Jmjd3 Controls Mesodermal and Cardiovascular Differentiation of Embryonic Stem Cells

Kisho Ohtani¹, Cong Zhao¹, Gergana Dobreva², Yosif Manavski¹, Britta Kluge¹, Thomas Braun², Michael A. Rieger³, Andreas M. Zeiher⁴ and Stefanie Dimmeler¹

¹Institute of Cardiovascular Regeneration, Centre for Molecular Medicine, University of Frankfurt, 60590 Frankfurt, Germany; ²Max-Planck-Institute for Heart and Lung Research, 61231 Bad Nauheim, Ludwigstr. 43, Germany; ³LOEWE Center for Cell and Gene Therapy, Department of Hematology/Oncology, Internal Medicine II, and; ⁴Department of Cardiology, Internal Medicine III, University of Frankfurt, 60590 Frankfurt, Germany.

K.O. and C.Z. contributed equally to this study.

Running title: Jmjd3 and Differentiation

Subject codes:
[139] Developmental biology

Address correspondence to:
Dr. Stefanie Dimmeler
Institute of Cardiovascular Regeneration
Centre for Molecular Medicine
University of Frankfurt
Theodor-Stern-Kai 7
60590 Frankfurt
Germany
Phone: +49-69-6301-7440
Fax: +49-69-6301-7113,
dimmeler@em.uni-frankfurt.de

This manuscript was sent to Benoit Bruneau, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

In June 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.67 days.
ABSTRACT

Rational: The developmental role of the H3K27 demethylases Jmjd3, especially its epigenetic regulation at target genes in response to upstream developmental signaling is unclear.

Objective: To determine the role of Jmjd3 during mesoderm and cardiovascular lineage commitment.

Methods and Results: Ablation of Jmjd3 in mouse embryonic stem cells does not affect the maintenance of pluripotency and self-renewal, but compromised mesoderm and subsequent endothelial and cardiac differentiation. Jmjd3 reduces H3K27me3 marks at the Brachyury promoter and facilitates the recruitment of β-catenin, which is critical for Wnt signal-induced mesoderm differentiation.

Conclusion: These data demonstrate that Jmjd3 is required for mesoderm differentiation and cardiovascular lineage commitment.

Keywords: Jmjd3, mesoderm, Brachyury, embryonic stem cell, epigenetics, signaling pathways

Nonstandard Abbreviations and Acronyms:
αMHC α-myosin heavy chain
ChIP chromatin immunoprecipitation
C-terminal carboxyl-terminal
DAPI 4',6-diamidino-2-phenylindole
ESC embryonic stem cell
EB embryoid body
GO gene ontology
HEK293 cell human embryonic kidney 293 cell
HOX homeobox
LEF1 lymphoid enhancer binding factor 1
LIF leukemia inhibitory factor
Pecam platelet endothelial cell adhesion molecule
RSV rous sarcoma virus
VE-cadherin vascular endothelial-cadherin

INTRODUCTION

Posttranslational modifications of histone proteins represent essential epigenetic control mechanisms that can either allow or repress gene expression. Trimethylation of H3K27 is mediated by Polycomb group proteins and represses gene expression. The JmjC domain-containing proteins UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome) and Jmjd3 (jumonji-domain-containing protein 3, Kdm6b) act as demethylases to remove the repressive H3K27me3 marks but also exhibit additional demethylase-independent functions. Jmjd3 is induced and participates in HOX gene expression during development, neuronal differentiation, inflammation, and recent data suggest that Jmjd3 inhibits reprogramming by inducing cellular senescence. Since previous studies suggest that H3K27me3 regulates endothelial gene expression in adult pro-angiogenic cells, we addressed the function of Jmjd3 in cardiovascular lineage differentiation of ESCs.
METHODS

A detailed description of the experimental procedure is provided as Supplemental Information.

Cell culture.
Mouse ESCs were cultured and maintained on mitotically inactivated mouse embryonic fibroblasts. Differentiation of ESCs was induced without LIF in static or rotary suspension condition. ES cell differentiation into mesoderm was performed with a well-described OP9 coculture system\(^{15, 16}\). For cardiac differentiation, EBs were derived using the hanging drop method\(^{17}\). For endothelial differentiation, EBs were formed and cultured in medium supplemented with cytokine cocktail\(^{18}\).

Plasmid construction and stable transfection.
The full-length Jmjd3, the mutants and Brachyury were cloned into pEF1 vector (Invitrogen). The linearized plasmids were transfected in Jmjd3\(^{-/-}\) ESCs using the Amaxa nucleofection system (Lonza).

Chromatin immunoprecipitation (ChIP).
ChIP assays were performed as previously described\(^{14}\). Sheared chromatin was immunoprecipitated with antibodies as indicated. The purified chromatin was used in quantitative real-time PCR (Applied Biosystems).

RESULTS

Jmjd3 knockout ESCs were generated by two rounds of gene targeting (Online Figure IA/B). We obtained 7 Jmjd3\(^{-/-}\) ESC clones, which lacked Jmjd3 mRNA and protein expression. All of the clones showed slightly increased global H3K27me3, but the expression of pluripotency genes, the morphology, the growth kinetic and survival was indistinguishable from wild type (wt) ESCs (Figure 1A-C, Online Figure IC-F). No significant changes of repressive H3K27me3 marks at the promoters of pluripotency genes were detected in Jmjd3\(^{-/-}\) compared to wt ESCs (Online Figure IH). When spontaneous differentiation was induced by LIF withdrawal, Jmjd3 expression increased in wt ESCs (Figure 1D). EBs derived from Jmjd3\(^{-/-}\) ESCs were slightly smaller in size compared with wt EBs (Figure 1E). mRNA expression profiling of Jmjd3\(^{-/-}\) and wt ESC clones at day 4 after induction of differentiation showed a distinct expression pattern of lineage specific genes (Online Figure IIA). Gene ontology (GO) functional analyses revealed a significant repression of genes that are involved in mesoderm development (Figure 1F, Online Figure IIB). Moreover, repressed gene sets in Jmjd3\(^{-/-}\) EBs were shown to be related to cardiac and vascular development, consistent with impaired mesoderm differentiation (Figure 1F, Online Figure IIB).

