RESEARCH ARTICLE

Endoglin potentiates nitric oxide synthesis to enhance definitive hematopoiesis

Rabab Nasrallah1,2, Kathy Knezevic1, Thuan Thai3, Shane R. Thomas3, Berthold Göttgens4,5, Georges Lacaud2,*, Valerie Kouskoff2,* and John E. Pimanda1,6,*

ABSTRACT

During embryonic development, hematopoietic cells develop by a process of endothelial-to hematopoietic transition of a specialized population of endothelial cells. These hemogenic endothelium (HE) cells in turn develop from a primitive population of FLK1+ mesodermal cells. Endoglin (ENG) is an accessory TGF-β receptor that is enriched on the surface of endothelial and hematopoietic stem cells and is also required for the normal development of hemogenic precursors. However, the functional role of ENG during the transition of FLK1+ mesoderm to hematopoietic cells is ill defined. To address this we used a murine embryonic stem cell model that has been shown to mirror the temporal emergence of these cells in the embryo. We noted that FLK1+ mesodermal cells expressing ENG generated fewer blast colony-forming cells but had increased hemogenic potential when compared with ENG non-expressing cells. TIE2+/CD117+ HE cells expressing ENG also showed increased hemogenic potential compared with non-expressing cells. To evaluate whether high ENG expression accelerates hematopoiesis, we generated an inducible ENG expressing ES cell line and forced expression in FLK1+ mesodermal or TIE2+/CD117+ HE cells. High ENG expression at both stages accelerated the emergence of CD45+ definitive hematopoietic cells. High ENG expression was associated with increased pSMAD2/eNOS expression and NO synthesis in hemogenic precursors. Inhibition of eNOS blunted the ENG induced increase in definitive hematopoiesis. Taken together, these data show that ENG potentiates the emergence of definitive hematopoietic cells by modulating TGF-β/pSMAD2 signalling and increasing eNOS/NO synthesis.

KEY WORDS: Endoglin, Nitric oxide, FLK1, SMAD2, Hemogenic endothelium, Hematopoiesis

INTRODUCTION

Endoglin (ENG), also known as CD105, is a 190 kDa homodimeric transmembrane glycoprotein composed of disulfide-linked subunits (Gougos and Letarte, 1988). It was first identified on the surface of pre-B acute lymphoblastic cells (Zhang et al., 1996), and was later found abundantly expressed on the surface of vascular endothelial cells (Cheifetz et al., 1992). ENG functions as an auxiliary receptor that modulates TGF-β signaling and promotes endothelial cell proliferation (Barbara et al., 1999). During development, ENG is first detected at the onset of gastrulation in the extra-embryonic ectoderm and future amniotic fold at embryonic day (E) 6.5. At E7–7.5, expression extends to the amnion and allantois and is also observed in hematopoietic and endothelial cells of yolk sac blood islands (Ema et al., 2006). By E9.5, ENG is expressed throughout the developing vasculature and menenchyme, but is absent from epithelial cells (Jonker and Arthur, 2002). In addition to its ubiquitous expression on the surface of proliferating endothelial cells, ENG is also expressed on endothelial cells and hematopoietic clusters within the dorsal aorta (DA), which contain emerging hematopoietic stem cells (HSCs) in the developing embryo (Cheifetz et al., 1992; Perlino, 2007), and on the surface of adult bone marrow long-term HSCs (Chen et al., 2002).

The mesoderm that contributes to blood and endothelial cells of the yolk sac blood islands represents a rare population that migrates from the posterior primitive streak. Whether the earliest blood and endothelial cells originate from a shared mesodermal precursor, i.e. a hemangioblast, or from distinct mesodermal precursors is a moot point. Whereas in vitro differentiation of embryonic cell populations and in vivo labelling in zebrafish support the existence of a shared progenitor (Huber et al., 2004; Vogeli et al., 2006), in vivo labelling and cell tracing in mice support largely independent origins (Padrón-Barthe et al., 2014). However, labelling rapidly dividing heterogeneous cell populations in E5.5–7.5 mouse embryos runs the risk of reporter systems marking a mix of epiblast, mesodermal, blood and endothelial progenitors and a method to uniquely label epiblast cells and trace their progeny in vivo remains elusive. Nevertheless, a clonal assay that permitted isolation of murine blast colony–forming cells (BL-CFCs) has been used extensively to define the presence of and quantify hemangioblasts in vitro and in vivo (Choi et al., 1998; Huber et al., 2004; Kennedy et al., 1997).

