Dlk1 is a negative regulator of emerging hematopoietic stem and progenitor cells

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ABSTRACT

The first mouse adult-repopulating hematopoietic stem cells emerge in the aorta-gonad-mesonephros region at embryonic day (E) 10.5. Their numbers in this region increase thereafter and begin to decline at E12.5, thus pointing to the possible existence of both positive and negative regulators of emerging hematopoietic stem cells. Our recent expression analysis of the aorta-gonad-mesonephros region showed that the Delta-like homologue 1 (*Dlk1*) gene is up-regulated in the region of the aorta-gonad-mesonephros where hematopoietic stem cells are preferentially located. To analyze its function, we studied Dlk1 expression in wild-type and hematopoietic stem cell-deficient embryos and determined hematopoietic stem and progenitor cell activity in *Dlk1* knockout and overexpressing mice. Its role in hematopoietic support was studied in co-culture experiments using stromal cell lines that express varying levels of *Dlk1*. We show here that *Dlk1* is expressed in the smooth muscle layer of the dorsal aorta and the ventral sub-aortic mesenchyme, where its expression is dependent on the hematopoietic transcription factor Runx1. We further demonstrate that Dlk1 has a negative impact on hematopoietic stem and progenitor cell activity in the aorta-gonad-mesonephros region *in vivo*, which is recapitulated in co-cultures of hematopoietic stem cells on stromal cells that express varying levels of *Dlk1*. This negative effect of Dlk1 on hematopoietic stem and progenitor cell activity requires the membrane-bound form of the protein and cannot be recapitulated by soluble Dlk1. Together, these data suggest that *Dlk1* expression by cells of the aorta-gonad-mesonephros hematopoietic microenvironment limits hematopoietic stem cell expansion and is, to our knowledge, the first description of such a negative regulator in this tissue.

Introduction

Hematopoietic stem cell (HSC) generation initiates autonomously in the aorta-gonad-mesonephros (AGM) region of the mid-gestation embryo.[1] This process is associated with the appearance of intra-aortic cell clusters derived from hemogenic endothelial cells, which may be the progeny of earlier cells located in the ventral sub-aortic mesenchyme (reviewed by Medvinsky *et al.*[2]). Although HSC production is first detected in the AGM, it only occurs there transiently from embryonic day (E) 10.5 until E12.5 and never exceeds more than three HSCs at a given time.[3] From E11.5, AGM HSCs are thought to colonize the fetal liver, which also receives hematopoietic cells from the yolk sac and the placenta[2] and becomes the predominant hematopoietic tissue after E12.5. In contrast to the AGM, the fetal liver itself is not capable of *de novo* HSC generation from pre-HSCs but plays an important role in supporting cycling HSCs and generating differentiated blood cells for immediate use. Thus, each site of hematopoiesis during development appears to be optimized to support the relevant stage of HSC production and function.

Further localization of HSCs within the AGM has shown that these cells lie exclusively in the middle length of the dorsal aorta around the junction with the vitelline artery.[4] Hematopoietic regulation is achieved through the integration of intrinsic and extrinsic signals. Such extrinsic signals are usually derived from stromal cells that make up the microenvironment and may act directly or indirectly on HSCs. While much work has focused on understanding the bone marrow hematopoietic microenvironment, the AGM HSC niche is less well-characterized. To identify potential regulators involved in the production of HSCs, we determined the gene expression profile of this middle part of the aorta. The genes found to be up-regulated here in relation to the flanking regions included delta-like homologue 1 (*Dlk1*).

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Dlk1 is a paternally expressed, imprinted gene[5,6] that codes for the protein Dlk1 (also known as Pref-1, FA-1 and dlk). The full-length protein is membrane-bound and contains six epidermal growth factor (EGF)-like repeats in the extracellular region which, apart from lacking the DSL domain used by Notch ligands to interact with Notch, are homologous to those found in the Notch/Delta family of proteins. A proximal cleavage site allows production of a functional, soluble protein, and mRNA isoforms encoding both cleavable and non-cleavable forms of the protein exist. *Dlk1* is widely expressed during development and has been associated with cell proliferation and differentiation in a number of tissues.[7,8] Its final effect is cell context-dependent, as it can act as an inhibitor of differentiation as well as a differentiation-promoting factor, even in different cell types of the same organ, such as the developing adrenal gland, and during adipogenesis.[9-11] Overexpression of *Dlk1* has been linked to malignancies, including those of the hematopoietic lineage.[12] In normal hematopoiesis, *Dlk1* is expressed in megakaryocytes but is not generally expressed in adult hematopoietic stem or progenitor cells.[13,14] It has also been associated with preferential differentiation along the megakaryocyte rather than the myeloid lineage and B-cell development.[12,15,16] *Dlk1* expression has been reported in the fetal liver, yolk sac and placenta, all of which are sites of hematopoiesis in the embryo. In addition, it is expressed in hematopoiesis-supportive stromal cell lines derived from fetal liver,[17] fetal thymus[18] and bone marrow.[19] Particularly in fetal liver stromal cell lines, Dlk1 was shown to impart supportive activity.[17]

Given its role as a regulator of hematopoietic development and its expression in the HSC-rich region of the AGM, we investigated the function of Dlk1 in the AGM.

Design and Methods

Mice and embryo generation

Details of animal strains can be found in the *Online Supplementary Design and Methods*. Mice were bred to obtain embryos of specific stages with the day of vaginal plug detection considered as day 0. All mice were housed according to institute regulations, and procedures were carried out in compliance with UK Home Office licenses.

Aorta-gonad-mesonephros explant cultures

E11-11.5 AGMs were cultured on Durapore filters (Millipore, Watford, UK) at the air-liquid interface in M5300 long-term culture medium (Stem Cell Technologies, Grenoble, France) supplemented with 10^{-6} M hydrocortisone (Sigma Aldrich, Gillingham, UK). Where indicated, recombinant human Fc-IgG at 1 μg/mL, human Control:Fc-IgG (Thy-1 RLE mutant) at 1 μg/mL or mouse Dlk1:Fc-IgG at 0.5 or 1 μg/mL (all Enzo Life Sciences, Lörrach, Germany) were added to the culture medium. After 3 days, AGMs were dissociated with collagenase (Sigma Aldrich, Gillingham, UK) and single cell suspensions transplanted into irradiated recipients.

