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Endoglin is required for hemangioblast and early hematopoietic development

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Endoglin (ENG), an ancillary receptor for several members of the transforming growth factor (TGF)-beta superfamily, has a well-studied role in endothelial function. Here, we report that endoglin also plays an important role early in development at the level of the hemangioblast, an embryonic progenitor of the hematopoietic and endothelial lineages. *Eng*$^{-/-}$, *Eng*$^{+/-}$ and *Eng*$^{+/+}$ mouse embryonic stem (ES) cells were differentiated as embryoid bodies (EBs) and assayed for blast colony-forming cells (BL-CFCs). Our results showed a profound reduction in hemangioblast frequency in the absence of endoglin. Furthermore, cell-sorting experiments revealed that endoglin marks the hemangioblast on day 3 of EB differentiation. When analyzed for hematopoietic and endothelial activity, replated *Eng*$^{-/-}$ BL-CFCs presented limited hematopoietic potential, whereas endothelial differentiation was unaltered. Analysis of hematopoietic colony formation of EBs, at different time points, further supports a function for endoglin in early hematopoiesis. Taken together, these findings point to a role for endoglin in both hemangioblast specification and hematopoietic commitment.

KEY WORDS: ES cells, Endoglin, Hemangioblast, Primitive erythropoiesis, Mouse

INTRODUCTION

Endoglin (ENG) is an accessory receptor for several growth factors of the transforming growth factor-beta (TGF-β) superfamily, including TGF-β_1, TGF-β_3, activin, BMP7 and BMP2 (Barbara et al., 1999). This receptor is known mostly for its expression on the surface of vascular endothelial cells (Cheifetz et al., 1992). Conditions involving alteration in vascular structure – such as angiogenesis, wound healing and inflammation – are associated with increased expression of endoglin. More recently, it has been demonstrated that endoglin is also expressed in adult bone marrow hematopoietic stem cells (HSCs) (Chen et al., 2002; Pierelli et al., 2001), marking the adult long-term repopulating HSCs (Chen et al., 2002).

Mutations in endoglin that lead to haploinsufficiency are associated with an autosomal-dominant vascular disorder termed hereditary hemorrhagic telangiectasia (HHT) (McAllister et al., 1994) in humans, which is characterized by hemorrhagic bleedings, which are presumably secondary to vascular malformations. Mice lacking one copy of the endoglin gene also display vascular defects and hemorrhage, and represent a murine model for HHT (Bourdeau et al., 1999). Complete loss of endoglin is embryonic lethal. Embryos homozygous for the endoglin knockout (*Eng*$^{-/-}$) fail to progress beyond 10.5 days postcoitum (dpc), primarily due to vascular and cardiac abnormalities (Arthur et al., 2000; Bourdeau et al., 1999). Analysis of 9.5 dpc mouse *Eng*$^{-/-}$ embryos revealed abnormal vasculature and anemia of the yolk sac (Arthur et al., 2000). Although the anemia was suggested to be secondary to defective vasculature, a possible earlier effect in the hematopoietic lineage has not been investigated in these knockout mice. Besides being detected in endothelial cells between embryonic day (E)8.5 and E10.5 (Hirashima et al., 2004), endoglin is also present in early extraembryonic mesoderm from gastrulating embryos (Ema et al., 2006b; Hirashima et al., 2004), suggesting a potential role for endoglin in earlier developmental stages.

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By differentiating embryonic stem (ES) cells using the OP9 co-culture system, Cho and colleagues (Cho et al., 2001) have shown that myelopoiesis and definitive erythropoiesis is impaired in the absence of endoglin. However primitive erythropoiesis, reflective of yolk sac hematopoiesis, was not investigated in this study.

The close association between endothelial cells and primitive hematopoietic precursors in the embryonic yolk sac suggests that the endothelium plays a crucial role in early hematopoietic development, providing the microenvironment required for stem cell proliferation and differentiation. Endothelial and blood precursors also both express several of the same crucial regulatory genes and antigenic markers (Kabrun et al., 1997; Kallianpur et al., 1994; Orkin, 1992; Yamaguchi et al., 1993; Young et al., 1995). These observations support the hypothesis that a common progenitor, the hemangioblast, gives rise to both endothelial and hematopoietic cells (His, 1900; Murray, 1932; Sabin, 1920). A cell with properties of the hemangioblast has been identified during in vitro differentiation of ES cells into embryoid bodies (EBs) (Kennedy et al., 1997), and has more recently been identified in the primitive streak of the mouse embryo (Huber et al., 2004). This precursor, referred to as the blast colony-forming cell (BL-CFC) forms in response to vascular endothelial growth factor (VEGF) and stem cell factor (SCF, also known as KITL – Mouse Genome Informatics), and represents a transient population of cells that stands at the juncture of the endothelial and hematopoietic lineages (Choi et al., 1998).

To address whether endoglin is important at these early stages of development, endoglin-deficient mouse ES cells were evaluated for their hemangioblast activity as well as for their hematopoietic and endothelial potential. The results of this study point to an essential role for endoglin at the stages of hemangioblast specification as well as at hematopoietic commitment.

MATERIALS AND METHODS

Growth and differentiation of ES cells

Wild-type mouse E14 and CCE as well as *Eng*$^{+/-}$ and *Eng*$^{-/-}$ ES cells (generously provided by J. C. Zuniga-Pflucker and M. Letarte from the University of Toronto, ON, Canada) (Cho et al., 2001) were used in this

study. *Eng*$^{-/-}$ ES cells were generated from heterozygous *Eng*$^{+/-}$ ES cells following selection in a high concentration of G418 (Bourdeau et al., 1999). *Eng* knockout was made on the E14 background (Bourdeau et al., 1999).

