muscle tissue was significantly reduced (Figure 3E and 3F).

**JMJD8 Has an Extranuclear Localization and Binds to PKM2 in ECs**

JmjC domain–containing proteins have been predominantly found to be localized in the nucleus, associated with

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**Figure 3.** Function of JMJD8 in endothelial cells. 
A, siRNA-mediated knockdown of JMJD8 using 3 siRNAs was confirmed using qPCR and immunoblot (inlet). mRNA expression was normalized to RPLP0 (ribosomal protein, large, P0) expression using the 2−ΔΔCt method. B, Network formation on matrigel after siJMJD8 treatment using 3 siRNAs. Representative pictures are shown in top. C, Sprouting of spheroids after siJmjd8 treatment using 3 siRNAs. Representative spheroids are shown in upper panel. D, VE-cadherin expression in mES (murine embryonic stem cells) after shRNA-mediated knockdown of Jmjd8 after 0, 7, and 10 days of endothelial differentiation. E, Capillaries in skeletal muscle of Jmjd8−/− and wild-type mice. Wheat germ agglutinin (WGA) was used to stain plasma membranes and lectin to stain vessels. F, Quantification of capillaries in skeletal muscle of Jmjd8−/− and wild-type mice (N=4 each group). Statistical significance was determined with Student test; *P<0.05 vs Scr-1; #P<0.05 vs Scr-2.
chromatin. In contrast, we found JMJD8 localized extranuclear in ECs, as demonstrated by immunofluorescence staining of flag-tagged JMJD8 (Figure 4A). To gain further insight into the function of JMJD8, mass spectrometry analysis after immunoprecipitation of JMJD8 was performed. The proteins interacting with JMJD8 included proteins of cellular metabolism such as pyruvate kinase M2 (PKM2) and phosphofructokinase 1 (Figure 4B). Indeed, gene ontology enrichment analysis revealed that proteins related to metabolic processes account for substantial portion of proteins that bind to JMJD8 (Figure 4C). The protein interactions of the 4 selected proteins phosphofructokinase 1, JAK1, CANX, and PKM2 with JMJD8 were confirmed by coimmunoprecipitation in human umbilical vein ECs. Because overexpression of proteins is not efficient in ECs, we confirmed the interaction of PKM2 with JMJD8 in HEK293 (human embryonic kidney 293) cells with coimmunoprecipitation targeting Flag-tagged JMJD8. Lysates of cells transfected with untagged green fluorescent protein (GFP) were used as controls incubated with anti-flag-coupled magnetic beads. E, Cellular localization of JMJD8 and PKM2 was analyzed using immunoblot in cytoplasmic and nuclear separated protein lysates. Topo I and Gapdh were used as controls for nuclear (N) and cytoplasmic (C) proteins respectively. F, Flag-tagged JMJD8 and V5-tagged PKM2 were transfected into HEK293 cells using RNAiMAX. V5-PKM2 was immunoprecipitated using V5-tagged magnetic beads, and binding to JMJD8 was analyzed by immunoblotting.

**Figure 4.** Extranuclear localization of JMJD8 in endothelial cells and interaction with pyruvate kinase M2 (PKM2). A, Flag-tagged JMJD8 was transfected into human umbilical vein endothelial cells (HUVECs) and visualized using Cy3-coupled antiflag antibodies. B, Analysis of Jmjd8 staining light intensity along a single line drawn across a cell. C, Gene ontology analysis on biological processes involved in the top 50 proteins bound to Jmjd8 identified by MS. D, Proteins binding to JMJD8 were validated in HUVEC and HEK293 (human embryonic kidney 293) cells with coimmunoprecipitation targeting Flag-tagged JMJD8. Lysates of cells transfected with untagged green fluorescent protein (GFP) were used as controls incubated with anti-flag-coupled magnetic beads.