Long-term transplantations

AGM cell preparations, together with $2x10^5$ total spleen cells as carrier cells to ensure short-term survival, were intravenously injected into C57BL/6 recipients that had received a split dose of 9 Gy of γ-irradiation. After 1 and 4 months, the donors' contribution to the recipients' peripheral blood was determined by FACS analysis, using antibodies specific to the Ly5.1 or Ly5.2 alloantigens (BD Biosciences, Oxford, UK). Mice were considered positive for repopulation if the donor contribution exceeded 5% after at least 4 months.

Gene expression analysis

Tissues and cells were dissociated in Tri Reagent (Sigma Aldrich, Gillingham, UK), RNA was isolated, treated with RQ1 DNase I (Promega, Southampton, UK) and reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Paisley, UK). Details of primers used for conventional and real-time reverse transcriptase polymerase chain reaction (RT-PCR) can be found in the *Online Supplementary Design and Methods.*

Immunohistochemistry and in situ hybridization

Embryos were fixed in 2-4% paraformaldehyde for 2 h at 4°C, cryoprotected overnight in 30% sucrose at 4°C and frozen in Tissue Tek (Sakura Finetek, Alphen aan den Rijn, the Netherlands). Cryosections of 10 μm were prepared and used for immunohistochemistry and *in situ* hybridization; further details, including antibodies used, are provided in the *Online Supplementary Design and Methods*.

Transfection of aorta-gonad-mesonephros stromal cell lines

Details of transfections to obtain Dlk1 overexpression and knockdown can be found in the *Online Supplementary Design and Methods*. The percentage knockdown of *Dlk1* expression was measured by quantitative real-time RT-PCR and the absence of Dlk1 confirmed by western blotting.

Co-culture experiments

KH9, KH21 and KH23 stromal cell lines were derived from *Runx1*$^{-/-}$, *Runx1*$^{+/-}$ and *Runx1*$^{+/+}$ E11.5 AGMs, respectively, as described previously.[20] One day prior to co-culture, confluent stromal cells received 20 Gy of γ-irradiation. HSC-enriched cell populations were obtained from wild-type mouse bone marrow (aged 8-10 weeks), seeded on irradiated stromal cell lines and maintained at 33°C for 1 or 4 weeks. Further details of stromal cell culture conditions and enrichment of HSC populations can be found in the *Online Supplementary Design and Methods*. Where indicated, mDlk1:Fc-IgG was added to the culture medium at a dose of 1 μg/mL. At the end of the co-culture, cells were harvested and plated in triplicate in M3434 methylcellulose medium (Stem Cell Technologies, Grenoble, France) at 37°C. Colonies were scored after 1 week. *P* values were calculated using paired, two-tailed Student's t-tests.

Results

Dlk1 localizes to the smooth muscle layer of the dorsal aorta and to cells of the sympathetic nervous system

Recent expression profiling of the mid-gestation dorsal aorta revealed *Dlk1* as being more highly expressed in the middle third of the aorta, where HSCs are preferentially located.[4] Therefore, to determine whether Dlk1 is involved in HSC regulation in the AGM, its expression pattern in this region was analyzed more extensively. Full-length Dlk1 is a transmembrane protein composed of an N-terminal signal sequence, six EGF-like repeats, a juxtamembrane region, a membrane-spanning region and a short cytoplasmic domain (Figure 1A).[21] It can be proteolytically cleaved just N-terminally to the transmembrane domain, producing a biologically active soluble protein.[22] Additionally, *Dlk1* mRNA is subject to alternative splicing, yielding four major (A-D) and two minor (C2 and D2) iso-

forms. Only isoforms A and B retain the region coding for the proteolytic cleavage site and thus the ability to produce a soluble protein.[23] RT-PCR analysis revealed that all the isoforms were present in the E11.5 AGM, implying that Dlk1 may exert its functions both as a membrane-bound and as a soluble protein (Figure 1B).

In situ hybridization experiments with a riboprobe that recognizes all *Dlk1* isoforms were performed on E11.5 embryo sections (Figure 1D-G). Figure 1D shows *Dlk1* expression in the fetal liver, the section of the gut that is just ventral to the AGM, the neural tube and the myotome. Within the AGM region, *Dlk1* expression was detected in the coelomic epithelium of the urogenital ridges, in cells outlining the dorsal aorta and in patches of cells in the mesenchyme surrounding the aorta. The expression of *Dlk1* in the AGM was stronger in the middle part (Figure 1F) than in the more caudal (Figure 1E) and more rostral (Figure 1G) sections (the relative position of the sections is shown in Figure 1C), thus confirming the results of the microarray experiments.[4]

To determine the nature of the *Dlk1*-expressing cells around the dorsal aorta, co-antibody staining was performed. No overlap was observed between Dlk1 and CD34, a marker for endothelial cells (Figure 1H). Instead, Dlk1 staining was found in the perivascular smooth muscle layer, as confirmed by co-staining with an antibody against smooth muscle actin (Figure 1I). Close-up views are provided in *Online Supplementary Figure S1*.

The pattern of *Dlk1* expression in lateral patches of cells in the mesenchyme around the dorsal aorta (Figure 1D-G) is reminiscent of cells of the sympatho-adrenal lineage, which contribute to the developing sympathetic ganglia dorsally and the adrenal gland ventrally in a Gata3-dependent process.[24] Co-staining for the sympatho-adrenal marker tyrosine hydroxylase confirmed that some Dlk1-expressing cells were tyrosine hydroxylase-positive (*Online Supplementary Figure S2A-C'*). Furthermore, the expression of *Dlk1* in the sympathetic ganglia and the adrenal anlage was absent in *Gata3*$^{-/-}$ embryos, which have a profound sympathetic nervous system defect (*Online Supplementary Figure S2D,D'*).[24] Interestingly, the expression of Dlk1 in the smooth muscle layer and the hindgut remained unchanged in the Gata3-deficient embryos.

