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

Richard Martin1,2, Rachid Lahlil1, Annette Damert3,*, Lucile Miquerol3,†, Andras Nagy3, Gordon Keller4 and Trang Hoang1,2,5,‡,§

1Laboratory of Hematopoiesis and Leukemia, Clinical Research Institute of Montreal, Montreal, Canada
2Department of Medicine, Division of Experimental Medicine, McGill University, Montreal, Canada
3Mount Sinai Hospital, Samuel Lunenfeld Research Institute, Toronto, Canada
4Institute for Gene Therapy and Molecular Medicine, Mount Sinai School of Medicine, New York, USA
5Departments of Pharmacology, Biochemistry and Molecular Biology, University of Montreal, Montreal, Canada

*Present address: University Hospital Charite, Institute of Pharmacology and Toxicology, Berlin, Germany
†Present address: Laboratoire de génétique et physiologie du développement, IBDM- campus de Luminy, Marseille, France
‡Present address: Institute of Research in Immunovirology and Cancer (IRIC), University of Montreal, PO Box 6128, Downtown Station, Montreal, Quebec H3C 3J7, Canada
§Author for correspondence (e-mail: trang.hoang@umontreal.ca)

Accepted 28 October 2003
Development 131, 693-702
Published by The Company of Biologists 2004
doi:10.1242/dev.00968

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−/− 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 Vegfalo/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 Vegfalo 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 Drosophila (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; Shvidrasani 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; Shvidrasani 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., 1996). 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

Vegfα hypomorph mice (Damert et al., 2002) were maintained on a mixed CD-1/129 background. We were unsuccessful in our attempt to breed the Vegfα allele onto a C57BL/6 background. The Scl transgenic line A(S5)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 Vegfα hypomorph mice and VegfαSclβ 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 (Kroslo et al., 1998). Included was 7-amino actinomycin D (7-AAD, Calbiochem) to detect dead cells. Cells were analyzed on a FACS Calibur 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 peroxidase activity was blocked with 1% H2O2, 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 peroxidase (NEN), both incubated for 1 hour at room temperature. Positive cells were revealed with the peroxidase substrate 3,3’-diaminobenzidine (Sigma) and counterstained with methyl green.

Growth and differentiation of ES cells

Parental wild-type R1 (Nagy et al., 1993), Vegfα−/− 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 0.3×105–1.0×105 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 (PFHMII, Gibco) and 3×10–4 M MTG. When indicated, recombinant human VEGF165 (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 (Eryβ) 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 4×105 ml–1 or half of dissociated ES.8 yolk sac in IMDM containing 1% methylcellulose, 10% FCS (Gibco), 5% PFHMI, 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 IL-5, 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′-TTGTACAGCTCTGCA-3′ and reverse primer 5′-CCCAAAAGTCAGTTTATT-3′; βmajor: 135-bp fragment immediately upstream of the polyA tail. Gata1: 443-bp fragment was amplified by PCR using forward primer 5′-GGAGCGATTGCTGGTGTTG-3′; reverse primer 5′-AGGGCATTGTTGCTGGTCA-3′; reverse primer 5′-GCCCAGCCCCTTATAGAAAATTC-3′; Mouse Scl: forward primer, 5′-CACTTGACACTGA-3′ and reverse primer 5′-AGGTGTTTGTGTCA-3′; reverse primer, 5′-CACTTGACACTGA-3′.

Quantification of ScI mRNA from ScI yolk sacs was accomplished using the Quantitect SYBR Green PCR kit (Quiaigen), 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′-AGGGCATTGTTGCTGGTCA-3′; reverse primer, 5′-AGGGCATTGTTGCTGGTCA-3′; reverse primer, 5′-GCCCAGCCCCTTATAGAAAATTC-3′; Mouse Scl: forward primer, 5′-CACTTGACACTGA-3′; reverse primer, 5′-GATGTGTGGGGA-TAGCTT-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 EryP (Fig. 1G). Hence, the increase in EryP 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 EryP was assessed based on morphology and cytospin analysis (Fig. 1H-J). 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 occurred 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 EryP per EB. The increase in EryP 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
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 EryP. Indeed, data shown in Fig. 3A indicate that EryP 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 EryP 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. EryP that developed in the presence or absence

---

**Fig. 2.** Clonal analysis of the effect of VEGF during primitive erythropoiesis. (A) VEGF increases the number of EryP 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 EryP. (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.

