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

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 doi:10.3324/haematol.2012.070789

Online Supplementary Design and Methods

Mice and embryo generation

The following mouse strains were used for this study: wild-type C57BL/6, *Gata3* knockout,[1] *Runx1* knockout,[2] *Runx1-LacZ* knockin,[3] *Dlk1* transgenic[4] and *Dlk1* knockout.[5]

Gene expression analysis

For the detection of the different *Dlk1* isoforms by conventional reverse transcriptase polymerase chain reaction (RT-PCR), the following primers were used; forward: GCTGGATTCGTCGACAAGAC, reverse: CGCACTTGTTGAGAAAGACG. For the quantification of *Dlk1* transcript levels by real-time RT-PCR analysis, the following primers were used; *Dlk1* forward: GCTGGATTCGTCGACAAGAC, reverse: ATCCTTGCAGATGCACTGC; *Actb forward*: TCCTGGCCTCACTGTCCA, reverse: GTCCGCCTAGAAGCACTTGC.

Immunohistochemistry and in situ hybridization

Tissue sections were stained with antibodies specific for CD34 (1:50, BD Biosciences, Oxford, UK), Dlk1 (1:500, Millipore, Watford, UK), smooth muscle actin, alpha (1:200, Sigma Aldrich, Gillingham, UK), Runx1 (a kind gift from Thomas Jessell, Columbia University, New York, USA),[6] CD31 (1:30-1:100, BD Biosciences, Oxford, UK), tyrosine hydroxylase (1:500, Millipore, Watford, UK) and Ki67 (1:500, Abcam, Cambridge, UK) as described previously.[7] For the detection of apoptotic cells by the TUNEL assay, an ApopTag Red In Situ Apoptosis Detection Kit was used following the manufacturer's instructions (Millipore, Watford, UK). Images were taken using a Zeiss LSM510 META Confocal Microscope or a Radiance 2000 Confocal Scanning System (Bio-Rad, Hemel Hempstead, UK) and analyzed with Zeiss LSM image software (Carl Zeiss Ltd., Wellyn, UK) or Laser Sharp 2000 software (Carl Zeiss Ltd., Wellyn, UK). For the detection of *Dlk1* transcripts, a riboprobe was prepared by RT-PCR from embryonic day (E) 11 aorta-gonad-mesonephros (AGM) cDNA using the following primers; forward: GTGACCCCCAGTATGGATTC, reverse: GCTTGCACAGACACTCGAAG. *In situ* hybridization experiments were performed as described previously[8] and pictures were taken on a Zeiss AxioSkop2 Wide-Field Microscope and analyzed with Zeiss AxioVision software (Carl Zeiss Ltd., Wellyn, UK).

Transfection of aorta-gonad-mesonephros stromal cell lines

To obtain Dlk1 overexpression, KH9 stromal cells were stably transfected with either linearized empty pEGFP-N1 vector or with a linearized pEGFP-N1 vector containing full-length *Dlk1* (Open Biosystems, Rockford, USA). For the knockdown of *Dlk1*, UG26-1B6 cells were stably transfected with linearized pSUPER.retro.neo+GFP vector (Oligoengine, Seattle, USA) containing the siRNA sequence GCTGGATTCGTCGACAAGA, which targets *Dlk1* at nucleotides 743-761 (NM_010052). Empty pSUPER.retro.neo+GFP vector was used as a control.

Co-culture experiments

All stromal cell lines were maintained at 33°C in expansion medium containing 50% Myelocult M5300 (Stem Cell Technologies, Grenoble, France), 35% α-MEM (Invitrogen, Paisley, UK), 15% fetal calf serum (Sigma Aldrich, Gillingham, UK), 0.5% penicillin-streptomycin (Sigma Aldrich, Gillingham, UK) and 10 μM β-mercaptoethanol. Bone marrow hematopoietic stem cell-enriched cell populations were obtained by either isolating low-density cells using the discontinuous Ficoll gradient method by E. Schneider *et al.*,[9] followed by sorting for cells with the phenotype $CD31^{med}$ $Ly\text{-}6C^{-}$ $ckit^{high}$;[10] or by isolating cells with the Lin^{-} $Sca1^{+}$ $ckit^{+}$ (LSK) phenotype. These were seeded on irradiated stromal cell lines in Myelocult M5300/1% penicillin-streptomycin/10^{-6} M hydrocortisone or expansion medium.