Together, these results show that expression of Dlk1, which may be soluble or membrane-bound, coincides temporally and spatially with hematopoiesis in the dorsal aorta, where it is mainly found in the smooth muscle layer, and that its expression is downstream of Gata3 in the developing sympathetic nervous system at E11.

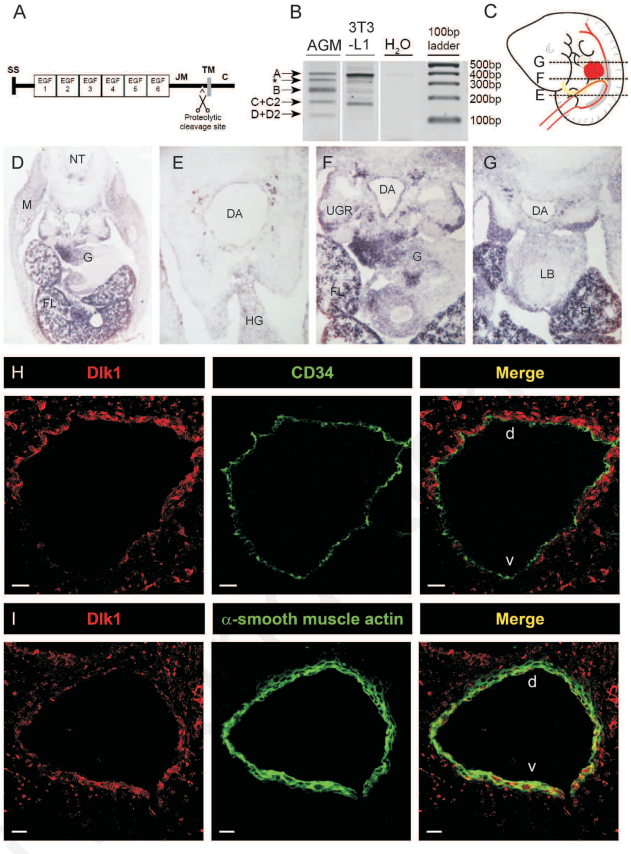

Figure 1. Expression analysis of Dlk1 in the mid-gestation embryo (A) Domain structure of the full-length Dlk1 protein. C, cytoplasmic domain; EGF, EGF-like repeat; JM, juxtamembrane domain; SS, signal sequence; TM, transmembrane domain. (B) Expression of *Dlk1* mRNA isoforms in the E11.5 AGM region. Expression in 3T3-L1 cells served as a positive control. The asterisk indicates an extra PCR product of unknown identity that is likely the product of polymerase slippage in the repeat region. (C) Schematic diagram of an E11.5 embryo showing the relative positions of the sections in E-G. (D-G) *Dlk1* transcript expression analysis by *in situ* hybridization on sections of an E11.5 embryo (D, 5x/0.15 objective) and the caudal (E), middle (F) and rostral (G) part of the AGM region (10x/0.25 objective). DA, dorsal aorta; FL, fetal liver; G, gut; HG, hindgut; LB, lung bud; M, myotome; NT, neural tube; UGR, urogenital ridges. (H,I) Immunohistochemical co-staining for Dlk1 [red, Cy3 in H and fluorescein isothiocyanate (FITC) in I] and (H) CD34 (green, FITC) or (I) smooth muscle actin, alpha (green, Cy3) on sections of E11.5 wild-type aortas. d, dorsal; v, ventral; scale bar is 20 μm.

Dlk1 expression is dependent on the transcription factor Runx1

The pattern of expression of *Dlk1* in the cell layers adjacent to the aortic endothelium is similar to that reported for known regulators of AGM HSC generation.[25,26] Furthermore, signals emanating from the gut, where *Dlk1* is expressed at high levels, have been shown to be important for HSC production.[27] This implies that Dlk1 may also be involved in the regulation of HSCs in the AGM. The transcription factor Runx1 is essential for HSC emergence in the AGM and is expressed in the ventral endothelium of the aorta, the ventral para-aortic mesenchyme and intra-aortic hematopoietic clusters at E11 (Figure 2A).[26,28] Co-antibody staining showed that, like Dlk1, Runx1 is also expressed by smooth muscle cells around the aorta (Figure 2B). We therefore examined the expression of *Dlk1* mRNA in comparable mid-aorta sections of wild-type and Runx1-null embryos. While *Dlk1* expression in the neural tube, the myotome and the sympathetic nervous system seemed unchanged, staining in the fetal liver appeared to be more intense in *Runx1*$^{-/-}$ embryos (Figure 2C-D). However, this may be due to the fetal liver having a more compact structure as a consequence of the disruption of definitive hematopoiesis. On close inspection of the AGM, decreased expression of *Dlk1* was observed in the ventral mesenchyme and the smooth muscle layer of the aorta, while *Dlk1* levels in sympatho-adrenal cells and the ventral gut area were unaffected (Figure 2E-F). This decrease in *Dlk1* expression was not due to a disruption of the smooth muscle layer, as we found no differences in smooth muscle actin staining in *Runx1*$^{+/+}$ and *Runx1*$^{-/-}$ embryos (Figure 2G-H). This suggests that Runx1 regulates *Dlk1* expression in