ES cells were maintained on gelatinized flasks in DMEM (Sigma) supplemented with 1000 U/ml leukemia inhibitory factor (LIF; Chemicon), 15% knockout serum replacement (Invitrogen), 0.1 mM non-essential amino acids (Sigma), and 0.1 mM of beta-mercaptoethanol (Sigma) in the absence of feeders for up to 15 passages. For differentiation cultures, the cells were dissociated with trypsin (0.25%; Invitrogen)/EDTA (1 mM; Sigma) to form a single-cell suspension. Cells were washed three times with phosphate-buffered saline (PBS) and EBs were generated by plating 5×10^4 cells per ml in EB media, which consists of IMDM (Sigma) with 15% fetal calf serum (FCS; Sigma), 50 μg/ml ascorbic acid (Sigma), 200 μg/ml iron-saturated transferrin (Sigma), 4.5×10^{-4} M monothioglycerol (MTG; Sigma) and 0.9% methylcellulose (M3120, StemCell Technologies) in 35 mm Petri dishes (StemCell Technologies).

Blast colony-forming cell (BL-CFC) assay

EBs were collected at 3 days post differentiation, washed in PBS and treated with 0.25% trypsin for 3 minutes at 37°C. EBs were disrupted to single cells by repeated pipetting and plated at 5×10^4 cells in 1 ml of methylcellulose medium (M3120) with 10% FCS, 50 μg/ml ascorbic acid, 200 μg/ml iron-saturated transferrin, 4.5×10^{-4} M MTG, in the presence of thrombopoietin (TPO, 25 ng/ml; Peprotech), vascular endothelial growth factor (VEGF, 5 ng/ml; Peprotech) and stem cell factor (SCF, 100 ng/ml; Peprotech), as previously described (Choi et al., 1998; Perlingeiro et al., 2003). Cultures were maintained in a humidified incubator at 37°C in an environment of 5% CO_2 in air. After 5 days developing, BL-CFCs were counted and picked for replating studies.

Generation of hematopoietic cells

Cells from EBs at different time points (days 3, 4, and 5), or individual BL-CFCs, were plated into methylcellulose media containing interleukin 3 (IL3), interleukin 6 (IL6), erythropoietin (EPO) and SCF (M3434; StemCell Technologies). Cultures were maintained as described above and primitive erythroid colonies were scored at 6 days of growth.

Generation of endothelial cells

Individual BL-CFCs were picked and transferred to gelatin-coated microtiter wells containing IMDM with 10% FCS, 10% horse serum (Biocell), VEGF (5 ng/ml), insulin-like growth factor 1 (IGF1, 10 ng/ml; Peprotech), EPO (2 U/ml; R&D Systems), fibroblast growth factor (bFGF, 10 ng/ml; Peprotech), interleukin 11 (IL11, 50 ng/ml; R&D Systems), SCF (100 ng/ml), endothelial cell growth supplement (ECGS, 100 μg/ml; Collaborative Research), L-glutamine (2 mM), and 4.5×10^{-4} MTG. After 3-4 days in culture, non-adherent cells were removed and adherent cells were cultured for an additional 1-2 weeks in IMDM with 10% FCS, 10% horse serum, VEGF (5 ng/ml), IGF-1 (10 ng/ml), bFGF (10 ng/ml), ECGS (100 μg/ml), L-glutamine (2 mM) and 4.5×10^{-4} MTG (Choi et al., 1998; Perlingeiro et al., 2003).

Rescue experiments

A CMV-endoglin transgene was inserted into the *Eng*$^{-/-}$ ES cells. The endoglin cDNA was obtained from Open Biosystems on a CMV expression vector, pSport6.1. The cDNA was subcloned from this construct to pcDNA3.1/zeo on a *Sal*I/*Not*I fragment. ES cells were electroporated with *Bst*Z17I/*Pvu*I digested plasmid and colonies selected on Zeocin. Five clones were tested for expression by flow cytometry. The best expressor was selected for differentiation studies.

Sprouting assay

Eng$^{+/-}$ and *Eng*$^{-/-}$ ES cells were differentiated into EBs at 2×10^3 ES cells per 35-mm Petri dish in EB media (as described above) supplemented with recombinant human (rh) insulin (10 μg/ml), human (h)VEGF (50 ng/ml), hEPO (2 U/ml), hIL6 (10 ng/ml) and bFGF (100 ng/ml) (Feraud et al., 2001; Vittet et al., 1996). After 11 days of EB differentiation, intact EBs were sub-cultured at 140-180 EBs per 35-mm Petri dish with the same cytokines on a collagen I matrix (Becton Dickinson). After 3 days at 37°C and 5% CO_2, the vascular spindle-like EBs were assessed for sprouting angiogenesis based on the following categories: I, no sprout formation; II, few sprouts; III, many sprouts but no network; and IV, many sprouts with network (Feraud et al., 2001). Scoring was performed in a blinded fashion.

Flow cytometry

For endoglin, a rat anti-mouse antibody was used (Pharmingen). For secondary staining, an R-phycoerythrin (PE)-conjugated goat anti-rat Ig (Pharmingen) was applied. For FLK-1 (also known as KDR – Mouse Genome Informatics), a biotinylated anti-mouse antibody was used (R&D Systems), followed by Streptavidin-Allophycocyanin (APC) (Pharmingen). We also used fluorescein-5-isothiocyanate (FITC)-conjugated anti-mouse GPIIB (also known as ITGA2B and CD41 – Mouse Genome Informatics) and PE-conjugated anti-mouse c-KIT (KIT; Pharmingen). For the staining of endothelial progenitors, we used APC-conjugated anti-mouse FLK-1 (eBioscience), biotinylated anti-mouse TIE-2 (also known as TEK – Mouse Genome Informatics; eBioscience) and rat anti-mouse VE-cadherin (also known as CDH5 – Mouse Genome Informatics; Pharmingen), followed by Streptavidin-APC and PE-conjugated goat anti-rat Ig.

