**Prdm6 Is Essential for Cardiovascular Development In Vivo**

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Abstract

Members of the PRDM protein family have been shown to play important roles during embryonic development. Previous in vitro and in situ analyses indicated a function of Prdm6 in cells of the vascular system. To reveal physiological functions of Prdm6, we generated conditional Prdm6-deficient mice. Complete deletion of Prdm6 results in embryonic lethality due to cardiovascular defects associated with aberrations in vascular patterning. However, smooth muscle cells could be regularly differentiated from Prdm6-deficient embryonic stem cells and vascular smooth muscle cells were present and proliferated normally in Prdm6-deficient embryos. Conditional deletion of Prdm6 in the smooth muscle cell lineage using a SM22-Cre driver line resulted in perinatal lethality due to hemorrhage in the lungs. We thus identified Prdm6 as a factor that is essential for the physiological control of cardiovascular development.


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Introduction

Prdm6 belongs to the PRDM family of transcriptional repressors which all possess an N-terminal PR domain and C-terminal Krüppel-type zinc finger motifs. While the zinc fingers are responsible for DNA binding, the PR domain is thought to mediate homodimerization and interaction with proteins such as the histone methyl transferase G9a and histone deacetylases [1-3]. Therefore, PRDM proteins are expected to play important roles as histone modifying factors that regulate gene transcription at the chromatin level. The most intensely studied PRDM member PRDM1 (also named BLIMP1) has been shown to mediate methylation of lysine residue 9 of histone 3 [2] and as a transcriptional repressor has been demonstrated to be essential for several physiological processes such as terminal B cell differentiation [4]. T cell
homeostasis and function [5,6], primordial germ cell formation [7] and regulation of proliferation and differentiation in the sebaceous gland [8]. Other members of the PRDM family were also reported to control developmental processes: Prdm5 regulates collagen gene transcription in developing bone [9]. Prdm9 defines hotspots of genetic recombination during meiosis [10]. Prdm14 was shown to be involved in the maintenance of embryonic stem cells in the mouse [11], and Prdm16 controls the bidirectional switch between skeletal myoblasts and brown fat cells [12]. While PRDM transcription factors control various developmental processes under physiological conditions, aberrant expression of PRDM proteins has been correlated with malignant disease and PRDM genes map to chromosomal regions frequently deleted in tumors [13-16]. Moreover, PRDM proteins can be expressed as PR domain-containing full length proteins or as amino-terminally truncated proteins lacking a functional PR domain by usage of an alternative internal promoter. Loss of PRDM full length expression or a shift in expression towards the truncated shorter form has been implicated in tumorigenesis [2,15,17-20].

Prdm6 was recently characterized as a transcriptional repressor that is expressed and plays a role in the vascular system. Davis and colleagues described Prdm6 as a transcription factor that plays a role in regulating the differentiation and proliferation of smooth muscle cells (SMCs) [3]. Moreover, Prdm6 was described as a factor that controls survival and differentiation of endothelial cells in the vascular system [21]. Furthermore, expression of Prdm6 has been reported in cells of the developing nervous system [22]. Finally, we identified PRDM6 to be transcriptionally deregulated and ectopically expressed from the rare, but recurrent chromosomal translocation t(5;14)(q23;q32) in B cell lymphoma patients (manuscript in preparation). To reveal physiological functions of Prdm6 in vivo, we generated and analyzed conditional Prdm6-deficient mice. We report here that Prdm6 is essential for embryonic development and for vital functions of the cardiovascular system.

Results

Generation of a conditional PRDM6 mutant mouse line

Because the physiological functions of PRDM6 are still largely unknown, we generated a gene-targeted mouse line that allows conditional Prdm6 ablation using Cre-loxP technology [23]. By homologous recombination in murine embryonic stem (ES) cells we flanked exon 3 of Prdm6 with loxP sites (see Materials and Methods and Figure S1 A,B). After injection of ES cells into blastocysts and removal of the neomycin selection cassette via flp-mediated deletion we eventually obtained Prdm6/lox/fox and Prdm6/fox/fox mice, which were born at expected Mendelian ratios and were phenotypically indistinguishable from their wild type littersmates (data not shown). Crossing Prdm6/fox/fox mice to Cre deleter mice [24] induced the deletion of the loxP-flanked exon 3 sequence in the germ line and resulted in Prdm6/DEL heterozygous mice. Correct homologous recombination events were confirmed by Southern blot analysis (Figure S1 C). Of note, deletion of Prdm6 exon 3 not only removes the central part of the PR domain but, due to a concomitant frame shift, it also prevents the expression of the complete Prdm6 reading frame downstream of the PR domain so that no functional protein can be expressed.