**Fig. 3.** VEGF increases the size and prolongs the life span of EryP. (A) Size distribution of EryP. 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 EryP. Day-3 EryP, 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 EryP 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 EryP (6/121) that developed in the absence of VEGF were viable 3 days after transfer. By contrast, 35% (42/121) of EryP 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** homozygous mice die by E9.5

The role of VEGF in vivo was defined further through reducing the VEGF dose in Vegf low (<Vegf>) hypomorph mice. These mice carry an internal ribosomal entry site (IRE)-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<sup> WT</sup>, Vegf<sup> WT/+</sup> and Vegf<sup>Welcome</sup>, indicating that lethality occurs later (Table 1). When embryos carrying the Vegf<sup>Welcome</sup> allele were analyzed between E8.5-E9.5 (8-26 somite pairs), all Vegf<sup>Welcome</sup> 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<sup>Welcome</sup> embryos had no obvious abnormalities, morphological and histological analysis of Vegf<sup>Welcome</sup> littermates showed similar defects to those seen in Vegf<sup>Welcome</sup> 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<sup>Welcome</sup> embryos are viable. The number of primitive erythropoietic precursors (Ery<sup>Welcome</sup>) per individual yolk sac of the different genotypes was defined using a colony assay. Analysis of four independent litters revealed that Ery<sup>Welcome</sup> are below detection in homozygous Vegf<sup>Welcome</sup> yolk sacs, whereas heterozygous Vegf<sup>Welcome</sup> embryos present an intermediate phenotype compared to wild-type litters (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<sup>Welcome</sup> hypomorph embryos

<table>
<thead>
<tr>
<th>Genotype</th>
<th>8.0 dpc</th>
<th>8.5-9.5 dpc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Total</td>
</tr>
<tr>
<td>Vegf&lt;sup&gt;Welcome&lt;/sup&gt;+/+</td>
<td>31</td>
<td>10</td>
</tr>
<tr>
<td>Vegf&lt;sup&gt;Welcome&lt;/sup&gt;lo/+</td>
<td>93</td>
<td>25</td>
</tr>
<tr>
<td>Vegf&lt;sup&gt;Welcome&lt;/sup&gt;lo/lo</td>
<td>53</td>
<td>16</td>
</tr>
</tbody>
</table>

Embryos are staged according to somite pairs (sp). Vegf<sup>Welcome</sup> embryos die before reaching 20 sp (E9.5).

---

**Fig. 4.** VEGF activity determines the hematopoietic output during primitive erythropoiesis. (A) Frequency of Ery<sup>Welcome</sup> per individual E8.5 (5-14 somite pairs) yolk sacs from Vegf<sup>+/-</sup> (+/+), Vegf<sup>Welcome</sup> (<lo/+>) and Vegf<sup>Welcome</sup> (<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 independent 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<sup>Welcome</sup> 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<sup>lo/lo</sup> 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<sup>lo</sup> hypomorph alleles (Fig. 4C). Interestingly, Flk1 was detected reproducibly in Vegf<sup>lo/lo</sup> yolk sacs, albeit at low levels. Thus, analysis of molecular markers indicates that low activity of VEGF, as found in Vegf<sup>lo/lo</sup> embryos, is sufficient for the migration of Flk1-positive hematopoietic precursors to the yolk sac. However, a higher threshold of VEGF, as found in Vegfr<sup>lo/+</sup> 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 (βHmajor) 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<sup>lo/lo</sup> 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 Annexin V -FITC (apoptosis) and TER119-PE for their expansion. TER119-positive cells undergoing proliferation or increased apoptotic death as hematopoietic precursors to the yolk sac. However, a higher threshold of VEGF, as found in Vegf<sup>lo/+</sup> 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 (βHmajor) 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.

![Image](311x402 to 557x719)

**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. Dead cells that stain with 7-ADD were excluded from the analysis. (B) Ki67 immunostaining of E9.0-E9.5 Vegf<sup>lo</sup> hypomorph yolk sacs. Homozygous Vegf<sup>lo/lo</sup> yolk sacs contain few blood islands, harboring rare primitive erythroid cells (FG). In contrast to heterozygote Vegf<sup>lo/+</sup> (D,E) and wild-type Vegf<sup>+/+</sup> (B,C) littermates, Ki67-positive (brown precipitate), proliferating, primitive, erythroid cells (arrows) are found at a slightly reduced frequency in Vegf<sup>lo/+</sup> and Vegf<sup>lo/lo</sup> yolk sacs. *n represents the number of primitive erythrocytes scored. Vegf<sup>+/+</sup>, n=137; Vegf<sup>lo/+</sup>, n=237; Vegf<sup>lo/lo</sup>, 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>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>TER119+ (% total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegf&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>4</td>
<td>77.5±6.3</td>
</tr>
<tr>
<td>Vegf&lt;sup&gt;lo/+&lt;/sup&gt;Scl&lt;sup&gt;tg&lt;/sup&gt;</td>
<td>8</td>
<td>79.1±11.4</td>
</tr>
<tr>
<td>Vegf&lt;sup&gt;lo/+&lt;/sup&gt;</td>
<td>8</td>
<td>71.5±8.5*</td>
</tr>
<tr>
<td>Vegf&lt;sup&gt;lo/+&lt;/sup&gt;Scl&lt;sup&gt;tg&lt;/sup&gt;</td>
<td>4</td>
<td>81.3±4.8</td>
</tr>
<tr>
<td>Vegf&lt;sup&gt;lo/+&lt;/sup&gt;Scl&lt;sup&gt;tg&lt;/sup&gt;</td>
<td>4</td>
<td>27.0±11.1</td>
</tr>
<tr>
<td>Vegf&lt;sup&gt;lo/lo&lt;/sup&gt;Scl&lt;sup&gt;tg&lt;/sup&gt;</td>
<td>2</td>
<td>38.2±5.1*</td>
</tr>
</tbody>
</table>