EB cells were collected after a short incubation with trypsin, washed twice, first with IMDM 10% FBS and then with blocking buffer (PBS 1% FBS), suspended in the same buffer containing 0.25 μg/10^6 cells of Fc block (Pharmingen) and placed on ice for 5 minutes. Antibody was added at 1 μg/10^6 cells and incubated at 4°C for 30 minutes before washing with blocking buffer. When secondary staining was required, cells were counterstained with the appropriate secondary antibody for 20 minutes at 4°C, followed by washing with blocking buffer. Stained cells were analyzed on a FACSAria cell sorter (Becton-Dickinson) after the addition of propidium iodide (Pharmingen) to exclude dead cells.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using Trizol (Invitrogen) as recommended by the manufacturer. First-strand cDNA was produced using Superscript II reverse transcriptase (Invitrogen) with Oligo dT. A total of 5% of first-strand reaction was used for each ensuing PCR reaction. Primer sequences and PCR conditions are described in Table 1. For real-time PCR, all probe sets were acquired from Applied Biosystems. For globins, we designed our own primer/probe sets (all shown 5′-3′):

Beta-major F, AGGGCACCTTTGCCAGC; Beta-major R, GGCA-GCCTCTGCAGCG; Beta-major probe, 6FAM-CGTGATTGTGCTGG-GCCACCACCT-TAMRA.

Beta-H1 F, CCTCAAGGAGACCTTTGCTCAT; Beta-H1R, CAGGCA-GCCTGCACCTCT; Beta-H1 probe, 6FAM-CAACATGTTGGTGATT-GTCCTTTCT-TAMRA.

Table 1. RT-PCR primer sequence information

Primer	Primer pair sequence (5′-3′)	Annealing temperature (°C)	Product size (bp)
beta-actin	GTGGGGCGCCCCAGGCACCA CTCCTTAATGTCACGCACGATTTC	50	520
endoglin	GGTGTTCCTGGTCCTCGTTT CAAAGGAGGTGACAATGCTGG	54	640
brachyury	TCCAGGTGCTATATATTGCC TGCTGCCTGTGAGTCATAAC	50	947
Tie-2	ATGGACTCTTTAGCCGGCTTA CCTTATAGCCTGTCCTCGAA	55	337

RESULTS

Defective hemangioblast development in the absence of endoglin

To assess whether endoglin plays a role in hemangioblast development, the ability of endoglin-deficient ES cells to produce BL-CFCs was tested. *Eng*$^{-/-}$, *Eng*$^{+/-}$ and *Eng*$^{+/+}$ ES cells were differentiated into EBs, and, at day 3, cells were disrupted and plated for blast colony formation. No difference in blast colony formation was found between heterozygous and wild-type ES cells (Fig. 1A). However, a significant reduction in the number of BL-CFCs was observed in *Eng*$^{-/-}$ cells (approximately 15-fold) when compared with *Eng*$^{+/-}$ or *Eng*$^{+/+}$ BL-CFCs (Fig. 1A; $P<0.001$). To evaluate whether the lack of endoglin was simply delaying the differentiation program of hemangioblast formation, BL-CFCs were also analyzed on day 4 of EB differentiation. Virtually no colonies were seen, independent of the genotype (data not shown). Conversely, to address whether lack of endoglin was accelerating the differentiation program, BL-CFCs were analyzed on days 2 and 2.5 of EB differentiation. On day 2.5, the number of BL-CFCs was also reduced in *Eng*$^{-/-}$ EBs when compared with *Eng*$^{+/+}$ EBs (data not shown). Day 2 EBs generated only secondary EBs and no BL-CFCs, irrespective of the genotype (data not shown).

Endoglin-deficient BL-CFCs have reduced hematopoietic and unaffected endothelial replating potential

To assess whether *Eng*$^{-/-}$ BL-CFCs, which were reduced in number as well as in size compared with those of *Eng*$^{+/-}$ or *Eng*$^{+/+}$ (Fig. 1A,B), had compromised bipotentiality, single *Eng*$^{-/-}$ and *Eng*$^{+/-}$ BL-CFCs were replated under hematopoietic and endothelial conditions. Whereas *Eng*$^{+/-}$ BL-CFCs generated secondary hematopoietic colonies and endothelial cells at a similar frequency, *Eng*$^{-/-}$ BL-CFCs were skewed towards the endothelial lineage (Fig. 1C). The lack of a defect in endothelial commitment agrees with the normal expression levels found for TIE-2 on EBs by RT-PCR (Fig. 2B). Taken together, these data suggest that, although endoglin is required for hemangioblast development and hematopoietic commitment, its absence does not affect, and might even enhance, endothelial potential at these early stages.

Endoglin marks the hemangioblast

FLK-1 marks nascent mesoderm (Ema et al., 2006a) and is the only known surface marker for the hemangioblast (D'Souza et al., 2005; Ema et al., 2003; Huber et al., 2004). To address whether the defective hemangioblast formation from endoglin-deficient ES cells was associated with an insufficient number of FLK-1-positive precursors, expression of FLK-1 and endoglin was evaluated over a time course of EB differentiation. As expected, endoglin was absent in *Eng*$^{-/-}$ cells (Fig. 2A, lower panel, and Fig. 2B). However, FLK-1 levels were equivalent to wild-type cells (Fig. 2A, upper panel), suggesting that a lack of endoglin does not interfere with the origin of mesoderm. This finding is corroborated by the normal levels of brachyury, a mesodermal marker, in differentiating *Eng*$^{-/-}$ cells (Fig. 2B). We also observed that endoglin was abundantly expressed in ES cells (Fig. 2A,B) and that, as differentiation proceeded, its expression was gradually reduced to approximately 13% of the total EB population by day 6 (Fig. 2A, upper panel). Interestingly, during differentiation, a fraction of ENG^{+} cells also became positive for FLK-1, reaching a peak for double-positives (ENG^{+}FLK-1^{+}) on day 3 of EB differentiation (approximately 30%), which coincided with the peak for hemangioblast activity during EB differentiation (Fig. 2A, upper panel). With the aim of examining which cell population contains the hemangioblast precursor (the BL-CFC), we carried out cell-sorting experiments on EBs derived from wild-type ES cells. In these experiments the four cell fractions (ENG^{+}FLK-1^{+}, ENG^{+}FLK-1^{-}, ENG^{-}FLK-1^{+} and ENG^{-}FLK-1^{-}) were purified and assayed for their hemangioblast activity (Fig. 2C). We observed a substantial enrichment for blast colonies in the double-positive fraction (ENG^{+}FLK-1^{+}) (Fig. 2C) when compared with the ENG^{-}FLK-1^{+}, ENG^{+}FLK-1^{+}, or ENG^{-}FLK-1^{-} cell fractions (enriched 3.4-, 5.3- and

