SCL interacts with VEGF to suppress apoptosis at the onset of hematopoiesis

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Summary

During development, hematopoiesis initiates in the yolk sac through a process that depends on VEGF/Flk1 signaling and on the function of the SCL/Tal1 transcription factor. Here we show that VEGF modifies the developmental potential of primitive erythroid progenitors and prolongs their life span. Furthermore, the survival of yolk sac erythrocytes in vivo depends on the dose of VEGF. Interestingly, in *Vegf*$^{lo/lo}$ embryos carrying a hypomorph allele, Flk1-positive cells reach the yolk sac at E8.5, but are severely compromised in their ability to generate primitive erythroid precursors. These observations indicate that during embryonic development, different thresholds of VEGF are required for the migration and clonal expansion of hematopoietic precursors. The near absence of primitive erythroid precursors in *Vegf*$^{lo/lo}$ embryos correlates with low levels of *Scl* in the yolk sac. Strikingly, gain-of-function of SCL partially complements the hematopoietic defect caused by the hypomorph *Vegf*^{lo} allele, and re-establishes the survival of erythroid cells and the expression of erythroid genes (*Gata1* and βH1). This indicates that SCL functions downstream of VEGF to ensure an expansion of the hematopoietic compartment.

Key words: VEGF, SCL, TAL1, Primitive erythropoiesis, Hematopoiesis, ES cell differentiation, Mouse

Introduction

Normal development is determined by interactions between a cell and its environment, and by the expression of tissue-specific genes, which allow the control of cell growth, survival and/or cell differentiation. Whereas definitive hematopoiesis, which is fairly well understood, involves signals from the environment and the expression of key transcription factors, the molecular mechanisms regulating primitive erythropoiesis remain to be determined.

Gene-targeting experiments indicate a prerequisite role for vascular endothelial growth factor A (VEGF) (Ferrara and Henzel, 1989; Gospodarowicz et al., 1989) signaling and for the basic helix-loop-helix (bHLH) transcription factor SCL (also known as TAL1) (Begley et al., 1989; Finger et al., 1989; Chen et al., 1990) during the establishment of the hematopoietic system. VEGF interacts with two tyrosine kinase receptors, Flt1 (Shibuya et al., 1990) and Flk1 (KDR – Mouse Genome Informatics) (Matthews et al., 1991; Millauer et al., 1993; Yamaguchi et al., 1993). The role of Flt1 during hematopoietic development is unclear because mice lacking the tyrosine-kinase domain of Flt1 have no obvious hematopoietic defects (Hiratsuka et al., 1998). In contrast, Flk1-deficient embryos die at midgestation (E8.5-9.5) because of the absence of blood islands (Shalaby et al., 1995). When differentiated in vitro, *Flk1*$^{-/-}$ embryonic stem (ES) cells retain the capacity to produce hematopoietic cells (Hidaka et al., 1999; Schuh et al., 1999), suggesting that Flk1 is not involved in hematopoietic commitment per se. In chimeras, *Flk1*$^{-/-}$ cells fail to contribute to primitive and definitive hematopoiesis (Shalaby et al., 1997). Instead, they accumulate aberrantly on the surface of the amnion, which indicates that VEGF might be involved in the migration of Flk1-positive precursors from the mesoderm to sites of hematopoiesis, as reported for *Drosophia* (Cho et al., 2002). As with *Flk1*$^{-/-}$ embryos, the loss of a single *Vegf* allele is lethal between E8.5 and E9.5 (Carmeliet et al., 1996; Ferrara et al., 1996) because of defects in blood island formation (Damert et al., 2002). This reveals a unique, tight dose-dependent regulation of embryonic vessel and hematopoietic development by VEGF.

SCL also plays a central role at the onset of hematopoiesis and vasculogenesis. SCL is first co-expressed with Flk1 at E7.0 in cells of the visceral mesoderm that are destined to generate blood islands. As blood islands develop, SCL expression is maintained in primitive erythrocytes and at low levels in

endothelial cells, whereas Flk1 becomes restricted to vascular cells (Shalaby et al., 1995; Shalaby et al., 1997; Elefanty et al., 1999; Drake and Fleming, 2000). Gene targeting and chimera analyses reveal that SCL is required for the generation of primitive and definitive hematopoietic lineages and for the remodeling of yolk sac vasculature (Robb et al., 1995; Shivdasani et al., 1995; Robb et al., 1996; Porcher et al., 1996; Visvader et al., 1998).

Evidence is accumulating to indicate that SCL might function downstream of VEGF/Flk1 signaling. First, SCL expression is subsequent to Flk1 in vivo (Elefanty et al., 1999; Drake and Fleming, 2000) and during the differentiation of ES cells in vitro (Robertson et al., 2000). Importantly, SCL expression is not detected in *Flk1*$^{-/-}$ embryos (Ema et al., 2003). Secondly, Flk1 and SCL are both required for blood island development (Robb et al., 1995; Shalaby et al., 1995; Shivdasani et al., 1995; Visvader et al., 1998). Moreover, gain-of-function studies indicate that SCL might act at the level of the putative, common hematopoietic and endothelial precursor, the hemangioblast, to specify and promote hematopoietic and endothelial fates at the expense of other mesoderm-derived tissues (Gering et al., 1998; Mead et al., 1998; Mead et al., 2001; Ema et al., 2003). Interestingly, ES cell-derived hemangioblasts (also known as blast colony-forming cells, or BL-CFCs) are found mostly in the Flk1/SCL double-positive population (Chung et al., 2002) and require VEGF (Kennedy et al., 1997; Choi et al., 1998) and SCL function (Faloon et al., 2000; Robertson et al., 2000; Ema et al., 2003). Finally, SCL can rescue the hematopoietic and vascular defect of the Zebrafish mutant *cloche* (Liao et al., 1998), which acts upstream of Flk1 (Liao et al., 1997), and allow blast colony formation in the absence of Flk1 signaling in vitro (Ema et al., 2003). However, it is not clear whether SCL rescues the multiple defects associated with Flk1 deficiency in vivo.

Hematopoietic cells have a finite life span in vitro and in vivo. When hematopoietic progenitors are plated in culture with the appropriate growth factors, they survive and first proliferate actively but eventually cease growth. It is not clear whether this growth arrest is determined intrinsically, or whether it can be influenced by environmental factors. Despite the importance of Flk1 signaling in hematopoiesis, it is not clear how VEGF/Flk1 regulates the development of hematopoietic cells. In the present study, we used cellular and genetic approaches to further define the role of VEGF and SCL at the onset of hematopoiesis.

Materials and methods

Mice

Vegf^{lo} hypomorph mice (Damert et al., 2002) were maintained on a mixed CD-1/129 background. We were unsuccessful in our attempt to breed the *Vegf*^{lo} allele onto a C57BL/6 background. The *Scl* transgenic line A(5)3SCL, in which the *Scl* transgene is placed under the control of the ubiquitous *Sil* promoter (Aplan et al., 1997), was maintained on a C57BL/6 background. Analysis of *Vegf*^{lo} hypomorph mice and *Vegf*^{lo}*Scl*^{tg} compound embryos were therefore performed on a mixed background. Animals were maintained under pathogen-free conditions according to institutional animal care and use guidelines.