**Figure 4.** Extranuclear localization of JMJD8 in endothelial cells and interaction with pyruvate kinase M2 (PKM2). A, Flag-tagged JMJD8 was transfected into human umbilical vein endothelial cells (HUVECs) and visualized using Cy3-coupled antiflag antibodies. B, Analysis of Jmjd8 staining light intensity along a single line drawn across a cell. C, Gene ontology analysis on biological processes involved in the top 50 proteins bound to Jmjd8 identified by MS. D, Proteins binding to JMJD8 were validated in HUVEC and HEK293 (human embryonic kidney 293) cells with coimmunoprecipitation targeting Flag-tagged JMJD8. Lysates of cells transfected with untagged green fluorescent protein (GFP) were used as controls incubated with anti-flag-coupled magnetic beads.
cells (Figure 4D). Consistently, we found JMJD8 outside the nucleus together with PKM2 after separation of cytoplasmic and nuclear proteins using immunoblot (Figure 4E). The interaction of JMJD8 with PKM2 was further confirmed by communoprecipitation using PKM2-V5 and Jmjd8-FLAG overexpression constructs (Figure 4F). To identify the interaction site, we generated deletion constructs of JMJD8 and coexpressed the constructs with V5-tagged PKM2. The N-terminal deletion of amino acids (aa) 1 to 124 (Δ3) and the deletion of aa 63 to 214 (Δ4) did not prevent binding of PKM2 (Figure IVA and IVB in the online-only Data Supplement). However, N-terminal deletion of aa 1 to 200 of JMJD8 (Δ2 construct) did prevent binding to PKM2 (Figure IVA and IVB in the online-only Data Supplement), suggesting that PKM2 interaction with JMJD8 occurs in the region between aa 63 and 124. Interestingly, PKM2 has recently been shown to promote angiogenesis in ECs,24,25 suggesting that cytoplasmic JMJD8 could regulate angiogenesis by controlling metabolic

Figure 5. JMJD8 regulates metabolism in endothelial cells. A, siRNA-mediated knockdown of PKM2 using 3 siRNAs was confirmed using qPCR, mRNA expression normalized to RPLP0 (ribosomal protein, large, P0) expression using the 2−ΔΔCt method. Inset: PKM2 knockdown on protein level, α-Tubulin was used as a loading control. B, Sprouting of spheroids after siPKM2 treatment using 3 siRNAs (n=3). *P<0.05 vs Scr-1; #P<0.05 vs Scr-2. Inlet: Representative spheroids. Bar=200 μm. C, Extracellular acidification rate (ECAR) profile demonstrating glycolytic function in (D) shCONTROL- and shJMJD8-transduced cells and (E) mock- and Jmjd8-transduced cells. Vertical lines indicate the time of addition of glucose, oligomycin, and 2-deoxy-D-glucose (n=4). F, Oxygen consumption rate (OCR) profile demonstrating mitochondrial respiration function in (G) shCONTROL- and shJMJD8-transduced Human umbilical vein endothelial cells (HUVECs) and (H) MOCK- and JMJD8-transduced (HUVECs). Vertical lines indicate the time of addition of oligomycin, carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone, antimycin A, and rotenone (n=4). Statistical significance was determined with Student t test comparing MOCK with JMJD8 cells and shCONTROL with shJMJD8 cells; n=4. *P<0.05.
functions of EC, a mechanism which was recently shown to play a key role in regulating angiogenesis.26,27

**JMJD8 Regulates Metabolism in ECs**

To investigate if the function of JMJD8 in endothelial sprouting is mediated by the interaction with PKM2, PKM2 was silenced with 3 siRNAs (Figure 5A). All 3 small interfering RNAs directed against PKM2 significantly reduced endothelial sprouting in the spheroid assays (Figure 5B). To investigate a potential influence of JMJD8 on metabolism in ECs, Seahorse Flux analysis was performed. The lactate production reflecting the glycolytic cell function was determined by the measuring extracellular acidification rate after shJMJD8 and JMJD8 transduction (Figure VA and VB in the online-only Data Supplement). The shRNA-mediated knockdown of Jmjd8 resulted in a decreased basal glycolysis, glycolysis, and oligomycin-induced maximal glycolysis, but only maximal glycolytic capacity was significantly reduced (Figure 5C and 5D). Additionally, cells isolated from Jmjd8−/− mice showed a significantly reduced glycolysis (Figure VIA and VIB in the online-only Data Supplement). Conversely, overexpression of wild-type JMJD8 enhanced the maximal glycolytic function of ECs, but these data failed to achieve significance (Figure 5E).