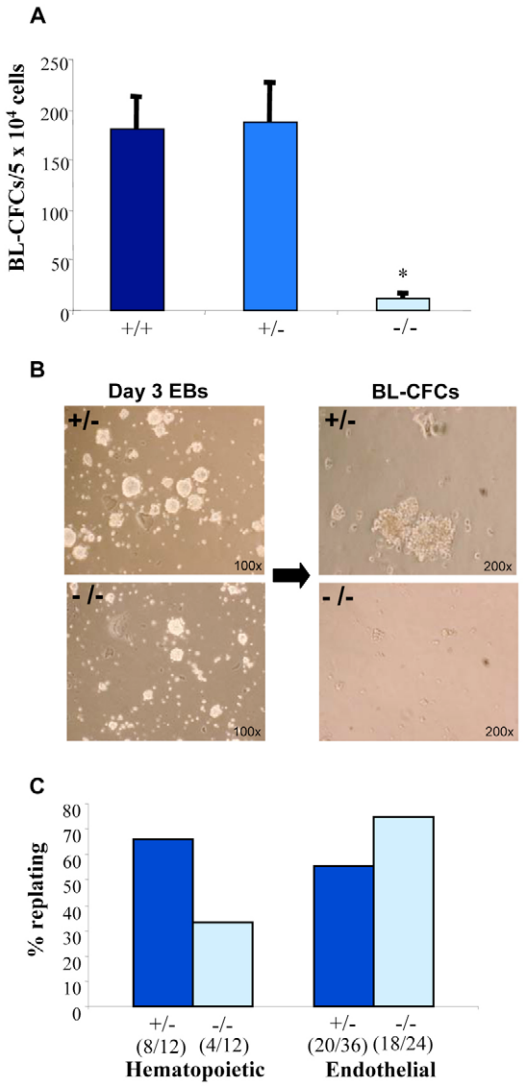

Fig. 1. Defective blast-colony formation in the absence of endoglin. (**A**) Number of BL-CFCs in EBs from *Eng*$^{+/+}$, *Eng*$^{+/-}$ and *Eng*$^{-/-}$ ES cells on day 3 of differentiation. Error bars indicate standard deviations from three independent experiments performed in duplicate. *, $P<0.001$ versus control (+/+ and +/–). (**B**) Morphologic aspect of EBs and resulting BL-CFCs in *Eng*$^{+/-}$ (upper panels) and *Eng*$^{-/-}$ (lower panels) ES cells. (**C**) Evaluation of the hematopoietic and endothelial potentials of BL-CFCs: individual BL-CFCs were picked, disrupted and replated under the hematopoietic (methylcellulose supplemented with IL-3, IL-6, SCF and EPO) or endothelial (gelatin-coated dishes with VEGF, bFGF, IGF-1 and ECGS) conditions to determine their potential to generate secondary hematopoietic colonies and to form adherent endothelial cells, respectively. The number of colonies that yielded secondary CFCs or adherent endothelial cells and the total number of replated colonies are indicated in parentheses.

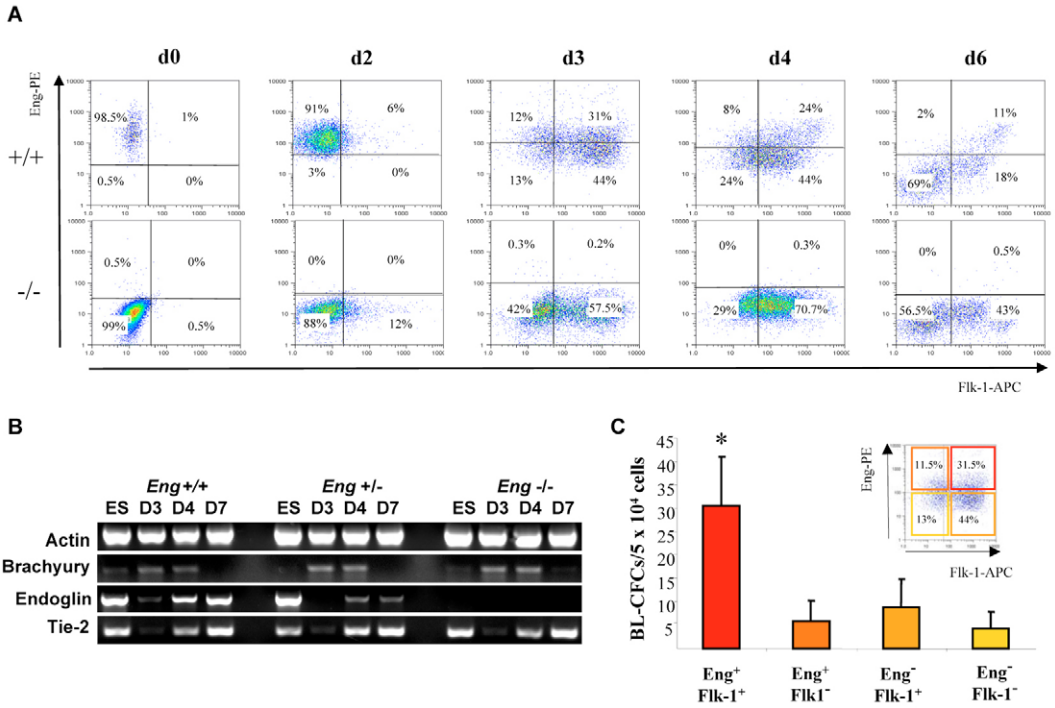

Fig. 2. Endoglin marks the blast colony-forming cell. (**A**) A representative FACS profile of a time-course analysis for endoglin and FLK-1 expression in *Eng*$^{+/+}$ and *Eng*$^{-/-}$ ES cells (day 0, d0) and EBs differentiated for 2, 3, 4 and 6 days. Fluorescence intensity for endoglin is indicated on the *y* axis and for FLK-1 on the *x* axis. (**B**) Gene expression analysis of *Eng*$^{+/+}$, *Eng*$^{+/-}$ and *Eng*$^{-/-}$ ES cells during EB differentiation. (**C**) Wild-type day 3 EBs were sorted based on expression of endoglin and FLK-1, according to represented gates, as follows: ENG^{+}FLK-1^{+}, ENG^{+}FLK-1^{-}, ENG^{-}FLK-1^{+} and ENG^{-}FLK-1^{-}. Resulting cell fractions were plated immediately for blast colonies (BL-CFCs). Error bars indicate standard deviations from two independent experiments performed in duplicate. *, $P<0.05$ versus the other three cell fractions.