Flow cytometry and antibodies

Yolk sacs were dissociated in 0.25% collagenase (Sigma-Aldrich) in PBS supplemented with 20% FCS. Cells were first immunostained as described (Herblot et al., 2000) using a phycoerythrin-conjugated TER119 antibody (Pharmingen BD Biosciences). Cells were then labeled with a fluorescein isothiocyanate-conjugated Annexin V (Pharmingen) as described previously (Krosl et al., 1998). Included was 7-amino actinomycin D (7-AAD, Calbiochem) to detect dead cells. Cells were analyzed on a FACScalibur flow cytometer (Becton-Dickinson).

Histology and immunohistochemistry

Yolk sacs were fixed in 4% paraformaldehyde in PBS. Tissues were washed with PBS and gelled in 2% agarose to facilitate transversal sectioning once embedded in paraffin. Agarose embedding did not hinder the staining of sections with dyes or antibodies. Samples were sectioned at 5 μm.

For immunohistochemistry, deparaffinized slides were placed in 1% SDS in PBS for 5 minutes then washed with water. Endogenous peroxydase activity was blocked with 1% H_2O_2, the fixative was quenched with 300 mM glycine and nonspecific binding was blocked with 10% horse serum (Sigma) in PBT (PBS supplemented with 0.2% tween-20). Sections were first incubated with a mouse antibody directed against KI67 (Pharmingen), overnight at 4°C. Slides were washed with PBT then incubated with a biotin-conjugated horse anti-mouse antibody (Vector Laboratories) followed by streptavidin-horseradish peroxydase (NEN), both incubated for 1 hour at room temperature. Positive cells were revealed with the peroxydase substrate 3,3′-diaminobenzidine (Sigma) and counterstained with methyl green.

Growth and differentiation of ES cells

Parental wild-type R1 (Nagy et al., 1993), *Vegf*$^{-/-}$ clones 36.7, 44.7 and 44.8 (Carmeliet et al., 1996) and the feeder-independent CCE (Robertson et al., 1986) ES cell lines have been described previously. ES cells were maintained on irradiated mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium (Gibco) supplemented with 15% FCS (Gemini Bio-Products), 1000 U ml^{-1} leukemia inhibitory factor (LIF) and 1.5×10^{-4} M monothioglycerol (MTG, Sigma). Prior to differentiation studies, feeder cells were diluted out following 3-4 sequential passages on gelatinized flasks.

Embryoid bodies (EBs) were generated as previously described (Keller et al., 1993). Briefly, dissociated ES cells were plated at a concentration of 0.3×10^4-1.0×10^4 ml^{-1} into Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 15% FCS (Gibco), 2 mM glutamine (Gibco), 50 μg ml^{-1} ascorbic acid (Sigma), 5% protein-free hybridoma medium (PFHM II, Gibco) and 3×10^{-4} M MTG. When indicated, recombinant human $VEGF_{165}$ (Sigma) was added 3 days after the initiation of EB differentiation (day 3). Day 7 EBs were dissociated into a single-cell suspension using 0.25% trypsin, 1 mM EDTA (Gibco). The size of EBs and primitive erythroid colonies (Ery^P) was assessed using imaging software (Northern Eclipse, Empix Imaging). Blast colonies were generated in methylcellulose cultures containing 10% FCS, 5 ng ml^{-1} interleukin 6 (IL-6) and 20% D4T endothelial conditioned medium as previously described (Kennedy et al., 1997). When indicated, 5 ng ml^{-1} VEGF was added to cultures.

Hematopoietic colony assays and growth factors

Hematopoietic colonies were generated by plating either dissociated day 7 EB cells at 4×10^5 ml^{-1} or half of dissociated E8.5 yolk sac in IMDM containing 1% methylcellulose, 10% FCS (Gibco), 5% PFHM II, 200 μg ml^{-1} transferrin, 100 ng ml^{-1} KL, 2 U ml^{-1} EPO, 5 ng ml^{-1} IL-6, 5 ng ml^{-1} IL-3, 5 ng ml^{-1} M-CSF, 30 ng ml^{-1} G-CSF (Amgen), 1 ng ml^{-1} LIF, 5 ng ml^{-1} VEGF and 1.5×10^{-4} M MTG. KL, EPO, IL-6, IL-3, M-CSF, and LIF were derived from media conditioned by COS cells transfected with corresponding expression vectors.

Gene expression

Representative amplification of total cDNA was carried out as

described previously (Sauvageau et al., 1994). Amplified cDNA was resolved on 1.2% agarose gels and transferred to nylon membranes (Pall Corporation) for hybridization. βH1: 269-bp fragment amplified by PCR using forward primer 5′-TTGTTACAGCTCCTGGGCA-3′ and reverse primer 5′-CCCAAAAAGTCAATGTTATT-3′. βmajor: 135-bp fragment immediately upstream of the polyA tail. *Gata1*, 443-bp fragment was amplified by PCR using forward primer 5′-GGAGACAGGATCTTCTGTAG-3′ and reverse primer 5′-CATGCTCCACTTGACACTGA-3′. *Scl*: 438-bp fragment was prepared by PCR using forward primer 5′-CATAACCACAGAGAGAATCCC-3′ and reverse primer 5′-ACACTATCATCACCACACTGG-3′ (Hoang et al., 1996). *Flk1*: 944 bp 3′ *Hin*cII fragment. Ribosomal *L32*: genomic 1.6 kb *Sac*I fragment encompassing the final exon. The *L32* probe was kindly provided by Dr N. Iscove and the βH1 probe by Dr G. Sauvageau. The hybridation signals were analyzed on a PhosphorImager apparatus (Molecular Dynamics).

Quantification of *Scl* mRNA from *Scl*^{tg} yolk sacs was accomplished using the Quantitect SYBR Green PCR kit (Quiagen), performed on a MX4000 apparatus (Stratagene) following the manufacturer's instructions. Briefly, cDNA was generated as described previously (Herblot et al., 2000), normalized for equal *S16* levels and either endogenous mouse or transgenic human *Scl* levels quantified using standard curves determined with known molar amounts of either mouse or human *Scl* templates, respectively. *S16*: forward primer, 5′-AGGAGCGATTTGCTGGTGTG-3′; reverse primer, 5′-GCTACCAGGGCCTTTGAGATG-3′. Mouse *Scl*: forward primer, 5′-GGGCAGTTGATGTGTTTGTGTCA-3′; reverse primer, 5′-GCCCAGCCCCTTTAGAAACTTTC-3′. Human *Scl*: forward primer, 5′-TCCCTATGTTCACCACCAAC-3′; reverse primer, 5′-GATGTGTGGGGATCAGCTT-3′.