We next analyzed the cellular oxygen consumption rate reflecting the mitochondrial respiration rate in cells. A significant decrease of the maximal respiration capacity induced by carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone in ECs was observed after shJMJD8 compared with shRNA control transduced cells, whereas overexpression of JMJD8 significantly increased the maximal oxygen consumption rate compared with mock-transduced cells (Figure 5E). These data suggest that JMJD8 controls glycolysis and respiration of ECs.

To finally test whether JMJD8 controls the enzymatic activity of PKM2, we measured pyruvate synthesis. Whereas silencing of JMJD8 significantly reduced pyruvate synthesis, overexpression significantly enhanced the formation of pyruvate in ECs (Figure VII in the online-only Data Supplement), suggesting that JMJD8 controls generation of pyruvate.

**Discussion**

When reducing the expression of NO66, JMJD4, and JMJD8 by siRNA-mediated silencing, only knockdown of JMJD8 impaired sprouting angiogenesis. It has recently been shown that several JmjC domain–containing proteins such as JMJD6 or JARID1B are essential for sprouting angiogenesis by modifying histone as well as nonhistone proteins.13–15 The JmjC domain–only subgroup was implicated in control of the hypoxic response6,7 and further was shown to regulate angiogenesis.13 The lysine demethylase No66 regulates osteoblast differentiation,28 whereas no functional role are described for JMJD4 and JMJD8 to date. It has recently been shown that many JmjC proteins regulate cellular proliferation and therefore are involved in the malignancy of cancer progression. In this regard, JMJD2b (Kdm4b) and Mina53 (Mina) regulate cellular proliferation in physiological states like spermatogenesis and in pathophysiological conditions like cancer.29,30 Although Jmjd8 did not affect proliferation of ECs, these findings do not exclude proliferation regulatory effects in other cell types. Most JmjC proteins analyzed to date have been found in the nucleus controlling histone states, only few JmjC proteins were reported to be outside of the nucleus like JMJD8.31,32 Analyzing proteins bound to JMJD8 revealed enzymes that have been shown to be important regulators of metabolism like PKM2 and phosphofructokinase. Metabolism controls angiogenesis. In this regard, endothelial migration and proliferation, as essential steps in angiogenesis, were recently shown to be regulated by metabolism. The outgrowth of new vessels is in particular dependent on glucose whose availability is controlled by glycolysis-regulating enzymes like PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3).26,27,33,34 Specifically PKM2, which is the rate-limiting enzyme of glycolysis, controlling the conversion of phosphoenolpyruvate to pyruvate, has recently been shown to promote angiogenesis in ECs.24,25 Indeed, we found that knockdown of PKM2 resulting in impaired angiogenic sprouting and JMJD8 stimulates the formation of pyruvate in ECs. The precise mechanism of how JMJD8 regulates PKM2 is currently unknown. However, we found that the region between aa 63 and 124 of JMJD8 is important for binding to PKM2. A subsequent analysis for this region using the SMART tool (http://smart.embl-heidelberg.de) revealed 2 low-complexity regions (aa72-88/aa92-106) as only protein motifs in this region. Indeed, low-complexity region motifs have been shown to modulate protein–protein interactions.35 Interestingly, JMJD5, a closely related family member which was previously shown to negatively regulate osteoclastogenesis and human circadian systems, was recently identified to regulate the nuclear translocation of PKM2 and thereby reprogramming HIF-1α–mediated glucose metabolism.31,36,37 This regulation is consistent with our observation that JMJD8 is enhancing the metabolic activity of ECs by increasing the oxygen consumption and the maximal glycolytic function. Of note, we also showed a modulation of maximal oxygen consumption by JMJD8, indicating that mitochondrial function may be affected beyond the level of pyruvate formation. Indeed, silencing of JMJD8 reduced the mitochondrial content in ECs (Figure VIII A in the online-only Data Supplement), suggesting an additional layer of modulation. JmjC domain–containing proteins have important roles in regulating stem cell plasticity and differentiation.8–11 Here, we show that endothelial and cardiac differentiation of ESCs significantly affect a variety of JmjC proteins. Most JmjC genes were upregulated during differentiation into the endothelial lineage, whereas cardiac differentiation reduced most JmjC proteins on mRNA level. Indeed, we found a significant reduction in cardiac differentiation of Jmjda (Kdm3a) and Jmjdc2 (Kdm4c), which have been shown to regulate self-renewal in ESCs.38 We further identified Jmjd8, which is the only gene significantly upregulated from the JmjC domain–only subgroup, implying a potential role in cardiovascular development. Mice lacking Jmjd8 proteins like Jmjd6 or the name-giving enzyme of the JmjC protein family Jarid2 (JMJ) have a disturbed cardiac development already in the embryonic stage, which potentially is the cause for pre- or postnatal lethality.39–41 However, Jmjd8−/− mice are viable and do not show an obvious phenotype, suggesting that Jmjd8 is not essential for embryonic vascular development. Nevertheless,
we found a significant reduction in glycolytic capacity in cells isolated from Jmjd8 knockout mice, supporting the results of our in vitro studies. Moreover, Jmjd8-deficient mice revealed a reduced number of capillaries in skeletal muscles in adulthood. To dissect the direct influence of Jmjd8 on the endothelium in adulthood, an endothelium-specific knockout would be necessary, which is currently unavailable.