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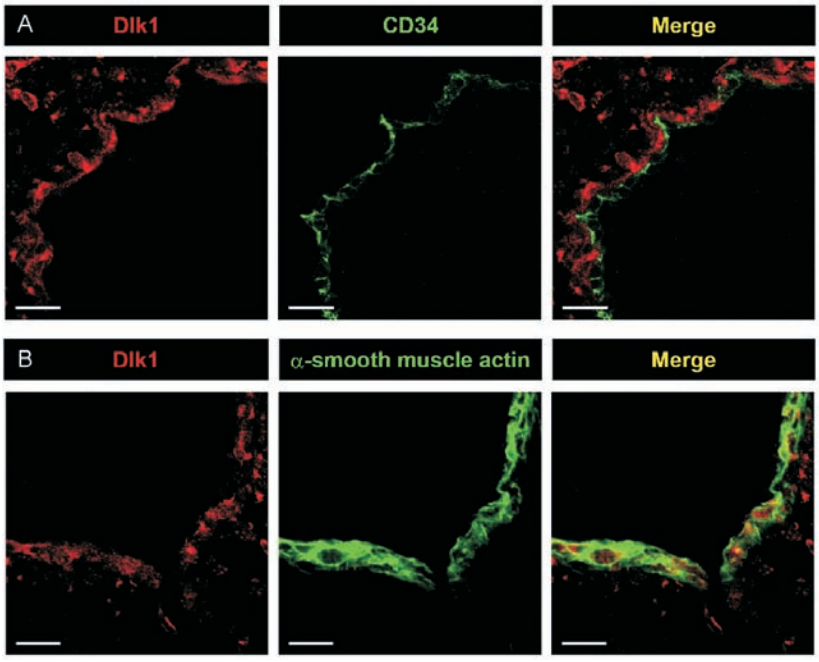
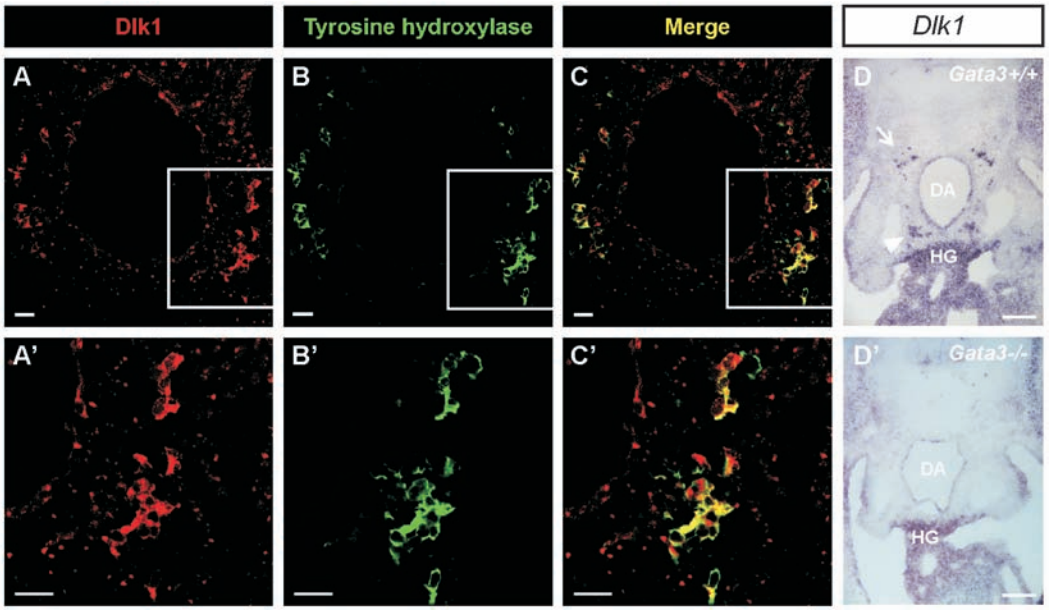
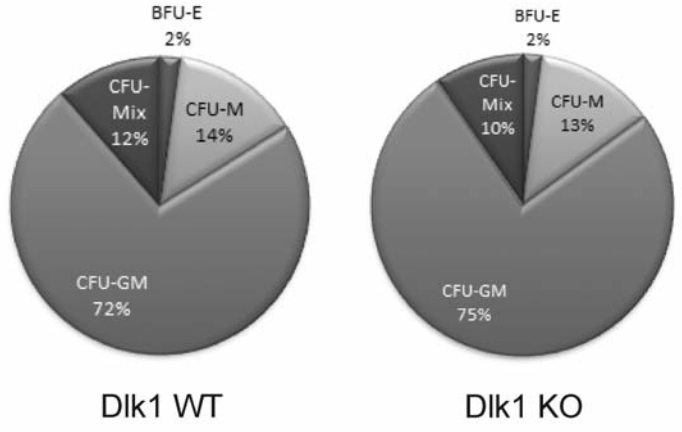


Online Supplementary Figure S1. Expression analysis of Dlk1 in the mid-gestation embryo. (A,B) Immunohistochemical co-staining for Dlk1 (red, Cy3 in A and FITC in B) and (A) CD34 (green, FITC) or (B) smooth muscle actin, alpha (green, Cy3) on sections of E11.5 wild-type aortas. The scale bar is 20 μm.



Online Supplementary Figure S2. Dlk1 is expressed in cells of the developing sympathetic nervous system. (A-C) Immunohistochemical co-staining of E11.5 embryo sections showing the dorsal aorta (ventral down) and Dlk1 in red (Cy3) and tyrosine hydroxylase in green (Alexa 488). Close-up of boxed region shown in A'-C'; the scale bar is 20 μm. *Dlk1* expression analysis by *in situ* hybridization on *Gata3*$^{+/+}$ (D) and *Gata3*$^{-/-}$ (D') E11.5 embryo sections. *Dlk1*-expressing sympathetic ganglia (arrow) and adrenal anlage (arrowhead) in wild-type embryos are indicated; the scale bar is 100 μm; DA, dorsal aorta; HG, hindgut.



Online Supplementary Figure S3. *Dlk1* knockout AGMs have a similar ratio of definitive hematopoietic progenitors. E11.5 wild-type and *Dlk1* knockout AGM cells were plated in methylcellulose assays. The total number of colonies was counted after 1 week and the colony types scored; n=3.