7.1-fold, respectively). These results indicate that endoglin is not only essential for hemangioblast development, but it actually also functions as a marker for this precursor on day 3 of EB differentiation.

Endoglin rescues hemangioblast activity in *Eng*$^{-/-}$ ES cells

To further confirm that the lack of endoglin was indeed the reason for the reduction in BL-CFC, we attempted to rescue the phenotype by inserting a CMV-endoglin transgene into the *Eng*$^{-/-}$ ES cells. ES cells containing the transgene (–/–:R5) expressed endoglin at similar levels to control ES cells (Fig. 3A, upper panel; 69% versus 67%, respectively). However, as differentiation into EBs proceeded, endoglin expression by the –/–:R5 clone was significantly reduced (Fig. 3A, lower panel; 3.7% versus 40.4%, respectively). This was probably due to epigenetic silencing occurring as differentiation progressed. Although *Eng*$^{-/-}$ rescue at the protein level at day 3 was only 4%, the hemangioblast phenotype incorporated effects of endoglin from day 0 to day 3, resulting in significantly higher numbers of BL-CFCs (77% rescue) (Fig. 3B,C).

Embryonic hematopoiesis is impaired in the absence of endoglin

Because anemia is a feature of yolk sacs from 9.5 dpc *Eng*$^{-/-}$ embryos (Arthur et al., 2000), we also examined early erythropoiesis using differentiating EBs. For this purpose, *Eng*$^{-/-}$, *Eng*$^{+/-}$ and *Eng*$^{+/+}$ ES cells were differentiated into EBs for 3, 4 and 5 days, at which time cells were disrupted and plated for primitive erythroid colonies (EryP) in methylcellulose medium containing IL-3, IL-6, SCF and EPO. Overall, a reduction in the number of EryP colonies was observed in *Eng*$^{-/-}$ EBs at all the EB time points studied (Fig. 4A; 8.3-, 6.7- and 25.8-fold reduction on days 3, 4, and 5 of EB differentiation, respectively). We also evaluated levels of both embryonic and adult globins as well as several hematopoietic-specific genes, including those encoding SCL, GATA2, RUNX1, GATA1, GPIIB (CD41), NFE2 and FMS (also known as CSF1R – Mouse Genome Informatics), on *Eng*$^{-/-}$ EBs, and observed an overall reduction in hematopoietic-specific gene expression (Fig. 4B). These results provide an explanation for the anemia observed in vivo in *Eng*$^{-/-}$ embryos as well as the reduced hematopoietic replating potential observed in *Eng*$^{-/-}$ BL-CFCs (Fig. 1C). Flow cytometry analyses of day 6 EBs for c-KIT and GPIIB (CD41) (the alpha component of $\alpha_{2b}\beta_3$ integrin), for which the double-positive fraction represents hematopoietic progenitors (Mikkola et al., 2003; Mitjavila-Garcia et al., 2002), revealed that this population is reduced by half in the absence of endoglin (Fig. 4C).

Lack of endoglin does not affect vasculogenesis but reduces branching ability (angiogenesis)

To investigate whether angioblast precursors are affected by the lack of endoglin, *Eng*$^{+/+}$ and *Eng*$^{-/-}$ day 6 EBs were analyzed for the expression of VE-cadherin in combination with FLK-1 or TIE-2 (Zhang et al., 2005). As evidenced by the percentage of cells that were double-positive for VE-cadherin/FLK-1 and VE-cadherin/TIE-2 (Fig. 5A), no differences were found in the frequency of vascular precursors between the two genotypes. These results suggest that vasculogenesis is not affected by the absence of endoglin, which corroborates our findings on the normal endothelial replating of *Eng*$^{-/-}$ BL-CFCs (Fig. 1C).