Prdm6 is essential during embryonic development

Heterozygous Prdm6/DEL mice were intercrossed to obtain homozygous Prdm6-deficient mutants (Prdm6/del/del). Prdm6/DEL and Prdm6/del/del mice were born at the expected Mendelian ratios. In contrast, we did not observe viable homozygous Prdm6/del/del offspring, indicating that functional Prdm6 expression is essential for the viability of mice. Thus, we next performed timed pregnancies and analyzed Prdm6/del/del embryos at different developmental stages. Up to E10.0, we observed Mendelian frequencies of morphologically intact Prdm6/del/del embryos. However, beyond E10.0 the frequency of viable Prdm6/del/del embryos declined significantly whereas Prdm6/del/DEL and Prdm6/del/del embryos were present at regular numbers (Figure 1A). RT-PCR analysis confirmed the absence of Prdm6 exon 3 and therefore the deficiency of functional Prdm6 mRNA in Prdm6/del/del embryos. An alternative exon2–exon4-spliced Prdm6 transcript is produced by the disrupted (del) allele in Prdm6/del/DEL and Prdm6/del/del embryos (Figure S1 D). However, since Prdm6/del/del heterozygous mice are born at Mendelian ratios and are viable, healthy and fertile, there is no indication for a dominant gain-of-function of the alternatively spliced Prdm6 transcript derived from the knockout (del) allele. The onset of embryonic lethality in the Prdm6/del/del embryos correlated with the onset of Prdm6 expression at E10.5 in wild type embryos (Figure 1B), a developmental stage at which the cardiovascular system undergoes critical developmental steps [25]. Macroscopic inspection of Prdm6/del/del embryos revealed that at E12.5 they eventually displayed pale and edematous bodies, implying cardiovascular insufficiency (Figure 1C). Analysis of the cardiac architecture of the Prdm6/del/del embryos via histological H&E staining revealed a thinning of the myocardial walls, indicating primary or secondary heart failure (Figure 1D).

Prdm6 affects vascular patterning

During the isolation of Prdm6/del/del embryos from the deciduae, we repeatedly observed vascular malformations exclusively on the yolk sacs of Prdm6/del/del embryos at stage ≥E13.5 (Figure 2A, left panel). Higher magnifications revealed that these malformations were composed of clusters of densely growing and partially dilated blood microvessels (Figure 2A, right panel). However, large vessel vascularization was present in the yolk sacs of Prdm6-deficient embryos, indicating regular overall vasculogenesis (Figure 2B). To investigate the role of Prdm6 in the development of the small blood vessel architecture in structural detail, we stained vascular endothelial cells with an anti-CD31 (anti-PECAM1) antibody and visualized the yolk sac vascular system via fluorescence microscopy. The yolk sacs of wild type control embryos displayed an organized vascular network with a hierarchy between vessels of higher and lower orders at the developmental stages E10.5 and E11.5 (Figure 2C, upper panels). Also in Prdm6/del/del yolk sacs a small vessel network was present (Figure 2C, lower panels).
Figure 1. *Prdm6* deficiency results in embryonic lethality. (A) *Prdm6*<sup>wt/wt</sup> mice were intercrossed. Pregnant mice were euthanized and embryos dissected and genotyped at defined developmental stages. The percentages of viable embryos of the respective genotypes at the different stages of embryonic development (dpc = days post coitum) are indicated; wild type *Prdm6*<sup>wt/wt</sup> (wt/wt) and heterozygous *Prdm6*<sup>wt/del</sup> (wt/del) mice are viable, whereas *Prdm6*-deficient *Prdm6*<sup>del/del</sup> (del/del) embryos begin to die after E10.0, with no *Prdm6*<sup>del/del</sup> embryos being found at developmental stages beyond E16.0. (B) Northern blot analysis of *Prdm6* expression using total embryonic RNA from different developmental stages from wild type embryos. *Gapdh* expression analysis served as a loading control. (C) Representative wild type control and *Prdm6*-deficient embryos (del/del) at the indicated developmental stages. White arrows indicate edematous swelling. (D) Transverse heart sections from wild type control and *Prdm6*-deficient embryos were stained with H&E and analyzed by microscopy. The thin myocardium of *Prdm6*-deficient embryos (del/del) is indicated by an arrow. Scale bars correspond to 200 µm.