Results

VEGF enhances primitive erythropoiesis during in vitro differentiation of Vegf$^{-/-}$ ES cells

We examined the effects of exogenous VEGF during the differentiation in vitro of *Vegf*$^{-/-}$ ES cells (Carmeliet et al., 1996). *Vegf*$^{-/-}$ ES cells were allowed to differentiate into EBs following LIF withdrawal. VEGF was added on day 3 of culture, at the time when its receptor, Flk1, is first detected (Kabrun et al., 1997), while control cultures were maintained in the absence of VEGF (Fig. 1A). On addition of VEGF, EBs harvested on day 7 appeared larger and more hemoglobinized (Fig. 1B-E). Size quantification using imaging software confirmed that VEGF-treated EBs were shifted in size compared to untreated EBs (P<0.001) (Fig. 1F).

To define whether the larger size of VEGF-treated EBs was caused by enhanced erythropoiesis, we first quantified the number of primitive erythroid precursors emerging from VEGF-treated and untreated EBs using a hematopoietic colony assay (Keller et al., 1993). When VEGF was added to differentiating *Vegf*$^{-/-}$ ES cells, we observed a dose dependent increase in the frequency of Ery^{P} (Fig. 1G). Hence, the increase in Ery^{P} was linear in the range 0.6-15.0 ng ml^{-1} and did not increase further beyond 15.0 ng ml^{-1}. The VEGF-induced increase in the number of primitive erythroid precursors was observed in three independent *Vegf*$^{-/-}$ ES clones and the parental ES line R1 (Nagy et al., 1993). The identity of Ery^{P} was assessed based on morphology and cytospin analysis (Fig. 1H,I). Together, these results demonstrate that VEGF enhances the hematopoietic output during primitive erythropoiesis.

To better characterize the effect of VEGF, EBs were analyzed individually. Although hemoglobinization occured in most EBs in the absence of VEGF, few if any of these EBs gave rise to hematopoietic colonies upon replating (Fig. 2A). In contrast, in the presence of VEGF, 15 out of 25 EBs gave rise to primitive erythroid colonies, with a mean of 10 Ery^{P} per EB. The increase in Ery^{P} cannot be accounted for on the basis of size expansion, because the average number of cells per EB was increased only by 1.6 fold (data not shown). Thus, our observations indicate that VEGF significantly enhances the erythroid potential of ES cells.

Hematopoietic markers are specifically induced in EBs exposed to VEGF

Next, we extended our analysis at the molecular level to ascertain the effects of VEGF. Day 7 *Vegf*$^{-/-}$ EBs, cultured either with or without VEGF, were harvested individually and

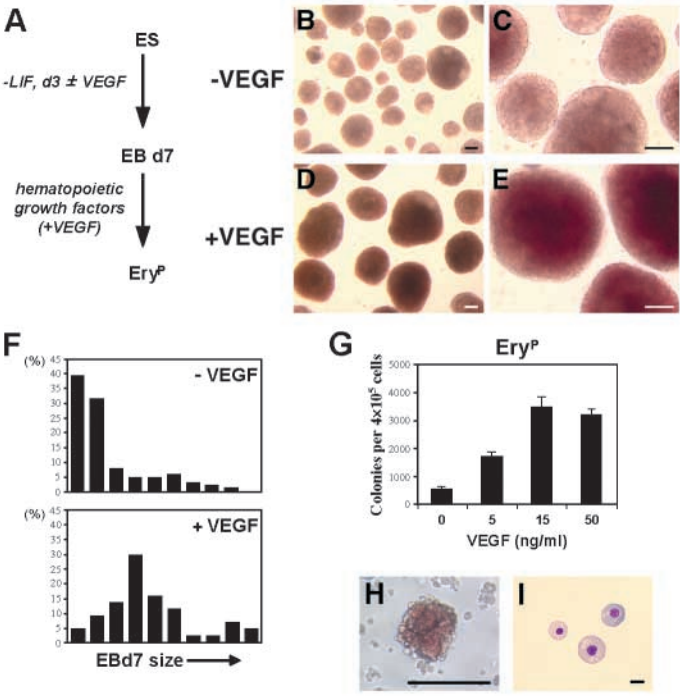

Fig. 1. VEGF stimulates primitive erythropoiesis. (A) ES cells were differentiated into EBs in either the presence or absence of VEGF, which was added on day 3 (d3). The hematopoietic precursor content of EBs was assessed by plating dissociated cells in the presence of hematopoietic growth factors (See Materials and methods). LIF, leukemia inhibitory factor; Ery^{P}, primitive erythroid colonies. (B-E) Morphology of day-7 EBs. *Vegf*$^{-/-}$ ES cells were differentiated for 7 days in either the absence (B,C) or presence (D,E) of VEGF (5 ng ml^{-1}). Note the larger size and the intensity of hemoglobinization in VEGF-treated EBs. (F) EB size was estimated by integrating individual surface area using Northern Eclipse software. Histograms illustrate the distribution of individual EB area. The range covered by columns is equivalent and determined arbitrarily. Data were analyzed by Student's *t*-test: –VEGF, n=102; +VEGF, n=44; P<0.001. (G) VEGF stimulates a dose-dependent increase in the number of primitive erythroid progenitors. *Vegf*$^{-/-}$ ES cells were differentiated in the presence of increasing concentrations of VEGF and assayed for primitive erythroid precursors (Materials and methods). Results are the mean±s.d. of duplicates and are representative of five independent experiments. (H) Morphology of day 4 Ery^{P}. (I) Morphology of colony cells revealed by Wright-Giesma stain. Scale bars: 100 μm in B-E,H; 10 μm in I.

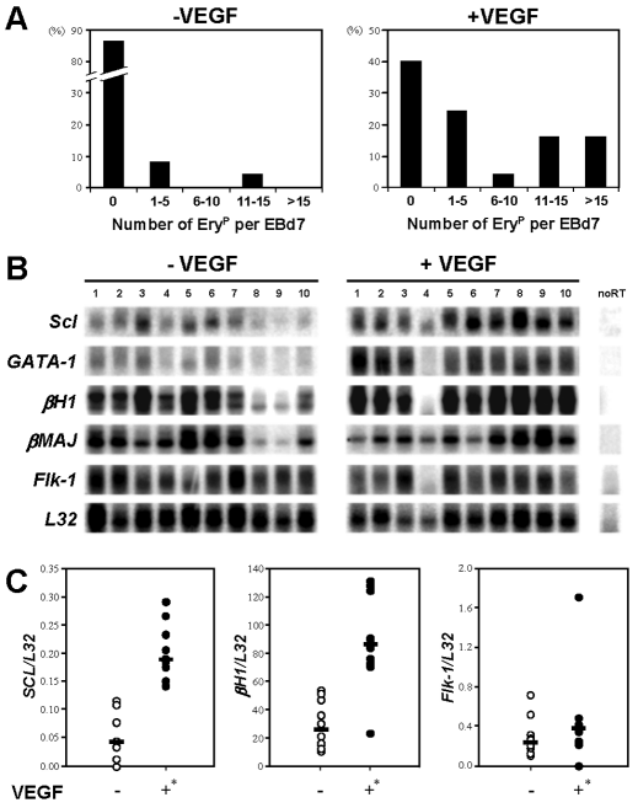

Fig. 2. Clonal analysis of the effect of VEGF during primitive erythropoiesis. (A) VEGF increases the number of Ery^P per single EB. On day 7 of culture either with or without VEGF (5 ng ml^{-1}), 25 EBs were picked at random and assayed individually into hematopoiesis. Histograms represent the frequency of EBs giving rise to the indicated number of Ery^P. (B) Gene-expression analysis of day-7 EBs treated with VEGF. Individual EBs (10) were dissociated as above and analyzed for hematopoietic-marker expression. No reverse transcriptase (RT) served as a control for genomic DNA contamination. Membranes were hybridized sequentially with the probes as shown. *L32* is a loading control. (C) Plots illustrate the level of gene expression in a single EB compared to *L32*. Horizontal bars represent median values. *P<0.05 compared to untreated cells.