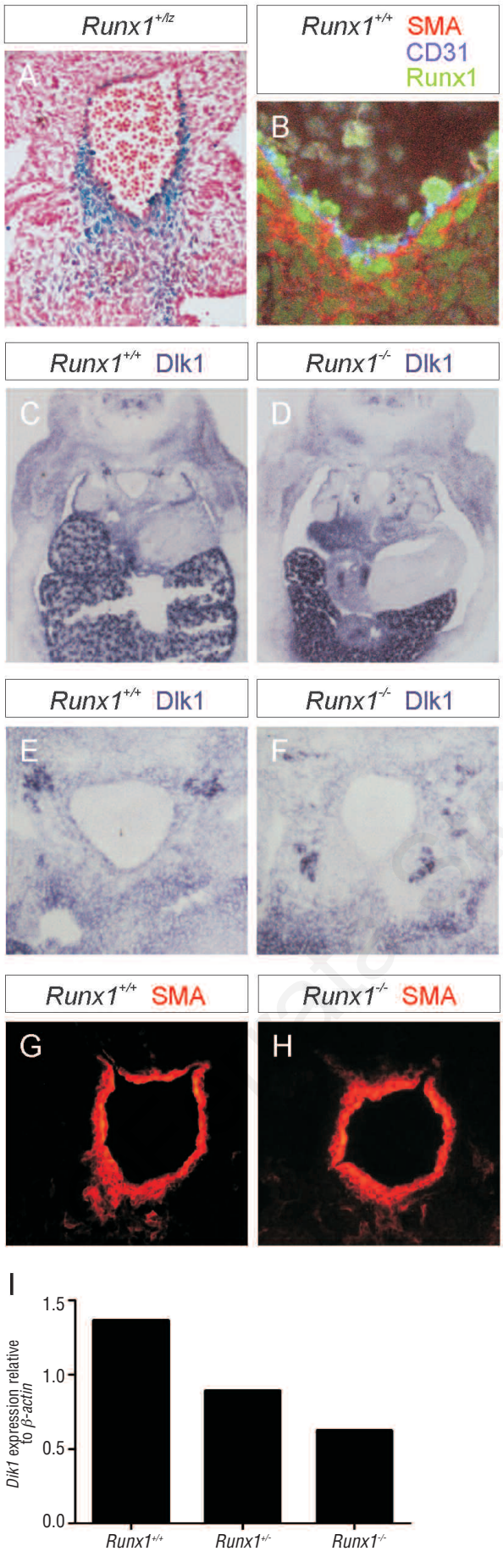

Figure 2. Dlk1 expression is downstream of Runx1. (A) X-gal staining (blue) around the dorsal aorta in an E11.5 *Runx1*$^{+/lz}$ embryo counterstained with Neutral Red (10x/0.25 objective). Ventral down. (B) Triple antibody co-staining on E10.5 *Runx1*$^{+/+}$ embryo section for smooth muscle actin (red, Cy3), endothelial CD31 (blue, Cy5) and Runx1 (green, FITC). A close-up of the ventral part of the dorsal aorta is shown. *In situ* hybridization with a *Dlk1*-specific riboprobe on sections of E11.5 *Runx1*$^{+/+}$ (C) and *Runx1*$^{-/-}$ (D) embryos (5x/0.15 objective, scale bar is 100 μm). A close-up of dorsal aorta is shown (E, F; 10x/0.25 objective). Ventral down. Smooth muscle actin staining (red, Cy3) around the dorsal aorta of E11.5 *Runx1*$^{+/+}$ (G) and *Runx1*$^{-/-}$ (H) embryo sections (10x/0.25 objective). Ventral down. (I) Real-time RT-PCR analysis of *Dlk1* expression in *Runx1*$^{+/+}$, *Runx1*$^{+/-}$, and *Runx1*$^{-/-}$ E11.5 AGM. Representative results of two experiments are shown.

smooth muscle cells. We have found Runx1 binding sites in the sequence upstream of the *Dlk1* open reading frame, which would support this observation (*data not shown*); however, this requires further investigation. Quantitative RT-PCR analysis also revealed decreasing levels of *Dlk1* in *Runx1*$^{+/-}$ and *Runx1*$^{-/-}$ AGMs as compared with wild-type (Figure 2I). These results demonstrate that *Dlk1* expression lies downstream (directly or indirectly) of Runx1 in ventral sub-endothelial cells in the AGM, implying a potential role for Dlk1 in Runx1-mediated HSC regulation.

Increased Dlk1 levels reduce the number of hematopoietic stem cells in the E11.5 aorta-gonad-mesonephros

Dlk1 is an imprinted gene expressed from the paternal allele.[5,6] The need for tight control of Dlk1 levels has been demonstrated in transgenic mice: hemizygous embryos expressing one extra copy of *Dlk1* under the control of endogenous regulatory sequences display overgrowth from E16, whilst homozygous embryos expressing two extra copies fail to thrive beyond this point and die perinatally.[29] We analyzed the expression of *Dlk1* in the E11.5 AGM region of these embryos and found an increase in the staining around the aorta in hemizygous (*Dlk1*$^{WT/TG}$) and a further increase in homozygous (*Dlk1*$^{TG/TG}$) over-expressing transgenic embryos as compared with wild-type ones (Figure 3A-C). The stronger intensity of the staining is not due to ectopic expression, as *Dlk1* expression around the aorta of transgenic embryos remained restricted to smooth muscle actin-expressing cells (Figure 3D).

To investigate the effect of increased Dlk1 levels on HSC numbers, AGMs were dissected from E11.5 *Dlk1*$^{WT/WT}$, *Dlk1*$^{WT/TG}$ and *Dlk1*$^{TG/TG}$ embryos and their HSC activity determined in transplantation assays. Interestingly, increasing the levels of Dlk1 had a negative impact on HSC activity in the AGM, with only 40% of recipient mice being repopulated with cells from *Dlk1*$^{TG/TG}$ AGMs (repopulation levels of 18%-81%) as compared with 67% showing repopulation after injection with wild-type cells (repopulation levels of 5%-73%) (Figure 3E). This suggests that there are fewer HSCs in *Dlk1*-overexpressing AGMs, but that the surviving HSCs can repopulate individual recipients to similar levels as HSCs from wild-type AGM.