In the presence of VEGF, BL-CFCs form blast colonies which upon re-plating give rise to primitive and definitive blood progenitors and endothelial cells (Choi et al., 1998; Kennedy et al., 1997). Blast colonies express a number of genes common to both hematopoietic and endothelial lineages, including Scl, CD34, and Flk1 (Kennedy et al., 1997).

The close spatio-temporal association between ENG expression and the emergence of hematopoietic tissues during development (Ema et al., 2006; Roques et al., 2012) led to investigations into a possible functional role for ENG in the embryonic emergence of blood and endothelium (Borges et al., 2012; Perlino, 2007; Zhang et al., 2011). These investigations showed that ENG null embryonic stem (ES) cells had a decreased ability in generating BL-CFC, and demonstrated reduced primitive erythroid and angiogenic

Received 13 January 2014; Accepted 14 April 2015
demonstrated that ENG expression in FLK1+ cells marks a population of cells with early hematopoietic potential. This was evidenced by an increase in the frequency of CD45+ hematopoietic colonies in FLK1+/ENG+ cultures compared with FLK1+/ENG− cultures at 24 hours (Fig. 2E). There was no skewing of differentiation towards any particular lineage (supplementary material Fig. S5). No hematopoietic colonies were detected in the FLK1− fractions regardless of ENG expression. It is salient to note however that FLK1+/ENG− cells do not remain ENG− for long and rapidly express ENG and at 24 hours there is little difference in ENG expression between pre-culture FLK1+/ENG− and FLK1+/ENG+ cells in liquid culture (supplementary material Fig. S6). Taken together with the decrease in BL-CFC potential in FLK1+/ENG+ cells compared with FLK1+/ENG− cells, these data suggest that surface expression of ENG in FLK1+ cells marks a population of cells that are more advanced in their development towards a hematopoietic fate.

**Induced ENG expression in FLK1+ mesoderm increases the frequency of hematogenic endothelial cells and enhances definitive hematopoiesis**

To establish whether ENG expression merely accompanies or drives the acceleration of hematopoietic commitment of FLK1+ cells, we generated an inducible ENG ES cell line (iEng) using parental LoxP-Cre Ainv18 ES cells (Kyba et al., 2002) in which ENG is linked to GFP via a 2A peptide to facilitate monitoring (supplementary material Fig. S7). The addition of Dox at day 0 of ES cell differentiation resulted in the induction of GFP and sustained surface expression of ENG (supplementary material Fig. S8Ai,ii). In addition to the proportion of cells expressing ENG, the abundance of ENG transcripts in FLK1+ cells was also increased (supplementary material Fig. S8Ai,ii). As previously reported, we also observed that Bry expression levels and the proportion of FLK1 expressing cells were largely unaltered with
ENG overexpression (Baik et al., 2012) (supplementary material Fig. S8B). When DOX was added at day 0 to differentiating iEng ES cells and BL-CFC assays were performed at days 2 and 3, we saw less BL-CFCs in DOX treated cells compared with untreated cells (supplementary material Fig. S9). As ENG is expressed in undifferentiated ES cells and expression falls during ES differentiation (supplementary material Fig. S1) forced expression of ENG prior to the emergence of FLK1+ mesoderm probably skews differentiation towards cell fates other than those derived from progenitors that give rise to blast colonies.