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However, visual inspection suggested subtle differences in the patterning of the vascular network of Prdm6\textsuperscript{del/del} yolk sacs compared to wild type control yolk sacs (Figure 2C). Indeed, quantitative analysis revealed that the small vessel network of Prdm6\textsuperscript{del/del} yolk sacs contains significantly less avascular space and exhibits a significant increase in the mean vessel diameter (Figure 2D and Figure S2). These findings indicate that Prdm6 function is involved in vascular patterning during embryonic development.

**Normal differentiation and proliferation of Prdm6-deficient smooth muscle cells**

It was recently reported that Prdm6 might play a role in SMC function [3]. We therefore next tested whether Prdm6 deficiency affects SMC differentiation and proliferation. To this end, we established Prdm6-deficient ES cell lines from the inner cell mass of early E3.5 Prdm6\textsuperscript{del/del} embryos and differentiated these ES cells into defined vascular cell lineages under specific culture conditions in vitro. Of note, the Prdm6\textsuperscript{del/del} ES cell lines differentiated regularly into smooth muscle alpha-actin (SMA)-expressing cells (i.e. pericytes or...
vascular SMCs [26]) at a rate and frequency comparable to wild type ES cell lines (Figure 3A), indicating that Prdm6 is dispensable for SMC-lineage differentiation. Moreover, Prdm6<sup>del/del</sup> ES cell lines also regularly differentiated into ECs and cardiomyocytes in vitro (data not shown). Immunohistochemical staining to SMA demonstrated that SMCs were regularly lining arterial vessel walls in Prdm6<sup>del/del</sup> embryos, thus indicating that recruitment of SMCs to the vasculature was intact (Figure 3B). To study the proliferation of embryonic vascular SMCs in vivo, we injected bromodeoxyuridine (BrdU) into pregnant mice and subsequently used immunohistology to determine the BrdU content within embryonic SMCs in different vascular regions. We did not observe significant differences in the frequencies of BrdU-positive SMCs between wild type control and Prdm6-deficient embryos, neither in SMCs in the aortic arch arteries nor in the yolk sac (Figure 3 C,D). In conclusion, Prdm6 does not seem to be required for either general SMC differentiation, recruitment to blood vessels or proliferation during embryonic development.

SM22-Cre-induced Prdm6 deletion results in perinatal death associated with pulmonary hemorrhage

We crossed the Prdm6<sup>floxed</sup> alleles into the SM22-Cre mouse line [27,28] to generate Prdm6<sup>flox/flox</sup>;SM22-Cre mice in which Prdm6 is selectively disrupted in the SMC lineage. Although SMC-conditional Prdm6 knockout mice were born at expected Mendelian frequency, we did not obtain viable adult SMC-conditional Prdm6 knockout mice (Figure 4A) because all newborn Prdm6<sup>flox/flox</sup>;SM22-Cre pups died within 2 days after birth (Figure 4B). Interestingly, perinatal death induced by SM22-Cre-mediated conditional deletion of Prdm6 was associated with massive hemorrhage in the lungs (Figure 4C).

Prdm6 regulates factors that are involved in angiogenesis

Since Prdm6 acts as a transcription factor [3], we were interested in the identification of target genes that are physiologically controlled by Prdm6. Therefore, we performed genome-wide cDNA microarray analysis and compared gene expression patterns between wild type Prdm6<sup>wt/wt</sup> and knockout Prdm6<sup>del/del</sup> embryos. Because we observed an impact of Prdm6 deletion on vascular development, we compared the mRNA expression patterns in the yolk sacs of day E10.5 embryos, which are highly vascularized and easily accessible, allowing high quality RNA isolation. A total of 51 genes were found to be differentially expressed in Prdm6<sup>del/del</sup> yolk sacs compared to wild-type tissue (Figure S3). Only two genes (Sfrp1 and Mtap1b) were upregulated, while all of the other deregulated genes displayed decreased expression levels in the absence of Prdm6. Several of the differentially regulated genes have been previously implicated in angiogenesis, such as those coding for the Wnt signaling inhibitor Sfrp1, the extracellular matrix protein F-Spondin, and the matrix metalloproteinase MMP2. Quantitative RT-PCR (qPCR) analysis of selected genes confirmed the microarray data (Figure 5), indicating that Prdm6 directly or indirectly controls the expression of a set of genes that are implicated in vascular development, and possibly also in other developmental processes.