Validation of the microarray results showed a similar reduction of pluripotency gene expression after LIF withdrawal in Jmjd3\(^{-/-}\) compared to wt ESCs (Figure 1G). However, depletion of Jmjd3 substantially compromised the induction of mesodermal genes (Figure 1G). Especially, the pan-mesoderm marker Brachyury and the early mesoendoderm marker Mxi1 were profoundly increased at day 4 of differentiation in wt ESCs, but not in Jmjd3\(^{-/-}\) ESCs (Figure 1G). Moreover, the mesoendodermal marker Eomes, and endodermal markers such as Sox17 and FoxA2 were significantly suppressed, which is consistent with a very recent study showing that Jmjd3 is required for endoderm differentiation\(^ {19}\). Ectodermal markers were not significantly changed in Jmjd3\(^{-/-}\) ESCs when using the spontaneous differentiation protocol (Figure 1G).

Since Jmjd3\(^{-/-}\) ESCs showed a prominent inhibition of mesodermal markers after LIF withdrawal, we next questioned whether this phenotype can also be observed when directing differentiation of mesoderm by using two different protocols. Consistent with our findings, Jmjd3\(^{-/-}\) ESCs showed a reduced
expression of mesodermal marker genes when using the protocol for mesoderm differentiation described by Gadue et al 20 (data not shown). Moreover, mesoderm differentiation was significantly suppressed when Jmjd3−/− ESCs were cultured on OP9 stromal cells which support mesodermal differentiation 21 (Figure 2A). Whereas wt ESCs showed the typical time-dependent increase in Brachyury+ cells, Jmjd3−/− ESCs generated significantly less Brachyury+ mesodermal cells (Figure 2B). Moreover, FACS analysis revealed that Flk1+ VE-cadherin mesodermal cells were generated in wt ESCs but were reduced when Jmjd3−/− ESCs were used (Figure 2C). Interestingly, the formation of VE-cadherin Flk+ cells was also significantly reduced by 96±1 % and 88±3 % in the two Jmjd3−/− ESC clones compared to wt ESCs (p<0.01), prompting us to further explore the role of Jmjd3 in vascular differentiation.

Endothelial differentiation was induced by a cytokine cocktail 18 and was associated with a significant up-regulation of Jmjd3 expression (Online Figure IIIA). Jmjd3−/− ESCs showed a marked reduction of endothelial differentiation as evidenced by significantly reduced mRNA levels of the endothelial marker VE-cadherin and Tie2 (Figure 3A). The formation of endothelial marker expressing vascular structures after induction of endothelial differentiation was abolished in Jmjd3−/− ESCs (Figure 3B, Online Figure IIIB). The impaired endothelial differentiation of Jmjd3−/− cells was partially rescued by the overexpression of Brachyury (Online Figure IIIC/D) suggesting that the inhibition of mesoderm formation at least in part contributes to the impaired endothelial commitment.

Since genes involved in heart development and morphogenesis were significantly down-regulated in Jmjd3−/− ESCs upon differentiation (Figure 1F, Figure II), we additionally determined the capacity of Jmjd3−/− ESCs to generate cardiomyocytes by inducing cardiac differentiation 17. Expression of cardiac progenitor cell markers Mesp1 and Pdgfra was inhibited in Jmjd3−/− ESCs compared to wt ESCs (Figure 3C). Moreover, after plating on gelatin-coated dishes, the Jmjd3−/− ESCs showed an impaired formation of EBs and only 20% of EBs were contracting (Figure 3D). Consistently, expression of the cardiac transcription factor Mef2c, the marker of working myocardium Nppa, and cardiac structural proteins TnT2 and αMHC were down-regulated in Jmjd3−/− ESCs (Figure 3E/F, Online Figure IIIE).

Next, we addressed whether the impaired mesoderm differentiation observed in Jmjd3−/− ESCs might be mediated by an increase of repressive H3K27me3 marks at the promoters of developmental regulators. Of the various promoters studied, only Brachyury and Mixl1 showed a significant augmentation of H3K27me3 marks in Jmjd3−/− ESCs upon differentiation (Figure 4A, Online Figure IVA). Consistently, the recruitment of RNA Polymerase II to the transcription start sites of the promoters of Brachyury and Mixl1 was also significantly reduced (Online Figure IVC). In addition, Jmjd3 deficiency repressed Polymerase II recruitment to the Flk1 and Mesp1 promoter but the inactivation of these promoters was not associated with changes in H3K27me3 marks (Online Figure IVA/C). These data were confirmed using protocols that induce mesoderm differentiation by addition of Wnt3a (data not shown) 20. Under these conditions, Jmjd3−/− ESCs showed a 1.81±0.23 fold (p<0.05) enrichment of H3K27me3 marks at the Brachyury promoter compared to wt ESCs.