examined for globin expression. When treated with VEGF, EBs expressed on average three-fold higher levels of embryonic βH1 globin, relative to an *L32* internal control, whereas the levels of adult globin (*βMAJ*) and that of *Flk1* was unchanged (Fig. 2B). As illustrated (Fig. 2C), VEGF-treated EBs showed on average a 4–6-fold increase in *Scl* and *Gata1* expression compared to control EBs. Given that both SCL and Gata1 are essential for primitive erythropoiesis, the observed increase in *Scl* and *Gata1* is consistent with an enhanced output of primitive erythroid progenitors per EB (Fig. 2A). Together, analysis of molecular markers demonstrates that VEGF treated EBs have higher levels of primitive erythropoietic markers and an increased output in the number of primitive erythroid precursors.

VEGF influences the developmental potential of primitive erythroid colonies

Results shown in Fig. 2 indicate that the expansion of the primitive erythroid compartment in response to VEGF might be attributable to an increase in the number of erythroid progenitors within each EB. In addition, it is possible that each erythroid progenitor exhibits an increased proliferation potential and extended life span. Therefore, we assessed the cellularity of primitive erythroid colonies derived from *Vegf*$^{-/-}$ EBs that developed in either the presence or absence of VEGF by integrating the area of individual colonies using imaging software. The distribution of colony size revealed that primitive erythroid precursors that developed in the presence of VEGF gave rise to larger Ery^P. Indeed, data shown in Fig. 3A indicate that Ery^P isolated from VEGF treated EBs are larger than colonies derived from untreated EBs, as seen by a shift to the right of their size distribution (P<0.003).

Our observations are consistent with the view that VEGF expands the primitive erythroid compartment by increasing the number and size of primitive erythroid colonies. Daily scoring of hematopoietic cultures indicated that the majority of Ery^P develop on day 3 and disintegrate by day 6 of culture (data not shown). We therefore addressed the question of whether VEGF also modulates their developmental potential by prolonging their longevity. Ery^P that developed in the presence or absence

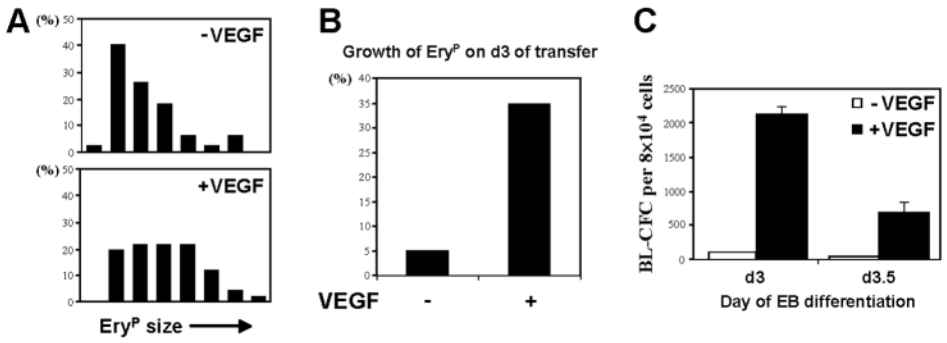

Fig. 3. VEGF increases the size and prolongs the life span of Ery^P. (A) Size distribution of Ery^P. EBs were dissociated on day 7 and assayed into hematopoiesis at 2×10^4 ml^{-1}. Colony size was determined by integrating the area of individual colonies using the Northern Eclipse software. The range covered by columns are equivalent and are determined arbitrarily. –VEGF, n=50; +VEGF, n=53; P<0.003. (B) VEGF prolongs the life span of Ery^P. Day-3 Ery^P, derived from either VEGF-treated (5 ng ml^{-1}) or untreated day-7 EBs, were transferred individually into 96-well plates containing fresh medium. Viability was assessed by visual inspection. Cells were considered nonviable when lysed or necrotic (n=121). Histogram represents the percentage of day-3 Ery^P that were viable 3 days after transfer. Histogram depicts pooled data from two independent experiments. (C) VEGF stimulates blast-colony formation (BL-CFC). Day-3 or day-3.5 EBs derived from R1 ES cells were assessed for BL-CFC in the presence or absence of VEGF. Results are the mean±s.d. of duplicates.

of VEGF were transferred individually on day 3 into fresh medium in microtiter wells, where the colonies were maintained and observed for a further 3 days. As expected, only 5% of Ery^P (6/121) that developed in the absence of VEGF were viable 3 days after transfer. By contrast, 35% (42/121) of Ery^P that differentiated in the presence of VEGF were viable at the same time point (Fig. 3B). Together, our results indicate that VEGF modifies the developmental potential of erythroid precursors and extends their life span.

Because primitive erythroid precursors do not express *Flk1* (Drake and Fleming, 2000), their expansion on addition of VEGF could be caused by an effect on earlier developmental stages. We therefore determined the effect of VEGF on BL-CFCs, which represent the earliest committed hematopoietic precursors and express *Flk1* (Kennedy et al., 1997; Chung et al., 2002). ES cells were allowed to differentiate into either day 3 or day 3.5 EBs and replated into hematopoiesis either with or without VEGF. As shown in Fig. 3C, VEGF strongly increased the number of blast colonies at both time points. These results imply that the expansion of the primitive erythroid compartment by VEGF might be attributable to an earlier effect on hemangioblast-like cells.

$Vegf^{lo}$ homozygous mice die by E9.5

The role of VEGF in vivo was defined further through reducing the VEGF dose in Vegf low *($Vegf^{lo}$)* hypomorph mice. These mice carry an internal ribosomal entry site (IRES)-lacZ insertion immediately downstream of the *Vegf* gene STOP codon, which disrupts the post-transcriptional processing of *Vegf* mRNA and renders a functionally hypomorph allele (Damert et al., 2002). Embryos dissected at E8.0 were viable and occurred at the frequency expected for $Vegf^{+/+}$, $Vegf^{lo/+}$ and $Vegf^{lo/lo}$, indicating that lethality occurs later (Table 1). When embryos carrying the $Vegf^{lo}$ allele were analyzed between E8.5-E9.5 (8-26 somite pairs), all $Vegf^{lo/lo}$ embryos had <20 somite pairs, whereas a significant proportion of wild-type and heterozygous embryos had >20 somite pairs. This analysis revealed that the mutation is homozygous lethal by E9.5 (n=220). Although heterozygous $Vegf^{lo/+}$ embryos had no obvious abnormalities, morphological and histological analysis of $Vegf^{lo/lo}$ littermates showed similar defects to those seen in $Vegf^{+/-}$ embryos (Carmeliet et al., 1996; Ferrara et al., 1996), that is a reduced dorsal aorta lumen, disorganized yolk sac vasculature and reduced numbers of blood cells in both the embryo proper and blood islands (Damert et al., 2002) (data not shown).