Discussion

In this study we reveal an essential role of Prdm6 for the development of the cardiovascular system. Prdm6 total knockout embryos (Prdm6<sup>del/del</sup>) die during development with an onset of about E10.5. At later stages, Prdm6<sup>del/del</sup> embryos display signs of cardiac insufficiency, i.e. edema and progressive heart defects. Anti-CD31 staining revealed that a vessel network is present in Prdm6-deficient yolk sacs, indicating that vasculogenesis is intact in Prdm6 deficient yolk sacs. However, the small vessel network of Prdm6-deficient yolk sacs displayed an altered patterning with increased vascular diameters and smaller avascular space when compared to wild type yolk sacs. Prdm6 deficiency therefore apparently affects aspects of angiogenesis. Embryonic lethal phenotypes involving intact vasculogenesis, but impaired angiogenesis have been described for mice that are deficient in a variety of genes, such as Fzd5 [29], Notch1 [30,31], Jagged1 [32], Hey1/Hey2 [33], Smoothened [34], Eph-B4 and Ephrin-B2 [35], Angiopoietin [36], Tie2 [37], Smad5 [38], Quaking [39], Hif2alpha [40], VE-PTP [41], SCL/Tal-1 [42], and PI3K p110-α [43]. Inactivation of those key regulators of angiogenesis results in embryonic death latest by E11.0. Compared to that, Prdm6<sup>del/del</sup> embryos start to die around E10.5 with clearly reduced but countable numbers still alive and without clear morphological defects at E12.5. All Prdm6<sup>del/del</sup> embryos that can be identified at E12.5 however are edematous, anemic pale and obviously are deceasing. While the above mentioned gene knockouts of angiogenic key regulators arrest angiogenesis in the yolk sac already at the level of the primitive primary plexus with defective development of the large vessel system, Prdm6<sup>del/del</sup> yolk sacs do possess large vessels and the observed angiogenesis defect of the small vessel network in Prdm6<sup>del/del</sup> yolk sacs is rather mild. Thus, it is questionable whether the observed subtle changes in vascular patterning can be responsible for the embryonic lethal phenotype of the Prdm6<sup>del/del</sup> embryos. It appears likely that Prdm6-deficiency might directly induce the observed heart defect that might be the primary cause of embryonic death. Further studies are required to resolve this issue.

Davis et al. proposed that Prdm6 is a transcriptional repressor that suppresses SMC differentiation and promotes SM proliferation [3]. We therefore investigated SMCs in Prdm6<sup>del/del</sup> total knockout embryos in order to test whether Prdm6 deficiency might have an effect on SMCs that potentially could contribute to the cardiovascular phenotype. However, we could not detect defects in the overall capacity of Prdm6-deficient SMCs to differentiate, proliferate and to be recruited to blood vessels in vivo. Moreover, Prdm6-deficient ES cells were able to differentiate into the pericyte lineage in vitro, to the same extent as wild type ES cells. These findings indicate that Prdm6 function might be required for alternative aspects of SMC function or in additional cell types during vascular development, e.g. the endothelial lineage as has been suggested by Wu et
Future experiments are required to address these questions.  

Even though we did not detect SMC defects in Prdm6\(^{\text{del/del}}\) total knockout embryos, we crossed our floxed Prdm6 allele to...
a SM22-Cre deleter line which induces Cre-mediated recombination in the SMC lineage [27] but also in other selected cell types such as mesothelial cells in the yolk sac and in cardiomyocytes during early heart development [44-46].