To investigate the effects of ENG induction in FLK1+ mesoderm, FLK1+ cells were sorted from day 3 iEng:GFP EBs and cultured in the presence or absence of Dox in four day liquid cultures (Fig. 3A). High ENG expression in FLK1+ cells resulted in comparatively lower frequencies of TIE2+/CD117+ and CD41+ cells (Fig. 3Ai,ii) but increased frequencies of CD45+ cells from day two onwards (Fig. 3Aiii). Cells collected at day 4 were also seeded into hematopoietic CFU assays, and showed a significant increase in the number of definitive hematopoietic colonies following Eng induction (Fig. 3B). As with endogenous ENG+ versus ENG− fractions in Fig. 2E and supplementary material Fig S5, there was no skewing of haematopoiesis towards any particular lineage (supplementary material Fig. S10). To investigate the relationship between the decrease in frequencies of TIE2+/CD117+ and CD41+ cells and the increase in CD45+ cell frequencies and definitive hematopoietic colonies, we quantified the frequency of functional HE progenitors following Dox treatment. To this end, a limiting dilution assay (LDA) was performed with sorted FLK1+ cells in the presence or absence of Dox. After four days, wells containing cells with hematopoietic morphology were counted. Following ENG induction in FLK1+ cells the frequency of functional HE cells increased from 1:341 (untreated) to 1:101 (treated) (Fig. 3C). These data show that high ENG expression in FLK1+ cells yields higher numbers of hemogenic progenitors within a diminished pool of TIE2+/CD117+ cells. Although ENG expression in FLK1+ mesoderm potentiates hematopoiesis, it is not a pre-requisite for HE commitment and haematopoiesis. FLK1+ mesodermal cells from day 3 EBs from ENG−/− ES are still able to generate HE and blood cells in liquid cultures but their numbers, as shown by LDA and colony counts are severely compromised (supplementary material Fig. S11).

**Induced ENG expression in hemogenic endothelial progenitors enhances definitive hematopoiesis**

To determine if ENG can similarly impact on the kinetics of blood emergence if overexpressed at later time points, we sorted...
Fig. 2. ENG expression on FLK1+ mesodermal cells is associated with early commitment to a hematopoietic fate. (A) Flow cytometry graphs showing surface expression of ENG and CD117 in day 3 BRY+/FLK1−, BRY+/FLK1+ and BRY−/FLK1+ cell populations. (B) (i) Representative flow cytometry graphs showing the expression of CD41 and TIE2 in FLK1+/ENG+ and FLK1+/ENG− sorted cell fractions during a four day liquid blast culture assay. (ii) Corresponding flow cytometry graphs showing the expression of CD117 and TIE2 in cell populations in Bi. (C) Phase-contrast photographs of FLK1+/ENG+, FLK1+/ENG− and FLK1+/ENG+ cells at day 3 of blast culture. (D) Histogram showing the percentage of cells expressing CD45 in cell populations in Bi. The P values represent the statistical significance of CD45 expressing cells between two different cell populations at the same time point. (E) Bar chart showing the number of hematopoietic colonies derived from the FLK1+/ENG+ and FLK1+/ENG− cell fractions collected at day 2 of liquid blast cultures. Each experiment was performed in triplicate. The error bars represent the SEM across three independent experiments. The P values were calculated using student’s t-tests from three independent biological replicates. *P<0.05.
Fig. 3. See the next page for legend.
TIE2+/CD117+ cells cultured in the presence or absence of Dox for EBs. TIE2+/CD117+ cells were sorted and re-plated in liquid blast media for a further 48 hours in the presence or absence of Dox. The flow cytometry graphs to the TIE2+ (28.7% and 66.2%) cells following ENG induction (Fig. 4A).