Different VEGF thresholds are required for hematopoietic precursor migration and for primitive erythropoiesis

Using a genetic gradient approach of VEGF activity, we assessed the effect of reduced VEGF function at E8.5 when $Vegf^{lo/lo}$ embryos are viable. The number of primitive erythropoietic precursors (Ery^P) per individual yolk sac of the different genotypes was defined using a colony assay. Analysis of four independent litters revealed that Ery^P are below detection in homozygous $Vegf^{lo/lo}$ yolk sacs, whereas heterozygous $Vegf^{lo/+}$ embryos present an intermediate phenotype compared to wild-type littermates (Fig. 4A). These results demonstrated a dose-dependent relationship between VEGF activity and the number of primitive erythroid precursors. This is most likely to be caused by a defect at the hemangioblast stage because of the low number of ES cell-

Table 1. Viability of *$Vegf^{lo}$* hypomorph embryos

	8.0 dpc	8.5-9.5 dpc		
Genotype	Total	Total	8-20 sp	>20 sp
$Vegf^{+/+}$	31	10	6	4
$Vegf^{lo/+}$	93	25	8	17
$Vegf^{lo/lo}$	53	16	16	0

Embryos are staged according to somite pairs (sp). $Vegf^{lo/lo}$ embryos die before reaching 20 sp (E9.5).

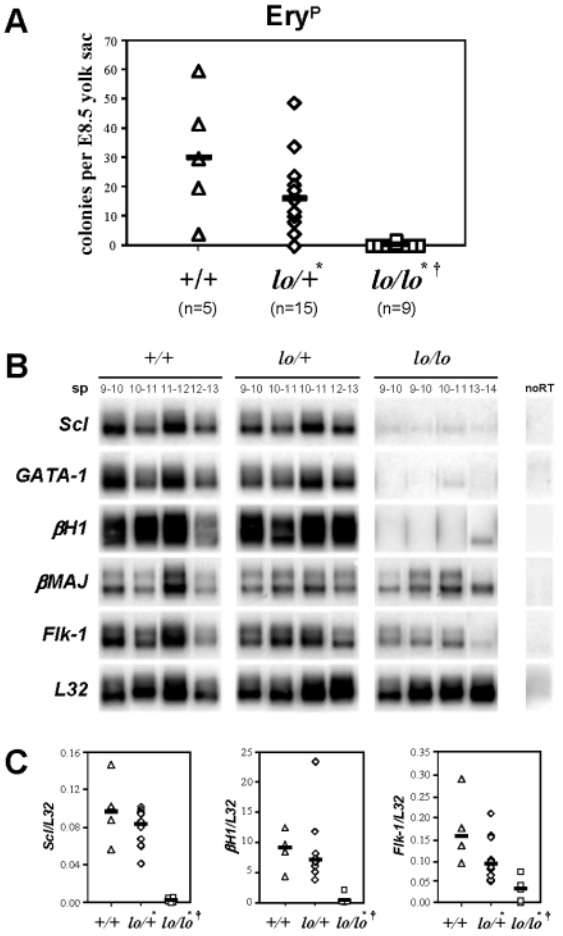

Fig. 4. VEGF activity determines the hematopoietic output during primitive erythropoiesis. (A) Frequency of Ery^P per individual E8.5 (5-14 somite pairs) yolk sacs from $Vegf^{+/+}$ (+/+), $Vegf^{lo/+}$ (*lo*/+) and $Vegf^{lo/lo}$ (*lo/lo*) embryos. Yolk sacs were isolated, dissociated into single-cell suspension and assayed for their content in hematopoietic precursors. Plots illustrate pooled data from four individual litters and *n* is the number of embryos of a corresponding genotype. Horizontal bars represent median values. $P<0.05$ compared to either heterozygous (†) or wild-type embryos (*). (B) Gene-expression analysis of E8.5 $Vegf^{lo}$ hypomorph yolk sacs. Globally amplified cDNA from single yolk sacs were probed for gene expression. The stage of development of the embryos, expressed in somite pairs (sp), is indicated at the top of each lane. No RT served as a control of genomic DNA contamination. (C) Plots illustrate the level of gene expression within individual yolk sacs as ratio of the indicated genes over *L32* taken as an internal control. Horizontal bars represent median values.

derived hemangioblasts (BL-CFCs) in the absence of VEGF (Fig. 3C).

In order to confirm our observation that VEGF increases the hematopoietic content of the yolk sac during primitive hematopoiesis, we analyzed the expression levels of molecular markers that are associated with the onset of hematopoiesis. *Flk1* and *Scl* are expressed at the earliest stages of blood-island formation (Shalaby et al., 1995; Shalaby et al., 1997; Elefanty et al., 1999), whereas erythroid genes *Gata1* (a zinc-finger transcription factor) and βH1 (embryonic globin) are upregulated at later stages. In heterozygous embryos, *Scl*, *Gata1* and βH1 expression levels were lower that those of wild type (Fig. 4B,C), confirming the analysis of hematopoietic colonies. Furthermore, *Scl*, *Gata1* and βH1 were either below or at the limit of detection in *Vegf*$^{lo/lo}$ embryos, which is consistent with a quasi-absence of primitive erythroid precursors when VEGF activity is reduced. Last, *Flk1* levels were elevated in wild-type yolk sacs, and decreased according to the number of *Vegf*^{lo} hypomorph alleles (Fig. 4C). Interestingly, *Flk1* was detected reproducibly in *Vegf*$^{lo/lo}$ yolk sacs, albeit at low levels. Thus, analysis of molecular markers indicates that low activity of VEGF, as found in *Vegf*$^{lo/lo}$ embryos, is sufficient for the migration of Flk1-positive hematopoietic precursors to the yolk sac. However, a higher threshold of VEGF, as found in *Vegf*$^{lo/+}$ embryos, is needed for the expansion and maturation of primitive erythroid progenitors. Previous work has shown that a considerable proportion of mature, primitive erythrocytes express both embryonic (βH1) and adult (βmajor) globins (Palis et al., 1999). Consistent with these results, we found low levels of βMAJ transcripts in the yolk sac. Together, the presence of hematopoietic markers at low levels indicates that low VEGF activity is sufficient for the migration of hematopoietic precursors to the yolk sac, but higher VEGF activity is required for their expansion.