Although previous studies reported a role of JmJC domain–containing proteins in EC biology, this study demonstrates that a JmJC domain–containing protein interacts with EC metabolism. Indeed, JmJC proteins have been proposed as metabolic sensors through their dependence on metabolites like 2-oxoglutarate or oxygen. Together, our data implicate a critical role of Jmjd8 in the metabolic control of angiogenesis.

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Disclosures
None.

References


**Highlights**

- Jmjd8 was significantly upregulated during endothelial differentiation of embryonic stem cells.
- Knockdown of Jmjd8 in endothelial cells significantly decreased in vitro network formation and sprouting in the spheroid assay.
- Mass spectrometry analysis revealed Jmjd8-interacting proteins with known functions in cellular metabolism like pyruvate kinase M2.
- Knockdown of Jmjd8 decreased pyruvate kinase M2 enzymatic activity, whereas overexpression of Jmjd8 led to increased pyruvate kinase M2 enzymatic activity.
- Knockdown of Jmjd8 caused a reduction of endothelial cell metabolism as measured. Conversely, overexpression of Jmjd8 enhanced cellular oxygen consumption rate of endothelial cells, reflecting an increased mitochondrial respiration.
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**Supplemental Methods**

**JMJD8 Regulates Angiogenic Sprouting and Cellular Metabolism by Interacting with Pyruvate Kinase M2 in Endothelial Cells**

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**Cell culture**

Endothelial cells were purchased from Lonza and cultured as previously described.$^{1}$

**mESCs culture**

Mouse ESCs (R1) were cultured on feeder cells (Primary Mouse Embryo Fibroblasts, Not Mytomycin C Treated, Strain CF1, Millipore) and gelatin-coated dishes in DMEM with 4500 Glucose, Glutamax and Pyruvat (Gibco, 31966-021) supplemented with 15% FBS, 150 \( \mu \text{M} \) monothioglycerol (Sigma Aldrich, M1753-500ml), 0.1 mM nonessential amino acids (Gibco, M11-003), penicillin-streptomycin (Roche), and 1000 U/ml leukemia inhibitory factor (rmLIF LIF12344253 Recombinant Mouse Leukemia Inhibitory Factor (rm LIF) 10\( \mu \text{g} \) ImmunoTools). The medium was changed every day and the ES-cells were splitted every 2-3 days.