Conditional deletion of Prdm6 by the SM22-Cre driver did not result in embryonic death, which is an additional indication that Prdm6-deficiency in SMCs might not be the main cause for the defect in embryonic development as we observe it in Prdm6^floxed^;SM22-Cre total knockout embryos. For the same reason, it is unlikely that Prdm6 plays essential roles in mesothelial cells or cardiomyocytes during early stages of embryonic development. Interestingly however, SM22-Cre driven conditional deletion of Prdm6 resulted in postnatal death associated with lung hemorrhage. In lung, the expression of SM22-Cre has been demonstrated to be confined to vascular smooth muscle cells [27]. Thus, it might be assumed that smooth muscle cells require Prdm6 for maintaining pulmonary vessel integrity. Alternatively, however, it cannot be ruled out that SM22-driven

<table>
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<th>Genotype</th>
<th>viable adult mice (%)</th>
<th>expected frequency (%)</th>
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<td>25</td>
</tr>
<tr>
<td>Prdm6^floxed^/del</td>
<td>29</td>
<td>25</td>
</tr>
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<td>Prdm6^wt^/flox;SM22-Cre</td>
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<td>25</td>
</tr>
<tr>
<td>Prdm6^floxed^/del;SM22-Cre</td>
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<table>
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<tr>
<th>Genotype</th>
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<th>viable on day 2 (total number)</th>
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<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Prdm6^floxed^/del</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Prdm6^wt^/flox;SM22-Cre</td>
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<td>5</td>
</tr>
<tr>
<td>Prdm6^floxed^/del;SM22-Cre</td>
<td>8</td>
<td>0</td>
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Figure 4. Selective disruption of Prdm6 in vascular smooth muscle cells results in perinatal lethality. (A) Prdm6^wt^/del;SM22-Cre mice were crossed with Prdm6^floxed^ mice, and the genotypes of the offspring were analyzed at three weeks of age. The Prdm6^floxed^;SM22-Cre genotype leads to deletion of Prdm6 in the SMC lineage. The frequencies of the resulting genotypes were calculated from a total of 28 offspring animals and compared to the expected Mendelian frequencies. (B) Newborn mice from the same crosses as in (A) were observed at day 1 and day 2 after birth and subsequently were genotyped. (C) Lungs from newborn Prdm6^floxed^ control animals (viable) and SMC-conditional Prdm6^floxed^;SM22-Cre animals (deceasing) were embedded in paraffin, and sections were stained with hematoxylin and eosin. Scale bars correspond to 100 µm.

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Moreover, Wnt/frizzled signaling plays a critical role in distinct transcriptional cofactors [47]. Our microarray data indicate that the related Prdm5 protein was reported to possess the capacity to mediate both negative and positive transcriptional regulation, presumably depending on its interaction with additional transcriptional coregulators [47]. Our microarray data indicate that Prdm6 could also act as both a positive and negative regulator of transcription. Interestingly, among direct or indirect Prdm6 targets, we recognized six genes that were previously associated with angiogenic processes: Sfrp1 [48], Spon1 [49], Rhob [50,51], Mmp2 [52,53], Arfb1 [54], and Amotl1 [55]. The upregulation of Sfrp1 in the Prdm6-deficient embryos might be of special interest, as Sfrp1 acts as an antagonist of the Wnt/frizzled pathway expressed in smooth muscle cells [56]. Moreover, Wnt/frizzled signaling plays a critical role in distinct steps of embryonic vascular development [57]. For example, disruption of Frizzled-5 leads to embryonic lethality by E11.5, accompanied by defects in yolk sac angiogenesis [29]. Moreover, deficiency of Wnt7b results in postnatal death due to lung hemorrhage caused by vascular leakage and subsequent respiratory failure [58], similar to what we observed upon selective Prdm6 deletion in SMCs using SM22-Cre. As an independent study has additionally revealed that Prdm6 can directly regulate Wnt4 expression [3], we speculate that Prdm6 might modulate angiogenesis partly through effects on the Wnt/frizzled pathway. Further mechanistic studies are required to test this hypothesis and to understand which target genes are directly or indirectly regulated by Prdm6, thereby also providing hints concerning a potential role of PRDM6 in lymphomagenesis. The present study is a starting point for future investigations of PRDM6 in vivo functions with our conditional knockout mouse model being a valuable tool to further define the role of PRDM6 in the cardiovascular system by its selective deletion in e.g. the endothelial lineage or in cardiomyocytes and to study the possible impact of Prdm6 in other physiological processes, such as neurogenesis with which Prdm6 expression has been reported to be associated [22].