To determine whether the demethylase activity of Jmjd3 controls Brachyury expression by reducing repressive H3K27me3 marks during differentiation, we overexpressed full length Jmjd3, the C-terminal part including the JmjC-domain (eJmjd3: amino acids 1141-1641) and a C-terminal mutant construct, which includes a point mutation (eJmjd3H1388A) to inactivate demethylase activity. Overexpression of full length Jmjd3 and the C-terminal part of Jmjd3 in Jmjd3−/− ESCs partially rescued the expression of Brachyury upon differentiation (Figure 4B/C). However, the inactive C-terminal part of Jmjd3 failed to rescue the impaired Brachyury expression in Jmjd3−/− ESCs (Figure 4C) suggesting that the demethylase activity of Jmjd3 is required for the activation of the Brachyury promoter.

Since canonical Wnt signaling regulates the expression of Brachyury during development 22, 23 and Wnt/β-catenin dependent genes were suppressed in Jmjd3−/− EBs compared to wt EBs (Online Figure
we further explored whether Jmjd3 might interact with β-catenin signaling. Indeed, β-catenin recruitment to the Brachyury promoter was significantly suppressed in Jmjd3−/− ESCs (Figure 4D) and was rescued by Jmjd3 overexpression (Figure 4E). Similar results were obtained when using the protocol for direct mesoderm differentiation described by Gadue et al 20 (data not shown). To determine whether Jmjd3 might interact with β-catenin, we performed co-immunoprecipitation studies and showed that Jmjd3 interacts with β-catenin in HEK293 cells and differentiated EBs (Figure 4F, Online Figure VI). To assess a direct effect of Jmjd3 on β-catenin responsive promoter activity we used a luciferase reporter assay. Co-expression LEF1 and the constitutive active form of β-catenin harboring a nuclear localization signal resulted in the activation of LEF1-luciferase reporter activity in wt ESCs, but this transcriptional activation was markedly impaired in Jmjd3−/− ESCs (Figure 4G).

DISCUSSION

The data of the present study demonstrate that deletion of Jmjd3 in ESCs does not affect self-renewal, but significantly impairs the formation of mesoderm upon induction of differentiation. The findings that Jmjd3 is not required for ESC maintenance is consistent with the dispensability of the polycomb complex and the related demethylase UTX for self-renewal 1. The requirement of Jmjd3 for mesoderm differentiation was shown in spontaneous differentiation as well as when more specifically inducing mesoderm differentiation by the OP9 co-culture system or under serum-free conditions followed by Wnt3a stimulation. Jmjd3 deficiency profoundly suppressed the expression of Brachury, which is essential for mesoderm differentiation. In the absence of Jmjd3, repressive H3K27me3 marks at the Brachyury promoter are significantly increased and the recruitment of β-catenin, which is a prerequisite for Wnt-induced mesoderm differentiation, is impaired. In addition, Jmjd3 is interacting with β-catenin and is contributing to β-catenin-dependent promoter activation. This is consistent with the recent findings that co-factors can form a complex with β-catenin/LEF1 at Tcf/Lef1 binding sites at β-catenin-dependent promoters and synerglize with canonical Wnt signaling 24. Interestingly, a demethylase-independent regulation of β-catenin-dependent gene expression was recently described for UTX 25. However, our data provide evidence that Brachyury expression in Jmjd3−/− ESCs is only rescued by catalytically active Jmjd3, which has maintained the demethylase activity. Based on these findings, we propose a model in which Jmjd3 is recruited to the Brachury promoter to remove repressive H3K27me3 marks and upon Wnt stimulation additionally promotes β-catenin-dependent promoter activation (Figure 4H). Such a model is similar to the recently described function of Jmjd3 in endoderm differentiation, whereby Jmjd3 associates with Tbx3 and is recruited to the poised promoter of Eomes, to mediate chromatin remodeling allowing subsequent induction of endoderm differentiation induced by activin A 19. The present study additionally demonstrates that Jmjd3 contributes to endothelial and cardiac differentiation. Particularly, endothelial differentiation was profoundly impaired, a finding that is consistent with previous findings in adult progenitor cells showing a high H3K27me3 at endothelial genes 14. The relatively modest inhibition of cardiomyocyte differentiation in Jmjd3−/− ESCs may be in part explained by a compensatory effect of UTX which was shown to regulate cardiac development 26. Together, our study provides first evidence for the regulation of β-catenin-dependent Wnt target genes by Jmjd3 during differentiation of ESCs.

However, the in vivo relevance of the findings is still unclear. The Jmjd3−/− mice that we have generated out of the ESCs used in the present study showed embryonic lethality before E6.5, suggesting a crucial role of Jmjd3 in early embryonic development (Online Figure VII). This is consistent with the requirement of Jmjd3 for blastocyst development 26, but is in contrast to other studies which showed that Jmjd3-deficient mice are perinatal lethal 12, 27. The discrepancy between the phenotypes of Jmjd3−/− mice is unclear but might be related to the different strategies that were used to generate the Jmjd3−/− mice. For example, the study of Burgold et al 27 resulted only in a half maximal reduction of Jmjd3 expression,
whereas Jmjd3 expression is fully absent in our mice. The early embryonic lethality of our mice, however, precluded the analysis of the effect of Jmjd3 on mesoderm development in vivo. Further studies involving conditional deletion of Jmjd3 using Sox2- and Brachyury-Cre lines are required.

ACKNOWLEDGEMENTS
We thank Marion Muhly-Reinholz, Susanne Heydt, Susanne Kreutzer, and Claudia Jourdan for excellent technical assistance.