Table 2. The Scl transgene partially rescues the frequency of TER119-positive cells in Vegf<sup>lo</sup> yolk sacs

Table 2. The Scl transgene partially rescues the frequency of TER119-positive cells in Vegf<sup>lo</sup> yolk sacs

Single cell suspensions of dissected E9.0-9.5 yolk sacs were stained for TER119 and 7-ADD as in Fig. 5. The frequency of TER119-positive cells decreased according to the number of Vegf<sup>lo</sup> alleles. *P<0.02 when compared with wild-type embryos, †P<0.001 when compared with heterozygous or wild type embryos. The Scl transgene (Scl<sup>tg</sup>) fully restores the frequency of TER119-positive cells in heterozygous Vegf<sup>lo</sup> embryos (†P<0.001), albeit partially in homozygous Vegf<sup>lo</sup> littermates (‡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<sup>lo/lo</sup> 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<sup>lo</sup> alleles (wild type, 53%, n=137; heterozygous 43%, n=237; homozygous 30%, n=80). Although primitive erythroid cells were scarce in Vegf<sup>lo/lo</sup> 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<sup>lo/lo</sup> embryos. To this end, heterozygous Vegf<sup>lo/+</sup> mice were bred with Scl transgenic mice that constitutively express Scl under the control of the Sil (Scl interrupting locus) promoter (Aplan et al., 1997) to generate compound-heterozygote Vegf<sup>lo/+</sup>Scl<sup>tg</sup> mice, that were then crossed to produce Vegf<sup>lo/+</sup>Scl<sup>tg</sup> 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 littersmates (Fig. 6A). Analysis of TER119 labeling indicated a modest increase in TER119-positive cells when the Scl transgene was introduced in a Vegf<sup>lo/+</sup> and Vegf<sup>lo/lo</sup> 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<sup>lo/lo</sup>, Vegf<sup>lo/+</sup> and Vegf<sup>lo+/+</sup> embryos, respectively (Fig. 6B). Strikingly, the Scl transgene reduced cell death by half in Vegf<sup>lo/lo</sup> and Vegf<sup>lo/+</sup> 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<sup>lo+/</sup> 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<sup>lo/x+Scl<sup>tg</sup></sup> embryos, whereas they were below the limit of detection in Vegf<sup>lo/lo</sup> embryos.

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; Damert et al., 2002). We used Vegf<sup>lo</sup> 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<sup>lo/+</sup> embryos are viable, homozygous Vegf<sup>lo/lo</sup> littersmates die by E9.5 because of hematopoietic and vascular defects that are similar to those of Vegf<sup>–/+</sup> embryos. From this, we infer that each Vegf<sup>lo</sup> allele provides <50% activity of a wild-type allele. Thus, by varying the number of Vegf<sup>lo</sup> alleles, we compared hematopoietic development in embryos exposed to a range of VEGF activity: homozygous Vegf<sup>lo/lo</sup> embryos provided ~50% and heterozygous Vegf<sup>lo/+</sup> littersmates, 75% of wild-type VEGF activity.

In Vegf<sup>lo/lo</sup> 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<sup>lo/lo</sup> yolk sacs, but it is diminished in comparison to heterozygous and wild-type littersmates because of the substantial loss of Flk1/Flk1-positive mature endothelial cells (Damert et al., 2002). However, in Vegf<sup>lo/+</sup> littersmates, 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<sup>–/–</sup> 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<sup>–/–</sup> clones (36.7, 44.7 and 44.8) and the parental R1 ES cells. However, we did not observe an increase in Ery<sup>P</sup> 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<sup>lo/lo</sup> 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<sup>b</sup> 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<sup>lo</sup> hypomorph embryos revealed a direct relationship between the number of Vegf<sup>lo</sup> 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<sup>lo</sup> allele, which correlated with an increase in TER119 staining and BH1 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). Similarly, 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.

The authors wish to thank Danielle Dubno for the analysis of blast colonies, Dr Peter Aplan for providing Sili-Scl transgenic mice and Dorothee Bégin for expert secretarial assistance. This work was supported in part by grants from the Canadian Institutes of Health Research (T.H. and A.N.) and by a studentship from the National Science and Engineering Research Council (R.M.). A.N. is a senior scientist of the CIHR.

References


upstream of a flk-1 homologue to regulate endothelial cell differentiation. Development 124, 381-389.