Materials and Methods

Ethics Statement

All animal work was conducted in accordance with German Federal Animal Protection Laws and approved by the Institutional Animal Care and Use Committee at the Technical University of Munich.

Figure 5. Deregulated expression of angiogenesis genes in Prdm6-deficient yolk sacs. Real-time RT-PCR analysis of selected transcripts identified via global gene expression profiling analysis (see Fig. S3). The expression values obtained from Prdm6-deficient (del/del) yolk sac samples were normalized to the expression values detected in wild type control samples. The housekeeping gene Tbp was expressed at equivalent levels in wild type and Prdm6-deficient yolk sacs.

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Prdm6 is a transcription factor that can associate with chromatin-remodeling enzymes, such as heterochromatin protein-1 (HP1-β), histone deacetylases HDAC1, -2, and -3, the histone acetyltransferase p300, and the histone methyl transferase G9a, to modulate gene expression [3]. In our microarray analysis, we identified approximately 50 significantly deregulated transcripts, among which only two genes were upregulated, while all of the others were downregulated in Prdm6-deficient yolk sacs. Considering that Prdm6 was assumed to function as a transcriptional repressor [3], we actually expected more transcripts to be upregulated rather than downregulated in the absence of Prdm6. However, also the related Prdm5 protein was reported to possess the capacity to mediate both negative and positive transcriptional regulation, presumably depending on its interaction with additional transcriptional cofactors [47]. Our microarray data indicate that Prdm6 could also act as both a positive and negative regulator of transcription. Interestingly, among direct or indirect Prdm6 targets, we recognized six genes that were previously associated with angiogenic processes: Sfrp1 [48], Spon1 [49], Rhob [50,51], Mmp2 [52,53], Arfb1 [54], and Amotl1 [55]. The upregulation of Sfrp1 in the Prdm6-deficient embryos might be of special interest, as Sfrp1 acts as an antagonist of the Wnt/frizzled pathway expressed in smooth muscle cells [56]. Moreover, Wnt/frizzled signaling plays a critical role in distinct steps of embryonic vascular development [57]. For example, deletion of Prdm6 in cardiomyocytes during early development [45] eventually causes heart failure in newborn mice with subsequent secondary blood congestion and hemorrhage in the lungs. This aspect needs to be addressed by future studies. The cardiovascular phenotype that we observe in the Prdm6 del/del total knockout embryos and pulmonary hemorrhage that we observe in the SM22-Cre driven conditional deletion of Prdm6 are in line with the reported physiological expression of Prdm6 within the vascular system [3,21]. However, further additional analyses will be necessary to clarify which cell types and which molecular mechanisms are contributing to the cardiovascular defects after total Prdm6 disruption during development or after conditional inactivation of Prdm6 by SM22-Cre.

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Generation of Prdm6 conditional knockout mice and flp/Cre deleter strains

Exon 3 of Prdm6 was flanked by loxP sites via homologous recombination in E14K ES cells according to standard procedures [59]. The embryonic stem (ES) cells containing the correctly recombined (rec) Prdm6 locus (Prdm6<sup>lox/lox</sup>) still also contained the FRT-flanked neomycin resistance selection cassette. Standard ES cell technologies were used to generate...
germline mutant Prdm6<sup>flHDLac!</sup> mice. Crossing with flip recombinase deleter mice [60] resulted in deletion of the neomycin resistance cassette and produced Prdm6<sup>flHDLac!</sup> mice. The following mouse strains were used: flip deleter mice (Jax human β-actin FLPe deleter strain B6;SjL-Tg(ACTFLPe)9205Dym/J), Cre deleter mice (Jax human CMV-Cre deleter strain B6.C-Tg(CMV-cre)1Cgn/J), and SM22-Cre (i.e. SM22alpha-Cre) mice (Jax Tg(Tagln-Cre)1Her/J). The mice were housed in a specific pathogen-free facility according to FELASA recommendations (http://www.felasa.eu). Littermates were used in all experiments.