SOURCES OF FUNDING
S.D. and M.A.R. were supported by the German Research Foundation (SFB834), the LOEWE Center for Cell and Gene Therapy Frankfurt (IIIL4-518/17.004 (2010)).

DISCLOSURES
None.

REFERENCES
1. Young RA. Control of the embryonic stem cell state. Cell. 2011;144:940-954


16. Fraser ST, Ogawa M, Nishikawa S. Embryonic stem cell differentiation as a model to study hematopoietic and endothelial cell development. *Methods Mol Biol.* 2002;185:71-81


**FIGURE LEGENDS**

**Figure 1.** Aberrant differentiation of Jmjd3<sup>−/−</sup> ESCs. (A) qPCR analysis of Jmjd3 in WT and Jmjd3<sup>−/−</sup> ESCs. (B) Western blot analysis of Jmjd3 and Histone marks in WT and Jmjd3<sup>−/−</sup> ESCs. Histone H3 is used as a loading control. Quantification is shown in the right panel (N=3-5). (C) Upper panel: Morphology of WT and Jmjd3<sup>−/−</sup> ESCs on feeder cells. Lower panel: Alkaline phosphatase staining of undifferentiated WT and Jmjd3<sup>−/−</sup> ESCs. (D) Western blot analysis of Jmjd3 and Oct4 in WT ESCs during differentiation. α-Tubulin is used as a loading control. (E) Bright field image of EBs at day 5. Scale bar indicates 200 µm. (F) Gene ontology analysis for more than 2-fold repressed genes in Jmjd3<sup>−/−</sup> ESCs compared to WT ESCs 4 days after differentiation. The most highly represented categories are presented with ontology terms on the y-axis and p-values for the significance of enrichment are shown on the x-axis. (G) Gene expression changes of pluripotency and lineage-specific markers in WT and Jmjd3<sup>−/−</sup> ESCs after spontaneous differentiation by LIF withdrawal. N=4.

**Figure 2.** Jmjd3<sup>−/−</sup> ESCs show an impaired ability to differentiate into mesoderm. (A) Schematic illustration of the experimental protocol. Differentiation of ESCs (WT and two Jmjd3<sup>−/−</sup> ESCs clones) on OP9 feeder cells was analyzed. (B) Left Panel: Representative FACS plots showing Brachyury expression of ESC-derived cells. Right Panel: Quantification of FACS analyses. N=3. (C) Left Panel: Representative FACS plots showing Flk1 and VE-cadherin expression on ESC-derived cells. Right Panel: Quantification of FACS analyses in Flk1<sup>+</sup> cells. N=3.

**Figure 3.** Jmjd3 is required for ESCs differentiation to the endothelial and cardiac lineage. (A) mRNA expression of endothelial markers at day 7 of endothelial differentiation. N=3. (B) Pecam-1 staining of WT and Jmjd3<sup>−/−</sup> ESCs at day 8 of endothelial differentiation. Phalloidin is used to stain F-Actin. Nuclei are stained with DAPI (blue). Scale bar indicates 20 µm. (C) Gene Expression of cardiac progenitor markers at day 3 of cardiac differentiation. (D) Number of beating embryoid bodies (EBs) at day 10 of cardiac differentiation. N=8. (E) Gene expression of cardiac markers at day 7 of cardiac differentiation. N=6. (F) αMHC staining of WT and Jmjd3<sup>−/−</sup> ESCs at day 9 of cardiac differentiation. Nuclei are stained with Hoechst (blue). Scale bar indicates 20 µm. *P<0.05, **P<0.01, ***P<0.001.

**Figure 4.** (A) ChIP against H3K27me3 at day 3.5 during differentiation of WT and Jmjd3<sup>−/−</sup> ESCs. ChIP enrichments are normalized to histone H3 density and are represented as fold change relative to WT. N=4. (B) Left panel: Western blot analysis of H3K27me3 in Jmjd3<sup>−/−</sup> ESCs after overexpression of Flag-tagged Jmjd3. Right panel: Brachyury expression at day 4 of differentiation in Jmjd3<sup>−/−</sup> ESCs after overexpression of Jmjd3. N=3 (C) Left panel: Western blot analysis of H3K27me3 in Jmjd3<sup>−/−</sup> ESCs after overexpression of the Flag-tagged C-terminal catalytically active or inactive domain of Jmjd3. Right panel: Brachyury expression at day 4 of differentiation in Jmjd3<sup>−/−</sup> ESCs after overexpression of catalytically active or inactive Jmjd3 C-terminal domain. N=3 (D) ChIP against β-catenin in WT and Jmjd3<sup>−/−</sup> EBs at day 3.5 of differentiation. ChIP enrichments are calculated as percentage of input signal and data are presented as fold changes compared with WT. N=3. (E) ChIP assay against β-catenin at day 3.5 differentiation in Jmjd3<sup>−/−</sup> EBs after overexpression of Jmjd3. ChIP enrichments are presented as fold changes compared to Jmjd3<sup>−/−</sup> EBs transfected with control vector. N=3 (F) Anti-Flag immunoprecipitates of nuclear extracts from Jmjd3-Flag overexpressing HEK 293 cells were analyzed by anti-β-catenin immunoblotting. Five percent of total lysates were loaded as input. (G) Luciferase reporter assay in WT and Jmjd3<sup>−/−</sup> ESCs transfected with LEF1 luciferase reporter construct, LEF1, β-catenin harboring a nuclear localization signal, and a RSV-β-galactosidase expression construct. The luciferase levels were normalized for the β-galactosidase activity of the cotransfected RSV-β-galactosidase reporter and represented as fold change relative to the luciferase level from WT ESCs transfected with the same constructs. N=3. (H) Summary of results. *P<0.05, **P<0.01, ***P<0.001.
Novelty and Significance

What Is Known?