Expression during four days of liquid blast cultures of FLK1+ cells with Dox (red) (A) (i) Representative flow cytometry graphs showing CD41 and TIE2 surface expression from the same cell populations in Ai. (ii) Representative flow cytometry graphs showing CD117 and TIE2 surface expression from the same cell populations in Ai. (iii) Histograms showing the percentage of cells expressing CD45 during 4 days of liquid blast culture. The error bars represent SEM values calculated from three independent biological replicates. (B) Bar chart showing the number of hematopoietic colonies in cells isolated from day 2 culture. (C) FLK1+ cells sorted from day 3 iEng:GFP EBs were seeded on to a 96 well plate in a limiting dilution assay (LDA) to quantify the effect of Dox induction on the frequency of HE. The histograms represent the data obtained from the LDA which shows that the frequency of HE increases from 1:341 (black) to 1:101 (red) following induction of ENG. The P values were calculated using t-tests from three independent biological replicates. *P<0.05.

Fig. 4. Induction of high ENG expression in FLK1+ cells in day 3 EBs enhances their hemogenic potential. iEng:GFP ES cells were differentiated into day 3 EBs and FLK1 positive cells were sorted and cultured (with or without Dox) for four days in media supporting blast colony differentiation. (A) (i) Representative flow cytometry graphs showing CD41 and TIE2 surface expression during four days of liquid blast cultures of FLK1+ cells with Dox (red) or without Dox (black). (ii) Representative flow cytometry graphs showing CD117 and TIE2 surface expression from the same cell populations in Ai. (iii) Histograms showing the percentage of cells expressing CD45 during 4 days of liquid blast culture. The error bars represent SEM values calculated from three independent biological replicates. (B) Bar chart showing the number of hematopoietic colonies in cells isolated from day 2 culture. (C) FLK1+ cells sorted from day 3 iEng:GFP EBs were seeded on to a 96 well plate in a limiting dilution assay (LDA) to quantify the effect of ENG induction on the hematopoietic differentiation potential of TIE2+/CD117+ HE cells. The histograms represent the data obtained from the LDA which shows that the frequency of blood emergence from HE cells increases from 1:479 (untreated) to 1:225 (treated) (Fig. 4C). Taken together, these data show that induction of ENG expression in FLK1+ cells or TIE2+/CD117+ promoted hematopoietic commitment and the emergence of CD45+ hematopoietic cells.

ENG induced acceleration of definitive hematopoiesis is mediated by increased eNOS/NO activity
There is also an extensive body of evidence showing that ENG increases eNOS expression by modulating Smad2 protein levels in endothelial cells (Santibanez et al., 2007). Shear stress induced expression of eNOS and NO production has been shown to enhance hematopoiesis in ES/EB differentiation assays and also in vivo (Adamo et al., 2009; North et al., 2009). Shear stress has also been shown to increase ENG expression in endothelial cells, which in turn mediates TGF-β/pSmad2 signaling and leads to the increase of eNOS (Lebrin et al., 2004; Seghers et al., 2012). Therefore, we speculated that the increase in definitive hematopoiesis that we observed with ENG induction in FLK1+ and TIE2+/CD117+ cells could be mediated by the ENG/pSmad2/eNOS/NO endothelial axis. Indeed, cellular levels of pSMAD2 (Fig. 5Ai) and expression levels of eNOS (Nos3) mRNA and protein (Fig. 5Ai,iii) were increased in ENG induced cells. Furthermore, there was no change in protein levels of SMAD2 (data not shown) and pSMAD1/5 (data not shown). Induction of ENG in FLK1+ cells was accompanied by an increase in intra-cellular NO levels, and this increase was blunted by...
the addition of NG-nitro-L-arginine methyl ester (L-NAME), a selective inhibitor of NOS enzymes (Fig. 5B). The addition of L-NAME to liquid cultures of FLK1+ cells also restrained the increase in ENG induced emergence of CD45+ cells and numbers of definitive hematopoietic colonies (Fig. 5Ci,ii).