Dlk1$^{-/-}$ *E11.5 aorta-gonad-mesonephros harbor increased numbers of definitive hematopoietic progenitor cells*

To obtain further evidence for the involvement of Dlk1 in AGM hematopoietic stem and progenitor cell regula-

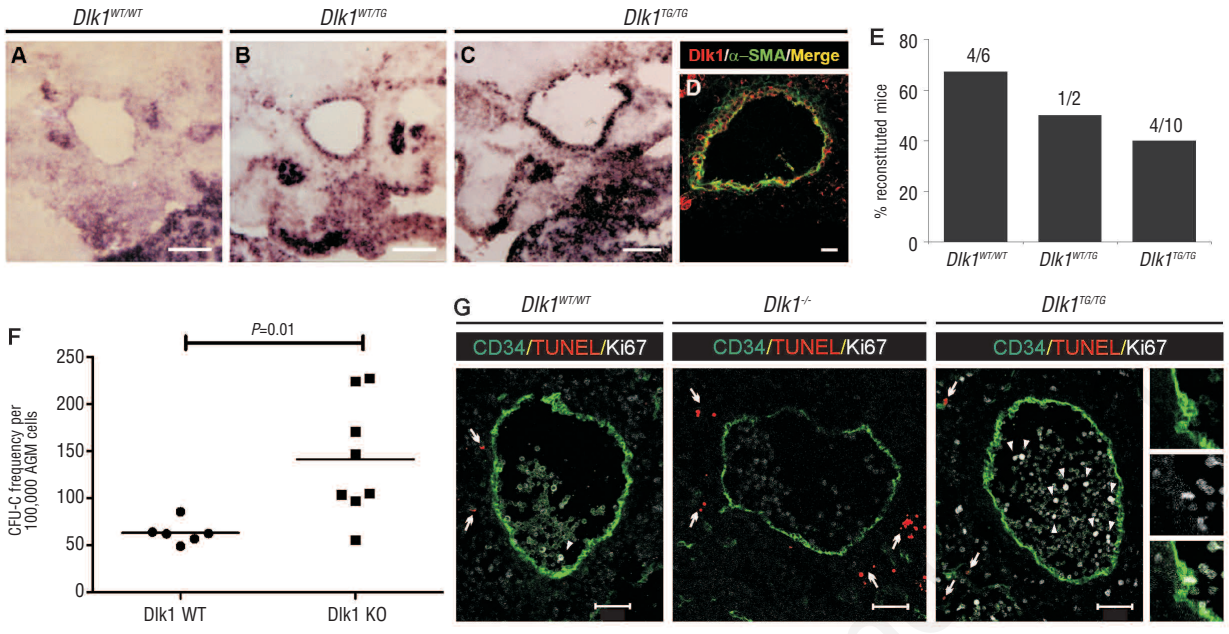

Figure 3. Increased *in vivo* levels of *Dlk1* reduce HSC activity in E11.5 AGMs, while *Dlk1* knockout AGMs have increased hematopoietic progenitor numbers. (A-C) *Dlk1* expression analysis by *in situ* hybridization in *Dlk1* transgenic E11.5 embryo sections; scale bar is 100 μm. (D) Immunohistochemical co-staining for Dlk1 (red, Cy5) and smooth muscle actin, alpha (green, Cy3) on a $Dlk1^{TG/TG}$ E11.5 embryo section; the dorsal aorta is shown; the scale bar is 20 μm. Ventral down. (E) Percentage of mice repopulated with E11.5 AGM cells from *Dlk1* transgenic embryos; number of positive mice per total number of transplanted mice shown above each bar; n=3. (F) E11.5 wild-type and *Dlk1* knockout AGM cells were plated in methylcellulose assays. The total number of colonies was counted after 1 week. n=3. (G) Immunohistochemistry for CD34 (green, FITC), Ki67 (white, Alexa647) and the TUNEL assay (red, Rhodamine) on $Dlk1^{WT/WT}$, $Dlk1^{-/-}$ and $Dlk1^{TG/TG}$ E11.5 embryo sections; the dorsal aorta is shown; the scale bar is 50 μm. White arrows indicate apoptotic cells and white arrowheads highlight proliferating cells. Smaller panels show an intra-aortic cluster stained for CD34 (top), Ki67 (middle) and merged stains (bottom). Ventral down.

tion, we also analyzed the effect that deletion of *Dlk1 in vivo* has on emerging definitive hematopoietic cells. *Dlk1* knockout mice have been generated, which suffer from pre- and post-natal growth retardation.[16,30] Hematopoiesis has also been analyzed in these mice and appears mostly normal in adult animals.[16] However, on closer inspection, an increase in granulocyte and megakaryocyte progenitors was observed, as well as alterations in B lymphocyte numbers, which appear to be partly due to a defect in the interaction of B cells with their stromal environment.

We obtained $Dlk1^{-/-}$ E11.5 embryos and compared the definitive hematopoietic progenitor numbers of their AGMs to those from wild-type embryos. Interestingly, the absence of Dlk1 caused a more than two-fold increase in the total number of progenitors (Figure 3F). This increase was not due to the selective expansion of one particular progenitor type since the percentage of each colony type was the same between wild-type and knockout embryos (*Online Supplementary Figure S3*). This may indicate that the absence of Dlk1 causes a similar expansion of both HSCs and progenitors or, alternatively, that HSCs alone are expanded, while maintaining their normal differentiation potential, thus giving rise to an expanded progenitor pool. To address this, we attempted to determine HSC numbers in $Dlk1^{-/-}$ embryos in transplantation assays; however, when E11.5 $Dlk1^{-/-}$ AGMs were transplanted into wild-type mice, there was such a severe rejection in the recipients that not only did almost 50% of them die within the first 2 weeks after transplantation (8 out of 17), but the remaining nine recipients that survived showed no donor contribution to the peripheral blood after 1 month (*data not shown*). Why the deletion of Dlk1 causes such a severe rejection will require further investigation.