- Cell fate decisions require well-controlled changes in gene expression that are tightly controlled by epigenetic modulators.
- The posttranscriptional modifications of histone proteins epigenetically regulate gene expression.
- Trimethylation of lysine 27 at histone K3 (H3K27me3) silences gene expression and can be reversed by the demethylase Jmjd3.

What New Information Does This Article Contribute?

- The histone demethylase Jmjd3 is required for mesoderm differentiation and cardiovascular lineage commitment of mouse embryonic stem cells.
- This effect is partially mediated by a silencing of the mesodermal regulator brachyury.
- Ablation of Jmjd3 further reduces β-catenin recruitment to the brachyury promoter, which interferes with Wnt signaling that is required for proper mesoderm differentiation.

The differentiation of stem cells to specific lineages requires a well-defined modulation of gene expression programs, which is often controlled by epigenetic mechanisms. Although several epigenetically active enzymes and complexes have been described, the function of the histone demethylase Jmjd3 for cardiovascular lineage commitment was unknown. Using mouse embryonic stem cells as a model, we now show that the demethylase Jmjd3 is required for mesoderm differentiation as well as for the differentiation of embryonic stem cells to the vascular and cardiac lineage. We further identified the mechanism and showed that ablation of Jmjd3 resulted in a silencing of the Brachyury promoter that is associated with an increase in H3K27me3 marks. In addition, Jmjd3 was shown to facilitate the recruitment of β-catenin to the Brachyury promoter, which contributes to the Wnt-dependent activation of mesoderm differentiation. Together these data describe a novel epigenetic mechanism that controls cell fate decision.
Figure 1. Aberrant differentiation of Jmjd3-/- ESCs.
(A) qPCR analysis of Jmjd3 in WT and Jmjd3-/- ESCs. (B) Western blot analysis of Jmjd3 and Histone marks in WT and Jmjd3-/- ESCs. Histone H3 is used as a loading control. Quantification is shown in the right panel (N=3-5). (C) Upper panel: Morphology of WT and Jmjd3-/- ESCs on feeder cells. Lower panel: Alkaline phosphatase staining of undifferentiated WT and Jmjd3-/- ESCs. (D) Western blot analysis of Jmjd3 and Oct4 in WT ESCs during differentiation. a-Tubulin is used as a loading control. (E) Bright field image of EBs at day 5. Scale bar indicates 200μm. (F) Gene ontology analysis for more than 2-fold repressed genes in Jmjd3-/- ESCs compared to WT ESCs 4 days after differentiation. The most highly represented categories are presented with ontology terms on the y-axis and p-values for the significance of enrichment are shown on the x-axis. (G) Gene expression changes of pluripotency and lineage-specific markers in WT and Jmjd3-/- ESCs after spontaneous differentiation by LIF withdrawal. N=4.
Figure 2. Jmjd3-/- ESCs show an impaired ability to differentiate into mesoderm. Schematic illustration of the experimental protocol. Differentiation of ESCs (WT and two Jmjd3-/- ESCs clones) on OP9 feeder cells was analyzed. (B) Left Panel: Representative FACS plots showing Brachyury expression of ESC-derived cells. Right Panel: Quantification of FACS analyses. N=3. (C) Left Panel: Representative FACS plots showing Flk1 and VE-cadherin expression on ESC-derived cells. Right Panel: Quantification of FACS analyses in Flk1+ cells. N=3.
Figure 3. Jmjd3 is required for ESCs differentiation to the endothelial and cardiac lineage. (A) mRNA expression of endothelial markers at day 7 of endothelial differentiation. N=3. (B) Pecam-1 staining of WT and Jmjd3-/- ESCs at day 8 of endothelial differentiation. Phalloidin is used to stain F-Actin. Nuclei are stained with DAPI (blue). Scale bar indicates 20 μm. (C) Gene Expression of cardiac progenitor markers at day 3 of cardiac differentiation. (D) Number of beating embryoid bodies (EBs) at day 10 of cardiac differentiation. N=8. (E) Gene expression of cardiac markers at day 7 of cardiac differentiation. N=6. (F) αMHC staining of WT and Jmjd3-/- ESCs at day 9 of cardiac differentiation. Nuclei are stained with Hoechst (blue). Scale bar indicates 20 μm. *P<0.05, **P<0.01, ***P<0.001.
Figure 4. (A) ChIP against H3K27me3 at day 3.5 during differentiation of WT and Jmjd3-/- ESCs. ChIP enrichments are normalized to Histone H3 density and are represented as fold change relative to WT. N=4. (B) Left panel: Western blot analysis of H3K27me3 in Jmjd3-/- ESCs after overexpression of Flag-tagged Jmjd3. Right panel: Brachyury expression at day 4 of differentiation in Jmjd3-/- ESCs after overexpression of Jmjd3. N=3 (C) Left panel: Western blot analysis of H3K27me3 in Jmjd3-/- ESCs after overexpression of the Flag-tagged C-terminal catalytically active or inactive domain of Jmjd3. Right panel: Brachyury expression at day 4 of differentiation in Jmjd3-/- ESCs after overexpression of catalytically active or inactive Jmjd3 C-terminal domain. N=3 (D) ChIP against β-catenin in WT and Jmjd3-/- EBs at day 3.5 of differentiation. ChIP enrichments are calculated as percentage of input signal and data are presented as fold changes compared with WT. N=3. (E) ChIP assay against β-catenin at day 3.5 differentiation in Jmjd3-/- EBs after overexpression of Jmjd3. ChIP enrichments are presented as fold changes compared to Jmjd3-/- EBs transfected with control vector. N=3 (F) Anti-Flag immunoprecipitates of nuclear extracts from Jmjd3-Flag overexpressing HEK 293 cells were analyzed by anti-β-catenin immunoblotting. Five percent of total lysates were loaded as input. (G) Luciferase reporter assay in WT and Jmjd3-/- ESCs transfected with LEF1 luciferase reporter construct, LEF1, β-catenin harboring a nuclear localization signal, and a RSV-β-galactosidase expression construct. The luciferase levels were normalized for the β-galactosidase activity of the cotransfected RSV-β-galactosidase reporter and represented as fold change relative to the luciferase level from WT ESCs transfected with the same constructs. N=3. (H) Summary of results. *P<0.05, **P<0.01, ***P<0.001.
Jmjd3 Controls Mesodermal and Cardiovascular Differentiation of Embryonic Stem Cells
Kisho Ohtani, Cong Zhao, Gergana Dobreva, Yosif Manavski, Britta Kluge, Thomas Braun, Michael A. Rieger, Andreas M. Zeiher and Stefanie Dammeler