VEGF is essential for the survival of primitive erythrocytes

The reduced number of primitive erythrocytes in *Vegf*$^{lo/lo}$ hypomorph embryos could be caused by either decreased proliferation or increased apoptotic death as hematopoietic cells undergo apoptosis in the absence of appropriate growth factors. Therefore, we assessed the effect of reduced VEGF activity on the survival and proliferation of differentiating primitive erythrocytes. Yolk sac erythroid cells were isolated between E9.0-E9.5 (12-26 somite pairs) and stained with TER119 and Annexin V, which recognize membrane phosphatidylserine residues that are exposed during the initial stages of apoptotic cell death. Consistent with colony assays and gene expression analysis, the frequency of TER119-positive cells correlated with the presence of either one or two hypomorph *Vegf*^{lo} alleles and was lowest in *Vegf*$^{lo/lo}$ embryos (Fig. 5A, Table 2). Furthermore, there was a direct correlation between VEGF activity and the survival of primitive erythrocytes. The proportion of TER119-positive cells that also stained for Annexin V was 2.3-fold and 4.8-fold higher in heterozygous *Vegf*$^{lo/+}$ and homozygous *Vegf*$^{lo/lo}$ embryos, respectively, compared to wild-type littermates. Interestingly, in heterozygous embryos, the level of apoptosis within the TER119-positive population increased as the embryos matured. The frequency of TER119-positive cells undergoing

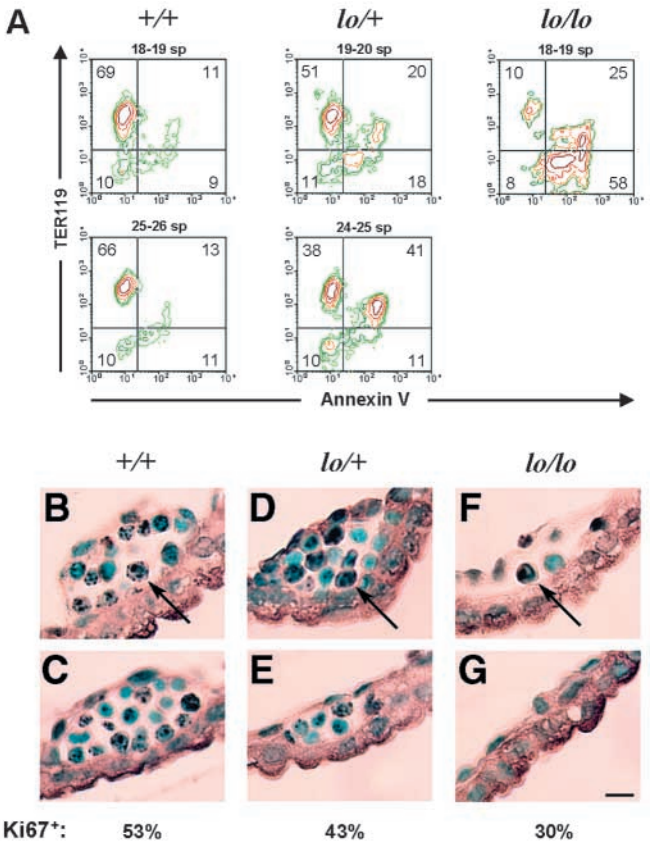

Fig. 5. VEGF is essential for the survival of primitive erythrocytes. (A) Single-cell suspensions of dissected E9.0-E9.5 yolk sacs were stained with Annexin V-FITC (apoptosis) and TER119-PE (erythroid). Dead cells that stain with 7-ADD were excluded from the analysis. (B) KI67 immunostaining of E9.0-E9.5 *Vegf*^{lo} hypomorph yolk sacs. Homozygous *Vegf*$^{lo/lo}$ yolk sacs contain few blood islands, harboring rare primitive erythroid cells (F,G) in contrast to heterozygote *Vegf*$^{lo/+}$ (D,E) and wild-type *Vegf*$^{+/+}$ (B,C) littermates. KI67-positive (brown precipitate), proliferating, primitive, erythroid cells (arrows) are found at a slightly reduced frequency in *Vegf*$^{lo/+}$ and *Vegf*$^{lo/lo}$ yolk sacs. *n* represents the number of primitive erythrocytes scored: *Vegf*$^{+/+}$, *n*=137; *Vegf*$^{lo/+}$, *n*=237; *Vegf*$^{lo/lo}$, *n*=80. No staining was observed when the primary antibody was omitted (data not shown). Nuclei were counterstained with Methyl Green. Scale bar: 10 μm.

Table 2. The *Scl* transgene partially rescues the frequency of TER119-positive cells in *Vegf*^{lo} yolk sacs

Genotype	*n*	TER119^{+} (% total)
Vegf$^{+/+}$	4	77.5±6.3
Vegf$^{+/+}$*Scl*^{tg}	8	79.1±11.4
Vegf$^{lo/+}$	8	71.5±8.5*
Vegf$^{lo/+}$*Scl*^{tg}	4	**81.3**±4.8†
Vegf$^{lo/lo}$	4	27.0±11.1‡
Vegf$^{lo/lo}$*Scl*^{tg}	2	**38.2**±5.1§

Single cell suspensions of dissected E9.0-9.5 yolk sacs were stained for TER119 and 7-AAD as in Fig. 5. The frequency of TER119-positive cells decreased according to the number of *Vegf*^{lo} alleles. *P<0.02 when compared with wild-type embryos, $^{\ddagger}P$<0.001 when compared with heterozygous or wild type embryos. The *Scl* transgene (*Scl*^{tg}) fully restores the frequency of TER119-positive cells in heterozygous *Vegf*$^{lo/+}$ embryos ($^{\dagger}P$<0.001), albeit partially in homozygous *Vegf*$^{lo/lo}$ littermates ($^{\S}P$<0.02). *n* represents the number of embryos of a corresponding genotype.

apoptosis was 19.7±7.7% for embryos between 18-21 somite pairs (n=3) and 48.6±7.3% between 22-25 somite pairs (n=3). These observations concur with the analysis of viability shown in Table 1, and indicate a requirement for high VEGF activity after 20 somite pairs. Finally, there was a significant increase in the level of apoptosis in TER119-negative cells in *Vegf*$^{lo/lo}$ embryos that was not observed in heterozygous and wild-type embryos, which was possibly caused by an increase in apoptosis in non-erythroid cells or by the loss of TER119 surface marker as erythroid cells die. Taken together, these results indicate that high VEGF activity is required for the survival of primitive erythrocytes.

Next, we examined the effects of reduced VEGF activity on the proliferation of primitive erythroid cells. Sections of E9.0-E9.5 yolk sacs were stained with an antibody directed against Ki67, a nuclear antigen present exclusively in proliferating cells (Fig. 5B-G). As expected, yolk sac cells in wild-type embryos proliferate actively. The frequency of Ki67-positive cells decreased slightly with the number of *Vegf*^{lo} alleles (wild type, 53%, n=137; heterozygous 43%, n=237; homozygous 30%, n=80). Although primitive erythroid cells were scarce in *Vegf*$^{lo/lo}$ yolk sacs, these cells still exhibited Ki67 staining. These results indicate that VEGF is essential for the survival of primitive erythrocytes whereas commitment to cell division appears to be VEGF-independent.