To determine how changing Dlk1 levels may affect HSC numbers, we stained sections of E11.5 $Dlk1^{WT/WT}$, $Dlk1^{-/-}$ and $Dlk1^{TG/TG}$ embryos for: (i) CD34 to check for integrity of the aortic endothelium and for intra-aortic cluster quantification, (ii) Ki67 to identify proliferating cells, and (iii) apoptosis by the TUNEL assay. There were very few apoptotic cells in the vicinity of the dorsal aorta in wild-type and homozygous transgenic embryos, while Dlk1-deficient embryos showed an increase in apoptotic cells (Figure 3G, white arrows). However, the apoptotic cells were outside the aorta and did not co-localize with CD34-expressing cells, indicating that HSC survival was not affected. The aortic endothelium of $Dlk1^{-/-}$ and $Dlk1^{TG/TG}$ embryos also appears to be normal with formation of intra-aortic clusters (Figure 3G). There also did not seem to be a real difference in the number of intra-aortic clusters with an average of 5 per $Dlk1^{WT/WT}$ section, 4 per $Dlk1^{-/-}$ and 6.5 per $Dlk1^{TG/TG}$ section (clusters were counted on 14-21 different sections per genotype). However, there seemed to be a striking difference in the number of $Ki67^+$ proliferating cells (Figure 3G, white arrowheads). While we counted an average of 2 $Ki67^+$ cells in $Dlk1^{WT/WT}$ sections, this number increased to 12 in $Dlk1^{TG/TG}$ sections. Most of the $Ki67^+$ cells were found amongst circulating cells inside the aorta and as scattered cells in the mesenchymal tissue surrounding the aorta, but occasionally we found $Ki67^+$ cells within intra-aortic hematopoietic clusters (Figure 3G, three smaller right pan-

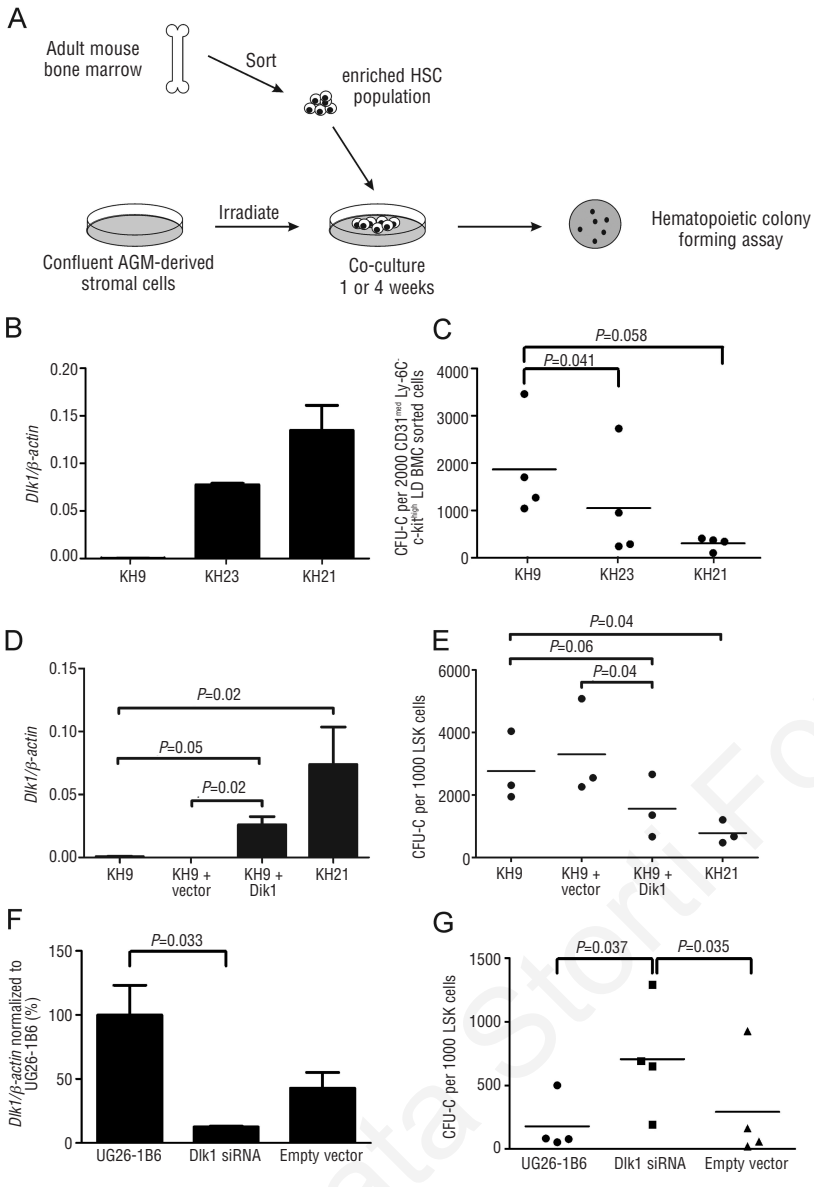

Figure 4. The supportive capacity of AGM-derived stromal cell lines correlates inversely with *Dlk1* levels. (A) Outline of co-culture experiments. (B) Real-time RT-PCR analysis of *Dlk1* expression in three AGM-derived stromal cell lines; n=2. (C) Number of colony-forming progenitors detected after 1 week of co-culture of HSC-enriched cells on KH9, KH23 and KH21 stromal cell lines; n=4. (D) *Dlk1* expression levels in untransfected KH9, KH21, KH9 transfected with a *Dlk1*-over-expressing vector and KH9 transfected with an empty vector; n=3. (E) Number of colony-forming progenitors detected after 1 week of co-culture of HSC-enriched cells on untransfected KH9 and KH21, *Dlk1*-overexpressing and empty vector-transfected KH9 stromal cell lines; n=3. (F) *Dlk1* expression levels in *Dlk1* siRNA and empty vector transfected UG26-1B6 cells relative to untransfected cells; n=4. (G) Number of colony-forming progenitors detected after 4 weeks of co-culture of HSC-enriched cells on untransfected, *Dlk1* siRNA-transfected and empty vector-transfected UG26-1B6 stromal cell lines; n=4.

els) and also in the perivascular layer and in rounded endothelial cells (*not shown*). We were unable to detect any Ki67$^+$ cells in the majority of *Dlk1*$^{-/-}$ sections.