Circ Res. published online July 15, 2013;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2013/07/15/CIRCRESAHA.113.302035

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/07/15/CIRCRESAHA.113.302035.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Methods

**Generation of Jmjd3 knockout ES cell lines**

Mouse genomic DNA encompassing the murine Jmjd3 gene region were isolated by PCR amplification and used to generate short (1.6kb) and long (6.2kb) arms of homology. The targeting vectors were constructed by inserting a loxP site together with an FRT flanked neomycin selection cassette within the intron 5 and a single distal loxP within the intron 3. This targeting strategy results in the deletion of 600bp coding sequences encoding for the ATG methionine codon and produces a frame shift of JmjC domain existing exon 19-21 required for Jmjd3 demethylase activity. The targeting vector was electroporated in 129Sv ES cells. G418 resistant ES cell clones were screened for homologous recombination by PCR analysis and targeting was verified by Southern blot analysis. Homozygous Jmjd3\textsuperscript{lox/lox} ES cells were generated by electroporation of heterozygous Jmjd3\textsuperscript{lox/+} ES cells with the same targeting vector as above except that the neomycin resistance gene was replaced by puromycin gene using the Nucleofector (Lonza). Double-allele-recombined ES cells were selected for puromycin (1.3µg/mL, Invitrogen). Correct targeting of homozygous Jmjd3\textsuperscript{lox/lox} ES clones were determined by PCR. To obtain Jmjd3\textsuperscript{-/-} ES cells, Jmjd3\textsuperscript{lox/lox} ES cells were electroporated with Cre-recombinase plasmid vector and loss of targeting cassettes was evaluated by loss of resistance of G418 and puromycin. Correct targeting of homozygous Jmjd3\textsuperscript{-/-} ES cells was determined by PCR.

**Generation of Jmjd3 knockout mouse**

The recombined ES cell clones were injected into blastocytes of C57BL/6 females to generate chimeric mice. The resultant chimeric mice were bred with C57BL/6J constitutively Flp recombinase expressing deleter mice to allow the germline excision of the neomycin cassette. These heterozygous floxed mice were mated with C57BL/6J constitutively Cre recombinase expressing delete mice to generate heterozygous mice carrying the constitutive Jmjd3 knockout allele. These mice were obtained on a mixed C57BL/6J background were backcrossed for at least five generations.

**Cell culture and differentiation**

Mouse ES cells were cultured and maintained on mitotically inactivated mouse embryonic fibroblasts (MEFs) in DMEM supplemented with L-glutamine, 15% FBS, leukemia inhibitory factor (LIF), non-essential amino acids, monothiol glycerol, and Penicillin/Streptomycin. For differentiation assay, MEFs were depleted by using Feeder Removal MicroBeads (Miltenyi Biotec). Differentiation of ES cells was induced without LIF in static or rotary suspension condition \textsuperscript{1,2}. For cardiac differentiation, embryoid bodies (EBs) were derived using the hanging drop method. In brief, ES cells are cultivated as EB in hanging drops at a final concentration of $3.3 \times 10^4$ cells/mL for 2 days and in suspension for 3 days. On the fifth day, EBs were plated separately onto gelatin-coated plates and checked daily for spontaneous contraction. For endothelial differentiation, EBs were formed and cultured in DMEM

1
supplemented with 15% FBS, 450µM Monothioglycerol, 10µg/mL Insulin (Merck Millipore), 50ng/mL human VEGF (Peprotech), 2U/mL human Erythropoietin (Merck Millipore), 100ng/mL human bFGF (Peprotech), 10ng/mL murine IL-6 (Peprotech) for indicated days.

Shortly, for the OP9 coculture system, OP9 cells (stably expressing GFP) were grown to confluence in 6-well culture dishes, then ESCs were seeded with a density of 15000 ESCs per well. The culture medium was α-MEM supplemented with 10% FCS and 5x10⁻⁵ M 2-mercaptoethanol. At day 3, 4 and 6, cells were dissociated and stained with antibodies against murine Flk1 (CD309, clone Avas12a1, PE-conjugated, eBioscienceBiolegend) and VE-cadherin (CD144, clone VECD1, Alexa 647 conjugated, Biolegend) as well as Fixable Viability Dye eFluor® 780 (eBioscience) at 4°C for 30 min for FACS analysis with a FACS Canto II (BD). Viable ES-derived cells were pre-gated for GFP negative cells and dead cell exclusion. For intracellular Brachyury staining, cells were dissociated, stained with Fixable Viability Dye eFluor® 780, fixed/permeabilized and stained with PE-conjugated anti-Brachyury antibody (R & D Systems) according to the manufacturer’s instruction. The corresponding PE-conjugated Goat IgG Isotype control (R & D Systems) was used.