**Endothelial differentiation of murine ES cells**

1.25\( \times 10^5 \) ES cells were seeded in 10 ml of mESC maintenance medium without LIF. The cells were cultured for 2 days on non-adhesive 100 mm dishes. After two days 30 ng/ml of hVEGF121 (PeproTech) was added. The cells were further cultivated and were harvested after 4 and 7 days of differentiation for analysis. The undifferentiated ES cells were collected at day 0 as described previously. $^{2}$

**Cardiac differentiation of murine ES cells**

Differentiation of mESCs into embryoid bodies (EBs) was performed using the suspension method as previously published.$^{3}$ Differentiation in hanging drops was achieved by first removing the feeder cells to obtain an enriched ES cell population. Next mESC (25.000 cells/ml) in differentiation medium (mESC Culture media without LIF) were spotted to the lid of a dish and cultured as hanging drops to allow aggregate formation the so-called embryoid body. The EBs were then (day 2) collected and cultured in suspensions for the next 3 days. At day 5 of differentiation protocol individual EBs were transferred to gelatine coated 12 wells plates (10 EBs/well). Between day 7-13 of differentiation protocol the monitoring of the EBs beating areas was done. The differentiated ES cells for mRNA extraction were collected at day 2, 5 and 8. The undifferentiated ES cells were collected at day 0 as previously described.
RNA Analysis

Total RNA was isolated using QIAzol (79306; Qiagen) and miRNeasy-kit (217004; Qiagen) with additional DNase I (79254; Qiagen) digestion according to the manufacturer’s protocol. 1 μg of RNA from each sample was reverse-transcribed into random hexamer primed single-strand cDNA (10 min at 25 °C, 15 min at 42 °C, 5 min at 99 °C) by MMLV Reverse Transcriptase (N8080018; Invitrogen). For qPCR cDNA was amplified using fast Fast Sybr Green Mastermix (Life Technologies,4385612) on a VIAA7 qPCR System (Life Technologies). Expression level of mRNAs were normalized to the housekeeping gene RPLP0 using the 2^ΔΔCt method.

PCR Primers:

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siRNA Transfection

3 x 10^5 HUVECs were seeded in a 60 mm cell culture dish. After 24 h, siRNA was transfected at a final concentration of 60 nM using GeneTransII transfection reagent.

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<th>siRNA</th>
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### Proliferation assay

Cell cycle analysis was performed using the FITC BrdU Flow Kit (557891, BD Pharmigen) and processed as previously described\(^4\).

### Cloning of Plasmids

Jmjd8 was amplified from HUVEC cDNA using primers directed against the protein coding sequence by KOD Polymerase and cloned into pFLAG-CMV™−6a expression vector with N-Terminal Flag-tag (E2275;Sigma Aldrich) using In-Fusion® HD Cloning Plus kit (638910, Takara Clontech) according to manufacturer’s instruction.

PKM2 was amplified using primers directed against the protein coding sequence by KOD Polymerase and cloned into pCMV6 protein expression vector using In-Fusion® HD Cloning Plus kit (638910, Takara Clontech) at the multiple cloning site according to manufacturer’s instruction. The reverse Primer used for amplification of PKM2 was extended with the sequence coding for V5-tag.

pLenti-Jmjd8-Flag plasmid was cloned using pFlagJmjd8-6a as a template using primers directed against the protein coding sequence and the flag-tag by KOD Polymerase with TOPO cloning kit (Thermo fisher) into pLenti6 according to the manufacturer’s protocol.

### Transfection of Plasmids in HUVECs

For Transfection of Jmjd8-Flag or empty mock plasmid 10 µg plasmid per 1x10\(^6\) HUVECs were used. HUVECs were electroporated using the Neon® Transfection System (Thermo Fisher Scientific) according to the manufacturer’s protocol.

### Transfection of Plasmids in HEK293

For Transfection of Jmjd8-Flag, PKM-V5 or empty mock plasmid 1x10\(^6\) HEK-293 cells were seeded in a 60 mm cell culture dish. After 24 h, cells were transfected with 3 µg of respective plasmids with the RNAiMax transfection reagent following the manufacturer’s instructions.

### Transduction of Lentiviral particles

Long term overexpression of Jmjd8\(^5\) and shRNA mediated silencing of Jmjd8 was done as previously described\(^6\). shRNA particles were obtained from Sigma Aldrich and are listed in the shRNA-table.