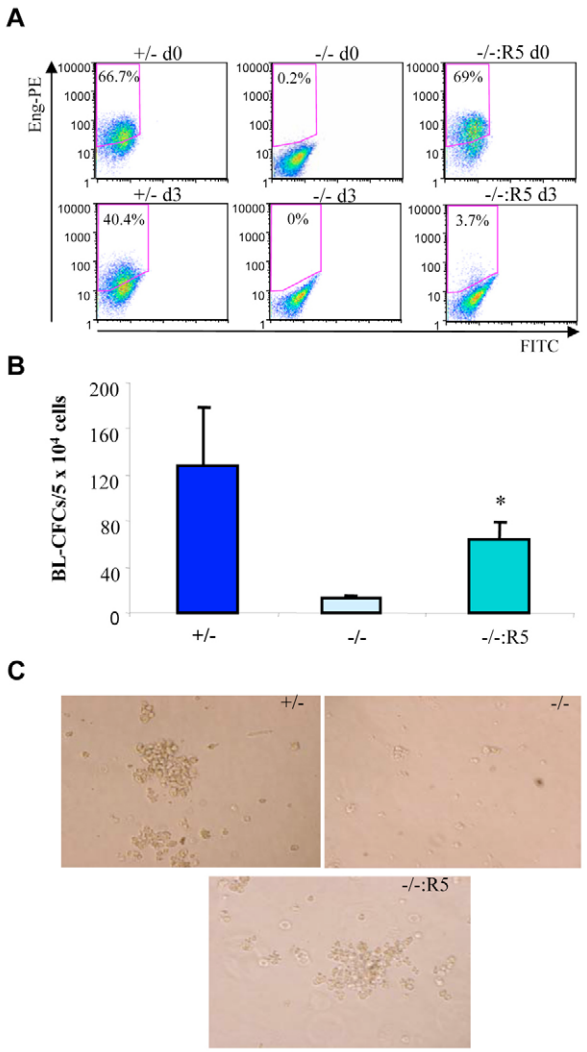

Fig. 3. A CMV-endoglin transgene rescues hemangioblast activity in *Eng*$^{-/-}$ ES cells. (**A**) FACS analysis for endoglin expression in *Eng*$^{+/-}$, *Eng*$^{-/-}$ and –/–:R5 (rescued clone) ES cells. Upper panels, undifferentiated cells (day 0); lower panels, day 3 of EB differentiation. Fluorescence intensity is indicated on the *y* axis. The *x* axis represents FITC, which provides a measure of autofluorescence. (**B**) Number of BL-CFCs on day 3 EBs for *Eng*$^{+/-}$, *Eng*$^{-/-}$ and –/–:R5 ES cells. Error bars indicate standard deviations from two independent experiments performed in duplicate. *, $P<0.01$ versus –/–. (**C**) Representative blast colonies shown at similar magnification (200×).

To assess the later role of endoglin in angiogenesis, we applied an in vitro angiogenic model, in which EBs are transferred into a collagen I matrix (Feraud et al., 2001). In this model, EBs develop endothelial sprouting in the presence of angiogenic growth factors (Vittet et al., 1996) and form a primitive vascular network. *Eng*$^{+/+}$, *Eng*$^{+/-}$ and *Eng*$^{-/-}$ ES cells were differentiated in methylcellulose for 11 days in the presence of VEGF, EPO, IL-6 and bFGF. At this point, intact EBs were sub-cultured with the same cytokines on a collagen I matrix for 3 days and sprouting angiogenesis was assessed. Whereas the majority of *Eng*$^{+/+}$ and *Eng*$^{+/-}$ EBs presented many sprouts with extensive network formation (class III and IV), the sprouts produced by *Eng*$^{-/-}$ EBs had reduced branching ability (Fig. 5B,C). This result agrees with the findings from endoglin-knockout mouse embryos (Li et al., 1999).

DISCUSSION

Origins of hematopoietic and endothelial progenitors

The spatial association between embryonic hematopoietic precursors and angioblasts in the yolk sac blood islands prompted the hypothesis that they might derive from a common precursor, the hemangioblast (His, 1900; Murray, 1932; Sabin, 1920). This remained a hypothesis for many decades, until a cell with the properties of the hemangioblast was identified in differentiating cultures of mouse ES cells (Kennedy et al., 1997). This system recapitulates the developmental program of yolk sac hematopoiesis. The BL-CFC forms in response to VEGF and, when replated, yields endothelial and hematopoietic progeny (Choi et al., 1998; Kennedy et al., 1997). Recently, the same investigators have detected this precursor in mouse embryos (Huber et al., 2004), where it was found at maximum frequency in the posterior streak region of the neural plate-stage embryo (Huber et al., 2004), indicating that the initial stages of hematopoietic and vascular commitment take place in posterior primitive streak mesoderm before blood island development in the yolk sac. Additionally, it has been suggested that hematopoetic cells in the early murine yolk sac might derive directly from endothelial cells (Li et al., 2005; Nishikawa et al., 1998). By using a combination of surface markers on 8.25 dpc yolk sac, Li and colleagues demonstrated that TIE-2^{+}FLK-1^{dim}CD41^{-} cells, which are devoid of hematopoetic activity, give rise to CD41^{+} hematopoietic progenitors and endothelial colonies upon co-culture of purified cells on OP9 stroma (Li et al., 2005). Although TIE-2 and FLK-1 are endothelial markers, it has recently been shown that early mesodermal precursors express these as well (Ema et al., 2006a; Ema et al., 2006b). Thus, whether these cells are truly endothelial in nature, or are early mesodermal progenitors, is not completely clear. Notably, endoglin is coexpressed with these markers in early extraembryonic mesoderm from gastrulating embryos (Ema et al., 2006b; Hirashima et al., 2004), providing support for the idea of an early role for endoglin in the specification of the hemangioblast.

Endoglin as a regulator of cell fate decision

Despite extensive research in the field, our understanding of the molecular mechanisms that govern the lineage decision from mesodermal cells to hemangioblast and how the hemangioblast selects between endothelial and hematopoietic fates is very limited. To date, a handful of genes have been reported to play a role in hemangioblast formation, including *Flk-1* (Faloon et al., 2000; Schuh et al., 1999), *Scl* (Faloon et al., 2000; Robertson et al., 2000), *Runx1* (Lacaud et al., 2002), *EphB4* (Wang et al., 2004) and *Mixl1* (Willey et al., 2006). In addition, a negative regulator, *Hex* (also known as *Hhex* – Mouse Genome Informatics), has been identified (Guo et al., 2003; Kubo et al., 2005).

The data shown here demonstrate that endoglin also plays a crucial role in hemangioblast development. Dramatically reduced numbers of BL-CFCs were observed in the absence of endoglin during EB differentiation. Unlike the *Ephb4* knockout (Wang et al., 2004), endoglin deficiency does not seem to affect the origin of mesoderm, because the levels of FLK-1 and brachyury were unaltered. This points to a later function, at the specification of mesoderm to hemangioblast.