Dlk1 is produced by cells of the aorta-gonad-mesonephros hematopoietic microenvironment

The expression of Dlk1 observed in the AGM (Figure 1) suggests that this protein might be produced by cells of the hematopoietic regulatory environment. Stromal cell lines are a well-established model for the HSC niche, and their study has resulted in the identification of a number of HSC regulators.[25] We therefore selected three AGM-derived stromal cell lines that express differing levels of *Dlk1* and analyzed their ability to support hematopoiesis in a co-culture system (Figure 4A). AGM-derived stromal cell lines are equally supportive for HSCs from the AGM or the bone marrow.[20] Therefore, in order to obtain sufficient numbers of HSCs for a quantitative analysis of hematopoietic support, we isolated HSCs from murine bone marrow and co-cultured these with AGM stromal cell lines.

While KH21 expressed almost twice the amount of *Dlk1* found in KH23, KH9 was virtually negative for *Dlk1* expression (Figure 4B). When these lines were tested in 1-week co-culture experiments, a negative correlation was observed between the levels of *Dlk1* expression in the cells and their hematopoiesis-supportive activity (Figure 4C). To ensure that the differences in supportive activity of the three cell lines were indeed due to differing levels of Dlk1 rather than to the fact that they are independently derived cell lines, we overexpressed *Dlk1* in KH9, the cell line with the lowest levels of *Dlk1*. Introduction of a *Dlk1*-expressing vector resulted in *Dlk1* levels that were almost halfway between those of the untransfected KH9 and KH21, while the introduction of the empty vector did not cause an up-regulation of *Dlk1* in KH9 (Figure 4D). We then repeated the co-culture experiments with KH9, KH21 and KH9 transfected with *Dlk1* or the empty vector and found that overexpression of *Dlk1* in KH9 decreased its

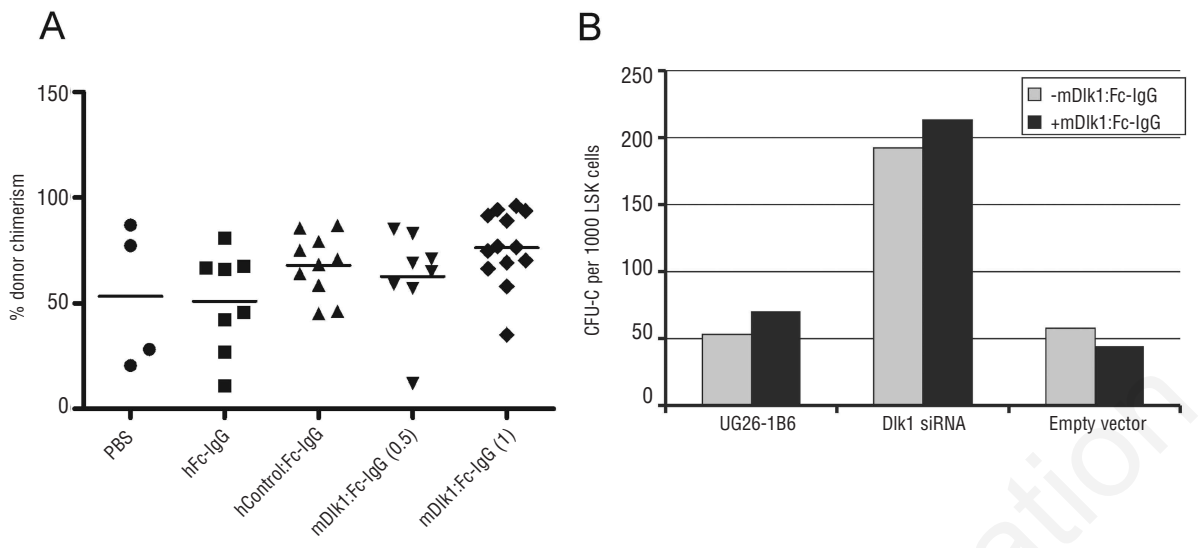

Figure 5. The effect on hematopoietic stem and progenitor cells requires the membrane-bound form of Dlk1. (A) Donor chimerism achieved with E11.5 AGMs cultured for 3 days in the presence of phosphate-buffered saline (PBS), hFc-IgG, hControl:Fc-IgG or mDlk1:Fc-IgG at 0.5 or 1 μg/mL. (B) Number of colony-forming progenitor cells detected after 4 weeks of co-culture of HSC-enriched cells on untransfected, *Dlk1* siRNA-transfected and empty vector-transfected UG26-1B6 stromal cell lines in the presence or absence of 1 μg/mL of mDlk1:Fc-IgG.

hematopoiesis-supportive activity to a level that was almost half-way between KH9 and KH21, while the empty vector had no effect (Figure 4E).

To provide further evidence for *Dlk1* expression in the AGM microenvironment having a negative influence on HSPC support, we knocked down the levels of *Dlk1* expression in the well-characterized, highly supportive stromal cell line UG26-1B6, which is also derived from the AGM.[20,25] Transfection of these cells with a *Dlk1*-targeting short-interfering RNA vector resulted in a decrease of *Dlk1* expression to 13% of wild-type (Figure 4F). When compared in a 4-week, long-term co-culture experiment, the knockdown cell line showed a four-fold increase in hematopoietic support (Figure 4G). *Dlk1* is therefore expressed by stromal cells found in the hematopoietic microenvironment and reduces their ability to support hematopoiesis. This further supports a role for Dlk1 as a negative regulator in the hematopoietic microenvironment of the AGM.