### Co-Immunoprecipitation

HUVEC or HEK were lysed in lysis buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA; 0.5% NP-40) (Chromotek) with addition of PMSF and proteinase

---

**Table: shRNA Targeting sequence**

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<th>shRNA</th>
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inhibitor cocktail (PIC) (Sigma Aldrich; P1860) for Input (I) = 10% of total lysate was used. First 25 µl Anti-FLAG® M2 Magnetic Beads (Sigma Aldrich; M8823) or 25 µl Anti-V5-tag mAb-Magnetic beads (MBL;M167-9) were equilibrated in washing buffer (10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA) (Chromotek). Lysates were incubated with beads over night at 4°C with constant rotation. Next, beads were washed 3 times with washing buffer and proteins were eluted with 30 µl (200 mM glycine pH 2.5) (Chromotek) for 1min at RT. Lysates from cells transfected with untagged GFP were used as a control and treated the same as the target samples.

Mass spectrometry analysis

Eluted proteins from the Co-immunoprecipitations were separated via SDS-PAGE (4-12% Novex-gels, Invitrogen, D-Darmstadt) and stained with colloidal Coomassie. The whole lane of the control and Jmjd8-flag immunoprecipitations were excised and subjected to in gel-digestion with trypsin. Proteins were measured by mass spectrometry with an LTQ-Orbitrap XL as previously described and label free protein quantification was performed with the MaxQuant Software tool8.

Immunofluorescence

HUVECs were transfected with JMJD8-Flag. After 24 h cells were fixed in 4% PFA and permeabilized using TritonX followed by incubation with monoclonal ANTI-FLAG® M2-Cy3™ antibody (Sigma Aldrich; A9594) for 1 h. After washing with TBST Dapi was used for nuclear staining.

Immunoblot

Eluted proteins from the Co-immunoprecipitations were separated via SDS-PAGE and transferred to nitrocellulose membranes. The Membranes were incubated with the following antibodies: PKM2 (3198, Cell signaling), HSP27 (2402, Cell signaling), CANX (2433, Cell signaling), PFK (5412, Cell signaling), JAK1 (3332, Cell signaling) or αFlag-HRP (A8592, Sigma Aldrich).

MTT Viability Assay

Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay as described previously 1.

Endothelial network-formation assay

Network formation assay were performed as previously described1. After 24 h, siRNAs targeting JMJD8 or PKM2 were transfected and another 24 h later transfected cells were seeded onto matrigel in a 6-well. After 24 h network-formation was analyzed by cumulative length in 5 random fields.

Spheroid sprouting assays

Spheroid assay were performed as previously described1. In vitro angiogenesis was quantified by measuring the cumulative length of all sprouts of each spheroid or the maximal distance of the migrated cells using digital imaging analysis software (AxioVision Rel. 4.8, Carl Zeiss). 10 spheroids were analyzed for each experiment.

Measurement of cellular metabolism/Extracellular flux analyses

Cellular bioenergetics was measured in real time by the Seahorse Bioscience Extracellular Flux Analyzer XF96 (North Billerica, MA, USA) with a modified protocol for HUVECs9,10. HUVECs were seeded overnight at 4x10^4 cells per well in 200 µL culture medium on XF96
Polystyrene Cell Culture Microplates (Seahorse Bioscience, 101085-004). 1 h before measurement the cells were washed with basal DMEM 0% and cultured for 1 h at 37 °C without CO₂ in Mito and Glycolysis Stress Assay Media according to the manufacturer’s protocol (Seahorse Bioscience). The oxygen consumption rate (OCR) was determined as a surrogate for mitochondrial respiration using the Mito Stress Test Kit (Seahorse Bioscience, 102308-400), extracellular acidification rate (ECAR) was determined as surrogate for glycolytic function using XF Glycolysis Stress Test Kit (Seahorse Bioscience, 102194-100). Metabolic profiles were determined by injection of following cell metabolism activators and inhibitors: Oligomycin (3 µM), Carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP) (1 µM), and Antimycin (1.5 µM) and rotenone (3 µM) for OCR; glucose (10 mM), Oligomycin (3 µM) and 2-DG (100 mM) for ECAR. Both, OCR and ECAR, were measured over 4 min periods followed by 2 min mixing in each cycle, 5 cycles per injection, 20 cycles in total. Each measurement was averaged from at least triplicate wells.