Alternatively, for direct mesoderm induction, EBs were formed in serum free system and were stimulated at day 2 with mouse recombinant Wnt3a (100ng/mL, Peprotech) \(^3\).

**Plasmid construction and stable transfection**

The full length Jmjd3, the C-terminal catalytic domain, the mutated catalytic domain or full length Brachyury were cloned into pEF1 vector (Invitrogen). The linearized plasmids were transfected in Jmjd3\(^{-/-}\) ES cells using the Amaxa nucleofection system (Lonza). The neomycin-resistant clones were selected with G418 (Invitrogen), were picked and screened by PCR. The full length Jmjd3 was also cloned into pcDNA3 Flag-HA vector for expression in HEK 293 cells. HEK 293 cells were transfected by using Lipofectamine 2000 (Invitrogen). The expression of all constructs was confirmed by western blotting.

**Proliferation assay**

For the proliferation assay, 2x10\(^3\) cells were seeded in triplicate in 12 well gelatin-coated plates and cell numbers were counted every other day.

**Chromatin immunoprecipitation (ChIP)**

ChIP assays were performed as previously described \(^4\). Sheared chromatin was immunoprecipitated with antibodies against H3K4me3 (17-614 Millipore), H3K27me3 (17-622 Millipore), histone H3 (ab1791 abcam), β-catenin (sc7199 Santa Cruz), and RNA polymerase II (sc-899X Santa Cruz). The
purified chromatin was used in quantitative real-time PCR (Applied Biosystems). ChIP-quantitative PCR data were normalized to the input DNA (percent input method) and ChIP signals of histone modifications were normalized to total histone H3 levels. The primers used to amplify the Brachyury promoter were designed based on previous publications. Primer sequences are available upon request.

Quantitative PCR
Total RNAs were extracted using RNeasy Kit (Qiagen) and reverse-transcribed to cDNA. Quantitative PCR was performed with StepOnePlusTM Real Time PCR System (Applied Biosystems). Gene expression levels were calculated in accordance with the \(2^{-\Delta\Delta C_t}\) method and normalized to GAPDH. Primer sequences are available upon request.

Gene Expression Microarray, GO, and GSEA Analyses
Total RNAs were isolated from wild-type or Jmjd3\(^{-/-}\) ES cells 4 days after differentiation (hanging drop method) and hybridized to Affymetrix GeneChip mouse genome 430 2.0 Array. Data analysis was performed using AltAnalyze. Relative gene expression was calculated as log2 ratio of gene expression in Jmjd3\(^{-/-}\) ES cells to the wild-type ES cells and heat map was generated. Gene Ontology analysis was performed using DAVID and Fisher's exact test. Gene Set Enrichment Analysis was performed using the GSEA software.

Co-Immunoprecipitation
For nuclear extraction, EBs were suspended in hypotonic buffer (10mM HEPES, pH 7.9, 1.5mM MgCl\(_2\), 10mM KCl, 1mM DTT, 1% NP-40) and nuclear pellet was resuspended in cell lysis buffer (Cell Signaling). Nuclear extracts were incubated with Flag M2 beads (Sigma). Immunoprecipitated proteins were analyzed by SDS polyacrylamide gel electrophoresis followed by Western blotting with the indicated antibodies.

Western blot
Whole cells were lysed in cell lysis buffer (Cell Signaling) and briefly sonicated. Equal amounts of proteins were separated in SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblotting was performed with the antibody against H3K27me3 (17-622 Millipore), H3K27me3 (CS200603 Millipore), H3K4me3 (CS200580 Millipore), H3K9me3 (07-442 Millipore), H3K36me3 (04-801 Millipore), acetyl-Histone H3 (06-599 Millipore), Histone H3 (ab1791 abcam), Jmjd3 (3457 Cell Signaling), Ezh2 (39639 Active Motif), β-catenin (sc7199 Santa Cruz), Brachyury (AF2085 R&D) and α-tubulin (DLN15413 Dianova).
**Immunohistochemistry**

For alkaline phosphatase staining, cells were stained with the Alkaline Phosphatase Staining Kit (Miltenyi Biotec). Apoptosis was detected by In Situ Cell Death Detection Kit (Roche). Immunofluorescence was performed as previously described ⁴ using antibody against Oct4 (2750 Cell Signaling), Nanog (ab80892 abcam), H3K27me3 (CS200603 Millipore), αMHC (sc-20641 Santa Cruz), Pecam-1 (550274 BD Biosciences) and Phalloidin (MFP-A1379 Mo Bi Tec).

**Reporter gene assays**

3xLEF1 reporter plasmid, LEF1 expression construct and NLS-β-catenin were kind gifts from Rudolf Grosschedl. Mouse ES cells were seeded (5×10⁴) on gelatin coated 24-well. After 24 hours of plating, 3xLEF1 reporter plasmid, LEF1, and NLS-β-catenin plasmids were transiently transfected with FugeneHD (Promega). β-galactosidase plasmid was co-transfected for normalization of transfection efficiency. Each group was transfected in triplicates. 48 hours after transfection, cells were harvested. Cell lysis and luciferase assay were performed following the protocol of Luciferase Reporter Assay System (Promega). β-galactosidase assays were performed using CPRG (Sigma) as substrate and the absorbance at 600nm was measured. Luciferase activity was normalized to β-galactosidase activity.