For genotyping of the Prdm6 wt, del and flox alleles, the following primer combinations were used: wt allele: fwd: 5'-agacagaacatcaagaagggtag-3' plus del rev: 5'-gacctcctggaactgattag-3' (260 bp band); del allele: fwd: 5'-agacagaacatcaagaagggtag-3' plus del rev: 5'-ccagattgtgtcaccctttaagc-3' (570 bp); and flox allele: 5'-agacagaacatcaagaagggtag-3' plus flox rev: 5'-ggatcctcgaggggaagttc-3' (380 bp). RT-PCR and qPCR were performed as described previously [61]. The following primer pairs were used: Prdm6 exon 3 wt allele specific primers (5'-taacagttgtgtcaccctttaagc-3' plus 5'-agaagaggagaattcctgctg-3'), Prdm6 knockout (del) allele specific primers detecting alternative exon2-exon4-splicing (fwd: 5'-gcatctctgggaggtcgaat-3' plus rev: 5'-ggtggaagggacgttcaagt-3'). To perform real-time quantitative PCR, the following primer pairs were designed to span exon-exon boundaries: Sfrp1 (5'-cctgagctacactataac-3' plus 5'-ggactacactataacactataac-3'), Spon1 (5'-tactctgtactgttgaactc-3' plus 5'-ttgtagatgtgtggtcgcatac-3'), Mmp2 (5'-tgctttcgcaggaatagta-3' plus 5'-ctctattgttacactataac-3'), Ama1 (5'-ccagccccgttggttga-3' plus 5'-cagagcagacgccctgtgtaa-3'), and Tbp (5'-caccacagagctgtgtagctgtgtaa-3' plus 5'-tgcttaccttttagcagactataac-3').

Southern blot and Northern blot analyses
Southern and Northern hybridizations were performed according to standard protocols [62,63]. For embryonic expression analysis, a Mouse Embryo Full Stage Blot (Seegene, Seoul, Korea) was used.

Histology, Immunohistochemistry, and BrdU incorporation assays
To obtain histological sections and perform H&E staining, standard protocols were used, as described previously [64]. For anti-CDS1 staining, primary rat anti-mouse CD31 Ab (BD Pharimingen, clone MEC 13.3) was used, followed by fluorescent labeling with a secondary anti-rat Cy2 Ab or anti-rat Cy3 Ab (both from Jackson Immuno Research). To determine the proliferative index in smooth muscle cells of embryos and yolk sacs, pregnant mice were injected intraperitoneally three times every 2 h with 1.5 mg BrdU in 150 µl PBS. Then, the mice were euthanized, and the embryos were sectioned. Epitope retrieval was achieved through boiling in citrate buffer (10 mM citrate, pH 6.0 ± 0.05% Tween-20) for 20 min and a subsequent DNase I digestion (DNase I, Roche, grade 2, 1 mg/ml in PBS-Tween) for 1 h at 37°C. The sections were double-stained with a rat anti-BrdU Ab (1:50 in PBS-Tween, Serotec) and mouse anti-SMA Ab (1:500, Sigma clone 1A4) and incubated with the secondary antibodies anti-rat Cy3 (Jackson ImmunoResearch) and anti-mouse IgG2a FITC (Southern Biotech). The samples were analyzed with a Zeiss Axioplan 2 fluorescence microscope.

ES cell generation and differentiation assays
ES cell clones were obtained according to standard protocols [65], with the addition of the MEK1 inhibitor PD98059 (50 µM, NEB). Differentiation assays were performed as described previously [66]. Briefly, ES cells were co-cultured with OP 9 cells in differentiation medium containing 10% FCS (PAN Biotech) and 10⁻⁴ M beta-mercaptoethanol (Sigma-Aldrich) in alpha-MEM (Gibco/Invitrogen) and then FACS sorted to detect Flk1-positive, Cadherin-negative lateral plate mesodermal cells (Flk1 Ab: clone AVAS12, eBioscience; Cadherin Ab, clone ECD2). To achieve mural cell differentiation, 2x10⁴ sorted mesodermal cells were cultivated for 4 days on Collagen IV-coated plates. The cells were fixed in methanol containing 5% DMSO and stained with a monoclonal anti-SMA-Cy3 Ab (Sigma-Aldrich, Clone 1A4).

cDNA microarray analysis
E10.5 yolk sacs from six Prdm6<sup>flHDLac!</sup> and six wild type control mice were dissected on ice in DEPC-treated PBS, shock frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated using RNeasy Mini kits (Qiagen), and 400 ng of RNA was amplified according to the instructions of the Target AMP<sup>TM</sup> 1-Round aRNA Amplification Kit 103 (Epicentre Biotechnologies). Genome-wide cDNA microarrays were generated, hybridized and analyzed as described recently [67]. The selection of significantly differentially expressed genes showing reproducible up- or down-regulation included less than 5% false positives (FDR) in combination with fold changes of >1.3. The expression data were submitted to the GEO database (GSE9065), where a full description of our microarray results is also available (GPL4937).