SCL interacts with VEGF to suppress apoptosis in primitive erythroid cells

The induction of *Scl* by VEGF in vitro and in vivo could either be a cause or a consequence of increased hematopoiesis. To distinguish between these two possibilities, we asked whether elevation of SCL could substitute for defective VEGF activity in *Vegf*$^{lo/lo}$ embryos. To this end, heterozygous *Vegf*$^{lo/+}$ mice were bred with *Scl* transgenic mice that consitutively express *Scl* under the control of the *Sil* (*Scl* interrupting locus) promoter (Aplan et al., 1997) to generate compound-heterozygote *Vegf*$^{lo/+}$*Scl*^{tg} mice, that were then crossed to produce *Vegf*$^{lo/lo}$*Scl*^{tg} embryos. In wild-type embryos, the *Sil-Scl* transgenic cassette allows 14.5-fold higher level of *Scl* in the yolk sac compared to non-transgenic littermates (Fig. 6A). Analysis of TER119 labeling indicated a modest increase in TER119-positive cells when the *Scl* transgene was introduced in a *Vegf*$^{lo/+}$ and *Vegf*$^{lo/lo}$ background, but not in wild-type embryos (Table 2). Annexin V labeling revealed that the survival of TER119-positive cells was dependent on the number of functional *Vegf* alleles. Thus, apoptotic death was 70%, 35% and 15% of TER119-positive cells, in *Vegf*$^{lo/lo}$, *Vegf*$^{lo/+}$ and *Vegf*$^{+/+}$ embryos, respectively (Fig. 6B). Strikingly, the *Scl* transgene reduced cell death by half in *Vegf*$^{lo/lo}$ and *Vegf*$^{lo/+}$ embryos, indicating an important anti-apoptotic function for the SCL transcription factor. To assess whether the suppression of cell death by SCL and VEGF occurred through parallel pathways or the same pathway, we examined apoptosis in *Vegf*$^{+/+}$ embryos. As shown in Fig. 6B, the anti-apoptotic effect of SCL was not additive to that of VEGF. We therefore surmise that SCL and VEGF operate within the same genetic pathway to determine the output in primitive erythroid cells. Consistent with a partial restoration of primitive erythropoiesis (Table 2), *Gata1* and βH1 transcripts (Fig. 6C,D) were present at low levels in the yolk sac of *Vegf*$^{lo/lo}$*Scl*^{tg} embryos, whereas they were below the limit of detection in *Vegf*$^{lo/lo}$ embryos.

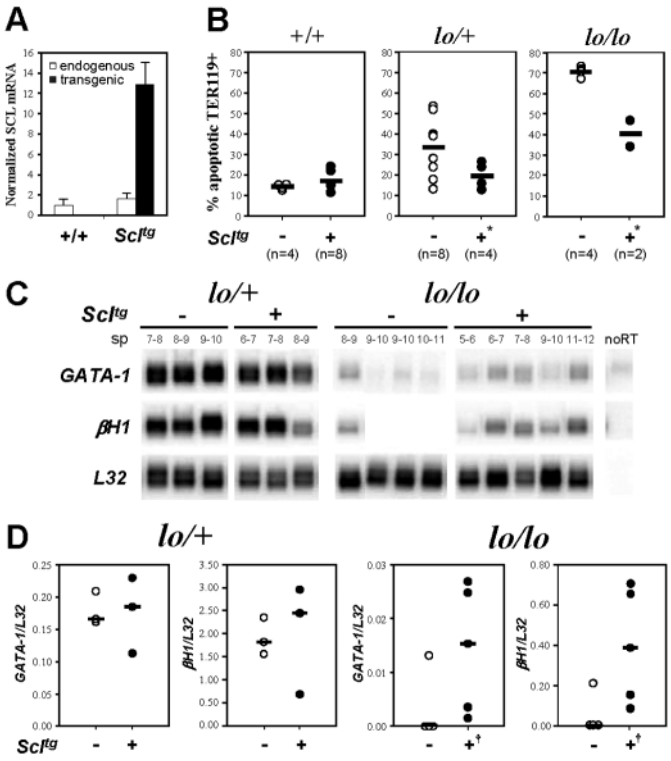

Fig. 6. Partial rescue of primitive erythropoiesis by the *Scl* transgene (*Scl*^{tg}). (A) Analysis of *Scl* expression in *Sil-Scl* transgenic E9.5 yolk sacs. Individual yolk sacs were analyzed for endogenous and transgenic expression of *Scl* using quantitative SYBR Green PCR. Histogram represents the average amount of *Scl* mRNA±s.d., normalized according to the internal control (*S16*). To estimate the relative levels of *Scl* expression from the transgene and endogenous source, the molar amount of endogenous *Scl* in wild-type mice was taken as 1. *Scl*$^{+/+}$, n=4; *Scl*^{tg}, n=6. (B) Apoptosis in TER119-positive cells. TER119, Annexin V and 7-AAD staining were performed as in Fig. 5. Data shown are from three independent litters and are expressed as % of TER119-positive cells. Note that the level of apoptotic death depends on the number of *Vegf*^{lo} alleles and is attenuated by the *Scl* transgene. *P<0.05; n is the number of embryos of a corresponding genotype. (C) Analysis of erythroid genes in individual yolk sacs was performed as described in Fig. 4. (D) Plots illustrate the level of gene expression within individual yolk sacs as ratio of the indicated genes and the *L32* internal control. Horizontal bars represent median values. Note that the *Scl*^{tg} increases the level of *Gata1* and βH1 in homozygous *Vegf*$^{lo/lo}$ yolk sacs. †P<0.01.

Together, our observations indicate that elevating *Scl* levels suppresses apoptosis and allows an expansion of Flk1-positive cells.

Discussion

In the present study, we show that different VEGF thresholds are required for recruitment of hematopoietic precursors to the yolk sac, expansion of the primitive erythroid compartment and survival of primitive erythrocytes. Furthermore, SCL can partially rescue the hematopoietic defects associated with loss of VEGF activity, thus providing evidence in vivo that during the initial stages of hematopoiesis, SCL acts downstream of

VEGF/Flk1 signaling to promote the survival of primitive erythrocytes.

Different VEGF thresholds are required during hematopoietic development

Knockout and chimera studies have linked VEGF function to the migration of hematopoietic precursors from the mesoderm to the yolk sac and for the generation of blood islands (Shalaby et al., 1995; Shalaby et al., 1997; Carmeliet et al., 1996; Ferrara et al., 1996, Damert et al., 2002). We used *Vegf*^{lo} hypomorph mice, developed by Damert et al. (Damert et al., 2002), to further define the effect of VEGF dose on cell-fate decisions involved with the development of the hematopoietic system. Although heterozygous *Vegf*$^{lo/+}$ embryos are viable, homozygous *Vegf*$^{lo/lo}$ littermates die by E9.5 because of hematopoietic and vascular defects that are similar to those of *Vegf*$^{+/-}$ embryos. From this, we infer that each *Vegf*^{lo} allele provides <50% activity of a wild-type allele. Thus, by varying the number of *Vegf*^{lo} alleles, we compared hematopoietic development in embryos exposed to a range of VEGF activity; homozygous *Vegf*$^{lo/lo}$ embryos provided ~50% and heterozygous *Vegf*$^{lo/+}$ littermates, 75% of wild-type VEGF activity.