The extent of reduction observed in these studies parallels that seen with *Flk-1*-knockout cells (Schuh et al., 1999). However, whereas the few BL-CFCs resulting from *Flk-1*-deficient EBs retain bipotentiality, the few BL-CFCs that formed from endoglin-null EBs were skewed towards the endothelial lineage. These findings were

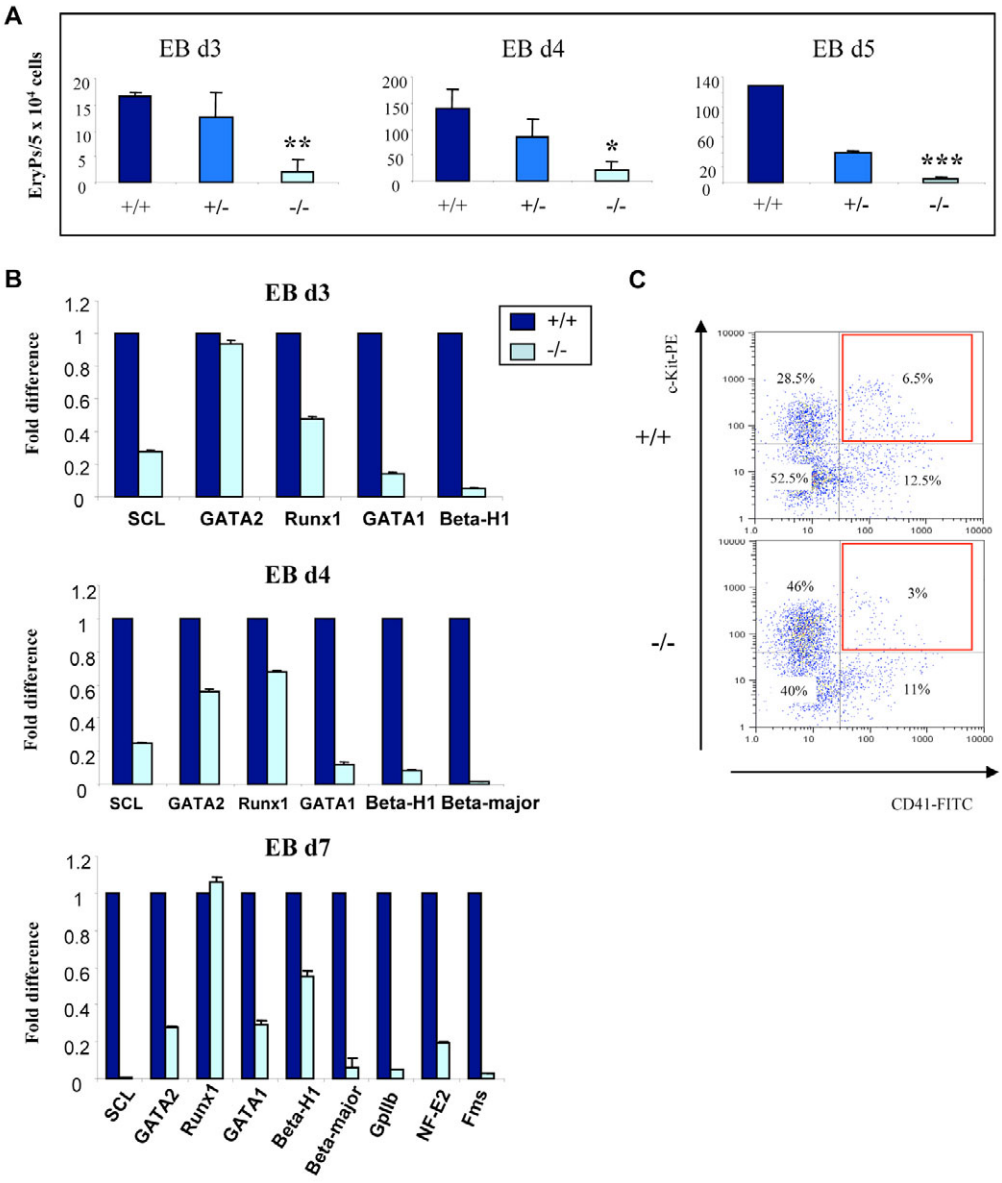

Fig. 4. Defective erythropoiesis in the absence of endoglin. (**A**) *Eng*$^{+/+}$, *Eng*$^{+/-}$ and *Eng*$^{-/-}$ ES cells were assayed for primitive erythroid development by plating cells from EBs differentiated for 3, 4 and 5 days (d3, d4 and d5, respectively) in methylcellulose (MCM) supplemented with IL-3, IL-6, SCF and EPO. (**B**) Relative levels of SCL, GATA2, RUNX1, GATA1, embryonic and adult globins, GPIIB (CD41), NFE2, and FMS from *Eng*$^{+/+}$ and *Eng*$^{-/-}$ day 3, 4 and 7 EBs by real-time RT-PCR. Transcripts are normalized to GAPDH. (A,B) Error bars indicate standard deviations from three independent experiments performed in duplicate. *, $P<0.05$; **, $P<0.005$; ***, $P<0.0001$; versus controls (+/+ and –/–). (**C**) FACS analyses of day 6 *Eng*$^{+/+}$ and *Eng*$^{-/-}$ EBs for GPIIB (CD41) and c-KIT. Fluorescence intensity for c-KIT is indicated on the *y* axis and for GPIIB (CD41) on the *x* axis.

further corroborated by the reduced number of primitive erythroid progenitors present at days 3, 4 and 5 of EB differentiation. The anemia of endoglin-knockout embryos has been interpreted as a secondary manifestation of improper vascularization. Our results suggest that anemia might result from a direct effect of endoglin on hematopoiesis. This assumption is supported by in vitro studies using day-9 and -12 *Eng*$^{-/-}$ ES/OP9 co-cultures, which show a reduction in myelo-erythropoietic progenitors in the absence of endoglin (Cho et al., 2001). Interestingly, in our studies, haploinsufficiency was observed for primitive erythropoiesis. Thus, it is reasonable to hypothesize that the anemia, a classic symptom of hereditary hemorrhagic telangiectasia (HHT) patients, might result from an intrinsic defect in erythropoiesis.