Supporting Information
Figure S1. Generation of a conditional Prdm6 allele. (A) Amino acid sequence of the murine Prdm6 protein according to GenBank accession number NP_001028453. Two methionine start residues are indicated by circles: the first corresponds to the sequence proposed by Wu et al. [21], the second was described by Davis et. al [3]. The PR domain in the central part of the sequence is indicated in bold, whereas the zinc finger region is underlined. Exon-exon borders are marked with dashed vertical lines, and exon numbers are given to indicate by which exons the different parts of the protein are encoded. (B) Targeting strategy for homologous recombination at the Prdm6 locus. The region containing exon 3 of the Prdm6 wt locus, the targeting vector and the distinct recombinant alleles...
(rec, flox, del) are shown. The restriction fragment lengths produced by Afl II digestion are indicated for the various wt and recombinant alleles. The homology arms for recombination are drawn as strong lines and the probe region used for Southern blot analysis is indicated. LoxP sites are represented by triangles, the neomycin selection cassette by NEO and the FRT sites by closed circles. Homologous recombination in ES cells produced the recombinant (rec) locus containing the LoxP-flanked exon 3 region and the FRT-flanked NEO cassette. ES cells carrying the rec locus were then transferred into the germ line of mice. Crossing mice with the rec locus with flp deleter mice resulted in deletion of the NEO cassette and generated the flox allele. Crossing flox mice with Cre deleter mice resulted in the deletion of exon 3, thus generating the del allele. (C) Southern blot analysis of genomic DNA from thymi of mice with the respective genotypes demonstrated the presence of the expected allele sizes, as defined in (B). Genomic DNA was digested with AflII and hybridized with the probe as depicted in (B). (D) RT PCR analysis using cDNA from yolk sacs of the indicated genotypes as templates. The wild type Prdm6 transcript was amplified using a forward primer that specifically binds to the Prdm6 exon 2-exon 3 splice fusion site and therefore cannot anneal to the exon 3-deficient knockout (del) allele. The Prdm6 knockout (del) transcript was amplified using a forward primer that specifically binds to the alternatively spliced exon2-exon 4 fusion site that is only present in the knockout (del) allele but not in the wild type allele.

(PDF)

Figure S2. Quantitative morphometric analysis of the yolk sac vasculature. The vascular networks of a representative wild type control (A) and a Prdm6 knockout (B) yolk sac were analyzed by measuring the avascular space (i.e. intercapillary space) and mean vessel diameters. The left panel shows the original image of the anti-CD31 stains of whole mount yolk sac vasculature. The white areas in the center panels indicate the avascular spaces as measured by the histogram function of the Photoshop CS6 software. The right panels indicate all points where vessel diameters were measured using the ruler function of Photoshop CS6 software. Vessel diameters were determined in between all branching points. Scale bars correspond to 200 μm.

(PDF)

Figure S3. Heat plot of gene expression profiles from yolk sacs of Prdm6-deficient embryos. One dye-flip pair represents two experimental replicates of each of the six analyzed E10.5 yolk sacs. Official gene symbols are given. The scale bar indicates the mean ratio of fold induction. Red indicates upregulated and green downregulated genes in Prdm6<sup>flx/flox</sup> yolk sacs compared to wild type control yolk sacs.

(PDF)

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Author Contributions

Conceived and designed the experiments: JR AG MJSD IF CP TS JB MHdeA HUD. Performed the experiments: AG MCV UF NJ KH UG SKHP MM OG MH ELK AM SA. Analyzed the data: AG JR UF NJ MM MJSD. Contributed reagents/materials/analysis tools: AG JR OG MH JB NJ HUD MJSD IF. Wrote the manuscript: AG JR MJSD.

References