**Statistical analysis**

Data are represented as mean ± standard error of the mean (SEM.). Data are analyzed by using unpaired Student’s t-Test when comparing two groups, or a one-way ANOVA with Bonferroni’s comparison test when comparing multiple groups. Probability values of less than 0.05 were considered statistically significant and tests were performed two-sided.
References


Online Figure I. Generation and characterization of Jmjd3−/− ESCs

(A) Targeting strategy to generate Jmjd3 mutant ESCs by homologous recombination. Primers used for PCR are shown. (B) Genotyping of Jmjd3−/− ESCs by using 2 different primers. Scale bar indicates 10µm. (C) Oct4 and Nanog staining in WT and Jmjd3−/− ESCs. Scale bar indicates 10µm. (D) Expression of Oct4 and Nanog in WT and Jmjd3−/− ESCs. Data are presented as fold changes compared with day 0 WT ESCs. N=6. (E) Tunel staining (green) of WT and Jmjd3−/− ESCs. Nuclei are stained with Hoechst (blue). Scale bar indicates 20µm. (F) Growth curves of WT and Jmjd3−/− ESCs. N=6-8. (G) H3K27me3 staining in WT and Jmjd3−/− ESCs. Nuclei are stained with Hoechst (blue). Scale bar indicates 20µm. (H) ChIP assay of undifferentiated WT and Jmjd3−/− ESCs for H3K27me3. ChIP enrichments are normalized to Histone H3 density and represented as fold change relative to WT. N=3. Data represent mean ± SEM.
Online Figure II. Jmjd3−/− ESCs show an impaired mesoderm differentiation. (A) Microarray gene expression heat map depicting expression of representative pluripotency and lineage markers 4 days after differentiation in Jmjd3−/− ESCs versus WT ESCs. Coloring illustrates log2 fold changes between Jmjd3−/− ESCs and WT ESCs. Green and red colors represent down-regulation and up-regulation, respectively. (B) Gene ontology analysis for more than 2-fold altered genes in Jmjd3−/− ESCs compared to WT ESCs 4 days after differentiation. Red and green colors represent down-regulation and up-regulation, respectively.
Online Figure III. Overexpression of Brachyury partially rescues endothelial differentiation defect of Jmjd3−/− ESCs.

(A) mRNA expression of Jmjd3 during endothelial differentiation in WT and Jmjd3−/− ESCs. The expression level of Jmjd3 in WT ESCs at day 0 is assigned to 1 and fold changes are shown. Data represent mean ± SEM. N=4

(B) Pecam-1 staining of WT and Jmjd3−/− ESCs at day 8 of endothelial differentiation. The mean fluorescence of Pecam-1 in WT is assigned to 1 and fold changes are shown. Data represent mean ± SEM. N=3

(C) Western blot analysis in Jmjd3−/− ESCs after overexpression of Brachyury. α-Tubulin is used as a loading control.

(D) Pecam-1 staining of Jmjd3−/− ESCs after overexpression of Brachyury at day 8 of endothelial differentiation. Phalloidin is used to visualize the cytoskeleton. Nuclei are stained with DAPI (blue). Scale bar indicates 20µm.

(E) Quantification of αMHC staining of WT and Jmjd3−/− ESCs at day 9 of cardiac differentiation. The mean fluorescence of αMHC in WT is assigned to 1 and fold changes are shown. Data represent mean ± SEM. N=3
Online Figure IV. Jmjd3 is required for proper mesoderm induction through reduction of H3K27me3 and recruitment of RNA polymerase II at mesodermal promoters.

(A) ChIP assay at day 3.5 during differentiation in WT and Jmjd3<sup>-/-</sup> ESCs for H3K27me3. ChIP enrichments are normalized to Histone H3 density and represented as fold change relative to WT. N=4 (B) ChIP assay at day 3.5 during differentiation in WT and Jmjd3<sup>-/-</sup> ESCs for H3K4me3. ChIP enrichments are normalized to Histone H3 density and represented as fold change relative to WT. N=3. (C) ChIP assay at day 3.5 during differentiation in WT and Jmjd3<sup>-/-</sup> ESCs for total RNA polymerase II. ChIP enrichments are calculated as percentage of input signal and data are presented as fold changes compared with WT. N=3. Data represent mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.
Online Figure V. Microarray analysis of the expression of Wnt target genes in WT and Jmjd3−/− ESCs (for further details of the experiment see Online Figure II).
Online Figure VI. Confirmation of Jmjd3-β-catenin interaction. Anti-Flag immunoprecipitates of nuclear extracts from Jmjd3-Flag overexpressing EBs were analyzed by anti-β-catenin immunoblotting. Five percent of total lysates were loaded as input.
Online Figure VII. Generation of Jmjd3 knockout mice

(A) Southern blot analysis of AflII-digested DNA isolated from Jmjd3 heterozygous (+/-) and wild type (+/+) mice. (B) Genomic DNA from mice embryos at E7.5 of the indicated genotypes was analyzed by PCR using primers specific for the targeted mutation. (C) Gross morphology of WT and Jmjd3 heterozygous embryos at E7.5. (D) Genotypes of offspring from intercrosses of constitutive Jmjd3+/- mice. Numbers of genotypes of live births, embryos of E9.5, 7.5, and 6.5 from constitutive Jmjd3+/- intercrosses.