Based on the extensive literature on the role of endoglin in endothelial cells, one could expect a defect in endothelial differentiation from hemangioblasts, and not the opposite. The data presented here show that the commitment from hemangioblast to endothelial cells is unaffected; however, there is a later defect in angiogenesis, shown by the impaired branching ability of endoglin-null EBs. This result agrees with the findings from endoglin-knockout mouse embryos, in which yolk sacs contain highly vascularized plexus but limited vessel branching (Bourdeau et al., 1999; Li et al., 1999). Therefore, in the endothelial lineage, lack of endoglin seems to affect mainly angiogenic processes, including the differential growth and sprouting of endothelial tubes as well as proper interaction among

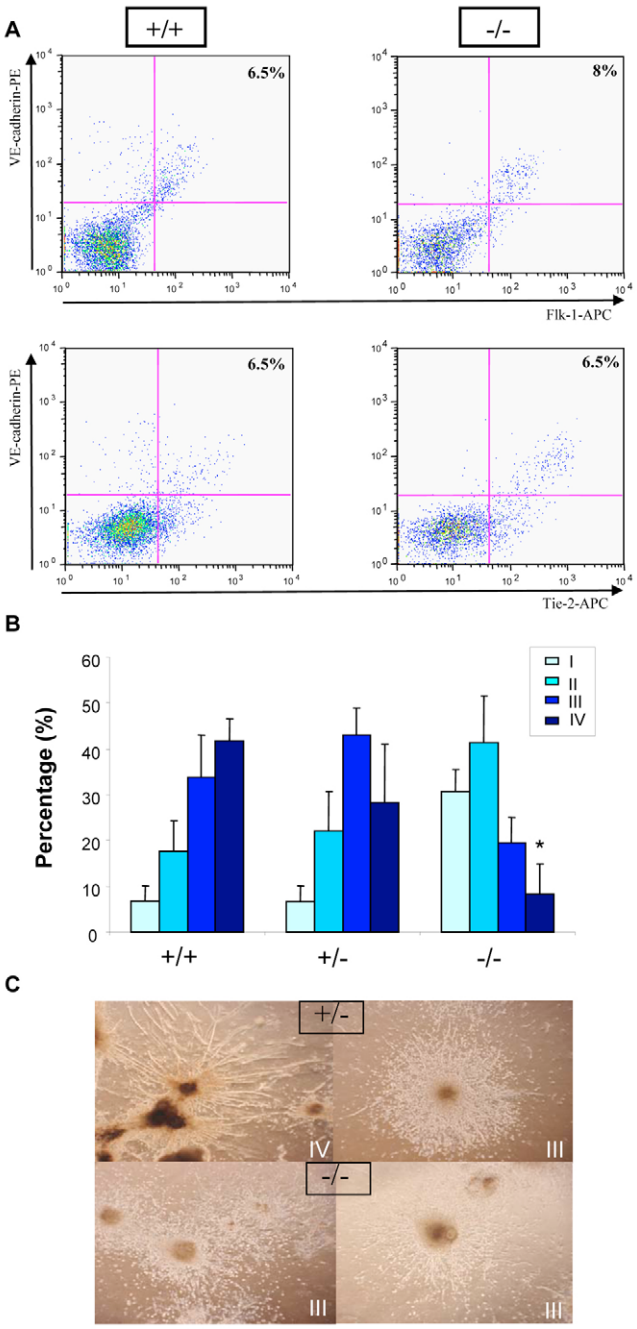

Fig. 5. The effect of endoglin deletion on in vitro vascular sprout formation in differentiating EBs. (**A**) FACS analyses of day 6 *Eng*$^{+/+}$ and *Eng*$^{-/-}$ EBs for VE-cadherin/FLK-1 (top) and VE-cadherin/TIE-2 (bottom). Fluorescence intensity for VE-cadherin is indicated on the *y* axis and for FLK-1 or TIE-2 on the *x* axis. (**B**) Percentage of EBs in each of the four classes of vascular sprout development in *Eng*$^{+/+}$, *Eng*$^{+/-}$ and *Eng*$^{-/-}$ ES cells. Categories: I, no sprout formation; II, few sprouts; III, many sprouts but no network; and IV, many sprouts with network. Error bars indicate standard deviations from two independent experiments performed in duplicate. *, *P*<0.0001 versus controls (+/+ and +/–) on category IV. (**C**) Morphologic aspect of sprouting in *Eng*$^{+/-}$ (upper panels) and *Eng*$^{-/-}$ (lower panels) EBs.

endothelial, vascular smooth muscle cells and pericytes, culminating in arrested endothelial remodeling and fragile vessel walls (Li et al., 1999).

Endoglin identifies the blast colony-forming cell

To date, the only cell-surface antigen known to mark the hemangioblast is FLK-1. However FLK-1 is currently known to be expressed in a broader variety of mesodermal cells, including smooth (Ema et al., 2003; Yamashita et al., 2000), cardiac (Ema et al., 2006a; Motoike et al., 2003; Schroeder et al., 2003) and skeletal muscle (Ema et al., 2006a; Motoike et al., 2003). Thus, only a subset of FLK-1^{+} cells possesses hemangioblast potential. In this regard, our cell-sorting experiments with wild-type EBs reveal that endoglin in combination with FLK-1 identifies the hemangioblast. Although it was also expressed in other cell types at day 3 of EB differentiation, approximately half of the FLK-1^{+} population expressed endoglin, and this cell fraction was significantly enriched for BL-CFCs.

Endoglin and TGF-β signaling

Because endoglin is a receptor for TGF-β, it is likely that this factor influences hemangioblast development, particularly in light of the fact that TGF-β1 is expressed in the yolk sac blood islands of 7.5 dpc embryos (Akhurst et al., 1990). TGF-β1 is well known for its inhibitory effect in endothelial differentiation (Basson et al., 1992; Muller et al., 1987) and in hematopoietic proliferation (Ohta et al., 1987; Sing et al., 1988). Accordingly, a reduction in the number of hematopoietic progenitors was observed when TGF-β1 was added to EB cultures (Park et al., 2004). Inhibition of BL-CFCs was also observed when TGF-β1 was added to the blast media (R.C.R.P., unpublished). Based on these in vitro results, one would expect that absence of this potent growth inhibitor in vivo would result in endothelial and hematopoietic hyperplasia. However, *Tgfb1* knockouts present compromised hematopoiesis and endothelial differentiation, a phenotype somewhat similar to the endoglin knockout (Dickson et al., 1995). These seemingly discordant findings might be due to the in vivo redundancy with other TGF-β isoforms or to a biphasic effect (Goumans et al., 2003), with high levels being inhibitory but low levels being necessary. Nevertheless, because endoglin is an accessory non-signaling receptor, it is most likely to function as a modulator of TGF-β responses in early mesoderm. We are currently investigating the precise mechanism by which endoglin plays a role in this early developmental process.

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