In *Vegf*$^{lo/lo}$ embryos, in which VEGF activity is presumed to be 50% of wild type, Flk1-positive mesodermal precursors reach the yolk sac but are severely compromised in their capacity to expand and differentiate into primitive erythroid cells. Indeed, Flk1 expression is detected clearly in *Vegf*$^{lo/lo}$ yolk sacs, but it is diminished in comparison to heterozygous and wild-type littermates because of the substantial loss of Flt1/Flk1-positive mature endothelial cells (Damert et al., 2002). However, in *Vegf*$^{lo/+}$ littermates, when VEGF activity is raised to higher levels, the embryos survive, thus setting a threshold for the development of blood islands and for the expansion of the primitive erythroid compartment. Moreover, we observed a direct relationship between the level of VEGF activity and the number of primitive erythroid precursors per yolk sac, indicating a tight dependence of the primitive erythroid lineage on the number of functional VEGF alleles. In contrast to the dose-dependent requirement for sustained VEGF activity during primitive erythropoiesis, inactivation of both *Vegf* alleles was needed to abrogate the survival of adult hematopoietic stem cells (Gerber et al., 2002). Consistent with studies in vivo, addition of VEGF to differentiating *Vegf*$^{-/-}$ ES cells in vitro increased the frequency of primitive erythroid precursors in a dose-dependent manner. This effect of VEGF on primitive erythropoiesis was observed using three independent *Vegf*$^{-/-}$ clones (36.7, 44.7 and 44.8) and the parental R1 ES cells. However, we did not observe an increase in Ery^{P} by VEGF using the feeder-independent CCE line, as previously described (Kabrun et al., 1997). CCE cells are efficient for hematopoietic differentiation, possibly because of a higher level of endogenous VEGF secretion. Furthermore, addition of VEGF to differentiating ES cells stimulated the clonal expansion of each precursor, giving rise to more primitive erythrocytes per colony and extended their life span in culture. Although not proven directly, extension of the life span of primitive erythrocytes by VEGF may be interpreted as a delay in their senescence. Mechanisms that underlie senescence are only beginning to emerge, and point to the importance of telomere erosion, cell-cycle control and growth conditions (reviewed by Rubin, 1998; Sherr and DePinho, 2000). Our results raise the question whether growth factors may also be involved in the senescence process, possibly by shaping the developmental potential of early progenitors long before the growth arrest of their progeny is observed in culture.

Our results indicate that VEGF enhances primitive erythropoiesis, but primitive erythrocytes do not express *Flk1* and *Flt1* (Shalaby et al., 1995; Shalaby et al., 1997; Fong et al., 1996; Drake and Fleming, 2000). There may be several possibilities. First, it is possible that a third, as yet unidentified, VEGF receptor is expressed on primitive erythrocytes. Second, VEGF might stimulate primitive erythropoiesis indirectly, through the secretion of a secondary hematopoietic growth factor from Flk1-positive vascular cells. Third, it is conceivable that VEGF affects the developmental potential of an earlier Flk1-positive precursor by promoting a hematopoietic fate. Although we cannot exclude the first and second possibilities, we favor the third. Given that reduced VEGF activity also affects endothelial development (Damert et al., 2002), we speculate that the reduction in the number of primitive erythroid precursors in *Vegf*$^{lo/lo}$ embryos is caused, for the most part, by the inability of Flk1-positive putative hemangioblasts to expand and differentiate into blood islands. Similarly, when VEGF is added to differentiating EBs, enhancement of primitive erythropoiesis might also occur at the hemangioblast stage. Indeed, ES cell-derived hemangioblasts, BL-CFCs, appear transiently after 3-4 days of differentiation (Kennedy et al., 1997; Choi et al., 1998) at the time when Flk1 is first detected (Kabrun et al., 1997) and at the time when we add VEGF to our cultures. In agreement with this interpretation, VEGF strongly enhanced the number of BL-CFCs isolated from day 3 and day 3.5 EBs. Thus, the increase in the number of Ery^{P} in day 7 EBs might, therefore, result from an in situ expansion of BL-CFCs in day 3-4 EBs. It is noteworthy that the only difference between VEGF-treated and control cultures is the presence of VEGF during days 3-7 of EB differentiation. The growth factor cocktail for the hematopoietic colony assay is identical and contains VEGF. Thus, the effect of VEGF must occur at the earliest stages of hematopoietic commitment.

VEGF is essential for the survival of primitive erythrocytes: partial rescue by SCL

VEGF has an established role in endothelial cell function, favoring the proliferation and survival of endothelial cells during development and in adults (Ferrara et al., 2003). VEGF is essential for the survival of hematopoietic stem cells, through Flk1 and possibly Flt1 signaling (Gerber et al., 2002), although the effect of VEGF during primitive erythropoiesis has not yet been defined. Analysis of *Vegf*^{lo} hypomorph embryos revealed a direct relationship between the number of *Vegf*^{lo} alleles and the frequency of apoptotic, TER119-positive, primitive erythroid cells, while reduced VEGF activity had little effect on the proliferation of the same cells. Strikingly, overexpression of SCL, using a *Scl* transgene under the control of the ubiquitous *Sil* promoter, partially alleviated the apoptosis of primitive erythrocytes associated with the *Vegf*^{lo} allele, which correlated with an increase in TER119 staining and βH1 expression in individual yolk sacs. We have shown previously that SCL functions downstream of the Flk1-related tyrosine kinase c-kit to promote the survival of definitive hematopoietic precursors (Krosl et al., 1998) (unpublished data). Simlarly, in

this study, we provide evidence that VEGF/Flk1 signaling enhances primitive erythropoiesis by promoting the survival of primitive erythrocytes through the anti-apoptotic function of SCL.

To date, few transcription factors have been identified that determine cell fate. For example, the expression of *MyoD*, a member of the bHLH family, is sufficient to induce muscle formation (Davis et al., 1987). Several groups have shown that SCL can specify and promote hematopoietic and vascular fates at the expense of other mesodermal tissues (Gering et al., 1998; Mead et al., 1998; Mead et al., 2001). However, it remains unclear how SCL potentiates hematopoietic and vascular fates. Ema et al. (Ema et al., 2003) have shown recently that SCL favors the endothelial lineage at the expense of smooth muscle in a VEGF dependent process. They have also shown that SCL acts downstream of Flk1, at the hemangioblast level, to rescue hematopoietic and vascular defects in vitro. Our work indicates that enhancement of the hematopoietic fate might result, at least in part, from the increased survival of primitive erythrocytes as a result of VEGF/Flk1-induced *Scl* expression.

Taken together, our data indicate that during the establishment of the hematopoietic system, in addition to guiding the migration of hematopoietic and endothelial precursors, VEGF enhances the hematopoietic fate by expanding the primitive erythroid compartment and potentiating the survival of primitive erythroid cells through SCL function in hemangioblasts.

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