**Jmjd8 knockout mouse**

The Jmjd8 mouse strain used for this research project was created from ES cell clone 13985A-A5, generated by Regeneron Pharmaceuticals, Inc. and obtained from the KOMP Repository (www.komp.org). Methods used to create the Velocigene targeted alleles have been published. 11

**References**


8. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein...


Supplemental Material

JMJD8 Regulates Angiogenic Sprouting and Cellular Metabolism by Interacting with Pyruvate Kinase M2 in Endothelial Cells

Jes-Niels Boeckel1,4, Anja Derlet1,4, Simone F. Glaser1,4, Annika Luczak1, Tina Lucas1,4, Andreas W. Heumüller1, Marcus Krüger2, Christoph M. Zehendner1,3,4, David Kalua1, Anuradha Doddaballapur1,4, Kisho Ohtani1, Karine Treguer1, Stefanie Dimmeler1,4

Supplemental Material
Supplemental figure I. Expression of JmjC domain-containing protein expression in cardiac differentiation of murine ES. A, Feeder cells were removed and mESC (25,000 cells/ml) transferred into medium without LIF, then embryoid bodies were formed using the hanging drop method. EBs were cultured on gelatine. The differentiated ES cells were collected at day 2, 5 and 8. The undifferentiated ES cells were collected at day 0. Total RNA was isolated and expression of 30 JmjC domain-containing proteins was assessed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). mRNA expression was normalized to Gapdh expression using the 2^(-ΔΔCt) method. Inset: Overview of the JmjC family subgroups, adapted from^3 B, Expression of the stemness gene Oct4 during cardiac differentiation of murine ES. C, Expression of cardiac marker gene Troponin (Tnnt2) in cardiac differentiation of murine ES. Data is shown as mean±SEM, fold change day 0. n=3, Statistical significance was determined using Student's t-test; *P<0.05
**Supplemental figure II.** A, Viability analysis using MTT assay after siJMJD8 treatment using 3 siRNAs. B, Cell cycle analysis by FACS after siJMJD8 treatment using 3 siRNAs. C, Number of cells invaded in a matrigel after siJMJD8 treatment using 3 siRNAs. All experiments are n≥3.
Supplemental figure III. A, Genotyping of Jmjd8 knockout mouse using PCR. B, Expression of Jmjd8 in wild type, Jmjd8+/− and Jmjd8−/− mice in lung, skeletal muscle and heart tissues (n=3).
Supplemental figure IV. A, Deletion constructs of human Jmjd8, blue indicates the location of the JmjC domain. B, Immunoblot showing the expression of the Jmjd8 deletion constructs in HEK cells using a FLAG tag for detection. C, Interaction of the Jmjd8 deletion constructs to PKM2 after immunoprecipitation of V5-tagged PKM2.
Supplemental figure V. A, Expression of JMJD8 after shControl and shJMJD8 treatment (n=3) B, or mock- and Jmjd8- transduced cells (n=3) *P<0.01 Data is shown as mean±SEM, (n≥3), Statistical significance was determined using Student's t-test; *P<0.05
**Supplemental figure VI.**

**A.** Extracellular acidification rate (ECAR) profile demonstrating glycolytic function in wild type and Jmjd8⁻/⁻ mice. **B.** Vertical lines indicate the time of addition of glucose, oligomycin, and 2-deoxy-D-glucose. (n=5). Statistical significance was determined with Student's t-test comparing wild type and Jmjd8⁻/⁻ mice. *P<0.05
Supplemental figure VII. A, Pyruvate synthesis rate after shJmd8 and B, JMD8 overexpression in endothelial cells (N=3). Statistical significance was determined with Student’s t-test; *P<0.05
Supplemental figure VIII. A, Mitochondrial content was determined after siJMJD8 using Mitotracker staining with FACS analysis. (n=6). Data is shown as mean±SEM. n=3, Statistical significance was determined using Student’s t-test; *P<0.05
Boeckel et al. *JMJD8 Regulates Angiogenic Sprouting and Cellular Metabolism by Interacting with Pyruvate Kinase M2 in Endothelial Cells*