The effect on hematopoietic stem and progenitor cells requires the membrane-bound form of Dlk1

Since Dlk1 can exist both as a soluble and a membrane-bound form, we asked the question whether soluble Dlk1 added to AGM explant cultures could recapitulate the negative effect on HSCs observed with overexpressing *Dlk1 in vivo* or in stromal cell lines. Interestingly, adding soluble Dlk1 at a concentration of up to 1 μg/mL did not decrease the repopulation activity of E11.5 wild-type AGMs when compared with AGMs exposed to phosphate-buffered saline or two different control IgG fusion proteins (Figure 5A). We also investigated whether adding soluble Dlk1 to co-cultures of HSCs on stromal cell lines in which *Dlk1* had been knocked down could reverse the enhanced maintenance. However, as was the case with the AGM explant cultures, adding soluble Dlk1 to the co-cultures had no effect on hematopoietic stem and progenitor cell (HSPC) support (Figure 5B). This suggests that Dlk1 needs to be in its membrane-bound form to act as a negative regulator of HSPCs. A differential effect of the soluble and transmembrane forms on HSC maintenance has also been reported for Kitl.[31]

Discussion

We have shown here that Dlk1 is a regulatory factor produced in the AGM region at the time of HSC production that has a negative impact on HSPC numbers. This effect was demonstrated by measuring HSPC content in AGMs from two different *in vivo* genetic models, a complete *Dlk1* knockout mouse line and a transgenic *Dlk1* overexpressing line. This HSPC inhibitory activity of Dlk1 does not appear to be related to a negative influence on cell survival, as we did not observe any changes in the number of apoptotic cells in the aorta in *Dlk1*-overexpressing or knockout embryos. There also does not seem to be a defect in HSC generation, as the number of intra-aortic clusters remained the same. The effect, therefore, may be at the level of HSC function. We saw more proliferating cells in the circulation as well as within the intra-aortic cell clusters in the *Dlk1*-transgenic embryos. However, since AGMs from these embryos had reduced stem cell activity, this increase in proliferation did not result in true HSC self-renewal, but rather seemed to be incompatible with HSC function and/or maintenance. Accordingly, a decrease in proliferating cells was observed in Dlk1 knockout embryos. In addition, we saw increased numbers of apoptotic cells in the mesenchyme surrounding the dorsal aorta of *Dlk1*$^{-/-}$ embryos. It is currently unclear whether these cells are part of the AGM hematopoietic microenvironment and whether this contributes to the increase in HSPC numbers.

The expression pattern of *Dlk1* and the experiments using AGM-derived stromal cell lines suggest that Dlk1 does not act cell autonomously, but is produced by cells of the AGM hematopoietic microenvironment. Very little is currently known about the cell types that make up the

HSC niche in the AGM. Mesenchymal stem/stromal cells have been shown to be crucial components of the HSC niche in adult bone marrow, where they are thought to reside in a perivascular location.[32,33] Cells with mesenchymal stem/stromal cell potential have also been identified in the AGM at the time of HSC emergence.[34] If these, in analogy with their adult bone marrow counterparts, are also located in the pericyte/smooth muscle layer of the dorsal aorta, then Dlk1 might be a regulatory factor produced by mesenchymal stem/stromal cells in the AGM as this is where we found *Dlk1* to be expressed. Since these cells are directly adjacent to the endothelial layer of the dorsal aorta, where HSCs are thought to emerge, they could interact directly with HSCs via cell surface Dlk1. Interestingly, a role for Dlk1 in post-natal neurogenesis in the subventricular zone was recently described, where Dlk1 secreted from niche astrocytes acts on neural stem cells that are required to express the membrane-bound version of Dlk1 on their cell surface.[35] However, this potential interaction between Dlk1 secreted from the niche and Dlk1 expressed on the surface of stem cells is unlikely to occur in (AGM) hematopoiesis since we did not detect Dlk1 on any blood cells in numerous sections of the aorta, and Dlk1 expression has not been found in adult HSPCs.[13,14] Dlk1 plays a role in controlling stromal cell differentiation and could, therefore, alter the hematopoietic microenvironment through this means. Interestingly, *Dlk1* has been reported to be expressed in bone marrow mesenchymal stem/stromal cells.[36]

Very little is currently known about interaction partners of Dlk1. Due to its EGF-like repeats, it has been classified as a protein homologous to members of the Notch/Delta family. However, Dlk1 lacks the DSL domain which is present in Notch ligands and which is required for interactions with Notch. Despite this, Dlk1 has recently been reported to act as an inhibitor of Notch signaling.[11,37,38] Considering the known role of Notch in promoting hematopoietic development,[39,40] it may be that Dlk1 negatively influences AGM hematopoiesis via this mechanism.

It may seem surprising that a negative regulator of emerging HSCs is up-regulated at the time and in the location where HSCs are detected and that it is downstream of the transcription factor Runx1, which is known to be essential for HSC production in the AGM. Both positive[17] and negative[41] effects of environmental Dlk1 on HSPCs have been described, which are likely to be dependent on the specific cellular context. The presence of physiologically important negative regulators of HSCs in the adult bone marrow niche has already been described,[42-44] and although no negative regulators have been identified in the AGM, it is known that HSC numbers are limited here.[3] The AGM appears to be primarily a site for HSC emergence, while the expansion of the HSC pool takes place in the fetal liver. Hence, in the AGM, Dlk1 may be part of a negative control mechanism that is initiated as soon as HSC generation commences and that restricts HSC expansion in this tissue, which may not be able to support large numbers of HSCs. This highlights the fact that biological processes are often the result of a fine balance between promoting and inhibiting control mechanisms. This fine tuning is especially important in the context of stem cells, where slight imbalances can lead to dramatic changes in the proliferation and differentiation output of these self-renewing, multipotential cells, and which is a major contributing factor to the development of malignancies.

Unlike the AGM, the fetal liver is well known for its remarkable capacity to expand HSCs. Interestingly, it has been reported that Dlk1 might be one of the components responsible for the supportive capacity of the fetal liver,[17] where it is highly expressed in cells of the hepatocyte lineage,[45] which we also observed in our embryo sections. The fetal liver microenvironment is functionally, and possibly also structurally, very different from the AGM microenvironment. Unlike the AGM, the fetal liver is not a site for *de novo* HSC generation from pre-HSCs, but it is here that HSC expansion occurs as well as differentiation into the different types of mature cells, thus resulting in the production of an almost adult-type hematopoietic system. These different demands on the fetal liver microenvironment might explain the requirement for a seemingly opposite action of Dlk1. The mechanism by which this is achieved may be due to the fact that *Dlk1* is expressed in a different cell type on which it may have a different effect. This, once again, indicates that the action of Dlk1 is highly context-dependent. This is further supported by work from Tetsuo Sudo's group who have demonstrated that the action of Dlk1 on hematopoietic progenitors is influenced by the specific cytokine milieu.[41]

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