**DISCUSSION**

We have used an ES cell differentiation system to evaluate the impact of ENG expression on hematopoietic commitment in mesodermal progenitors. We report that FLK1+ mesodermal cells that express ENG have reduced BL-CFC potential and are committed towards a hematopoietic fate. FLK1+ cells from day 3 EBs subjected to high ENG expression showed increased capacity to produce hemogenic endothelial, CD45+ hematopoietic cells and definitive hematopoietic colonies. Forced ENG expression in TIE2+/CD117+ cells from day 2 liquid cultures also showed similar properties. ENG mediates the acceleration of hematopoiesis from mesodermal and hemogenic progenitors by facilitating increased eNOS and NO levels.

The TGF-β superfamily of ligands signal by binding a complex of type 1 and type 2 receptors and mediating phosphorylation of downstream SMAD transcription factors to impact on gene transcription. The identity of the type 1 receptors (ALK1 or ALK5) that engage the ligand and its cognate type 2 receptor helps determine whether downstream TGF-β signaling occurs via the pSMAD1/5/8 pathway (ALK1) that promotes cell migration and proliferation or the pSMAD2/3 pathway (ALK5), which is associated with cell differentiation (Seghers et al., 2012). Shear stress induced increase in ENG expression facilitates ALK5 mediated pSMAD2 phosphorylation and eNOS expression (Santibanez et al., 2007; Seghers et al., 2012). Shear stress has also been reported to increase hematopoietic colony-forming potential and expression of hematopoietic markers in the para-aortic splanchnopleura/aorta-gonads-mesonephros of mouse
embryos and that abrogation of nitric oxide, a mediator of shear-stress-induced signalling, compromises hematopoietic potential in vitro and in vivo (Adamo et al., 2009; North et al., 2009). Here we show that stage specific overexpression of ENG was associated with increased pSMAD2/eNOS and NO levels and abrogation of eNOS activity blunted the increase in definitive hematopoiesis that was mediated by high ENG. eNOS molecules have been reported to bind the cytoplasmic region of the ENG receptor, where Hsp90 molecules reside. This ENG mediated proximity of eNOS and Hsp90 generates a complex that catalyzes the degradation of L-Arginine (L-Arg) to L-Citrulline (L-Cit) and generates NO molecules. This eNOS/Hsp90 association is impaired in ENG−/− ES cells, leading to decreased production of NO (Santibanez et al., 2007; Toporsian et al., 2005).

The most direct evidence to show that the diminished BL-CFC potential in a FLK1+ mesodermal cell that expressed ENG was accompanied by commitment towards a haemogenic fate would be to sort the same cell into both blast colony (BL-CFCs) and liquid culture (haemangionic endothelial and blood) assays. As this is beyond current limits of technology, we had to settle for sorting FLK1+/ENG− or FLK1+/ENG+ cells from the same EB differentiation assay into either BL-CFCs (Fig. 1B) or liquid cultures (Fig. 2B). The data showed that FLK1+/ENG+ cells produced fewer blast colonies but were more advanced in expression of haematopoietic markers (CD41/45; Fig. 2B,D), emergence of blood cells (Fig. 2C,E) than FLK1+/ENG− cells. The decrease in BL-CFCs that we observed in ENG expressing FLK1+ cells in day 3 EBs, contrasts with earlier reports that ENG marked a population of cells with increased BL-CFC activity (Perlingeiro, 2007). The number of BL-CFCs that can be generated from ES/EB cultures is closely linked to the emergence of FLK1+ cells. ES/EBs acquire FLK1 expression by day 2.5 (~6% of cells), and this progressively increases to reach 20–30% by day 4, after which it begins to fall (Fehling et al., 2003). The number of blast colonies on the other hand peaks at day 3 and falls sharply at day 4. There is therefore a window during ES/EB differentiation, where FLK1+ cell numbers increase but BL-CFC potential decreases. The numbers of BL-CFCs in the report associating ENG expression with increased BL-CFC activity (Perlingeiro, 2007) (10–30 BL-CFCs/5×10^4 FLK1+ cells from day 3 EBs) were considerably lower than those in this study (e.g. 2000–2500 BL-CFCs/2×10^4 FLK1+ cells from day 3 EBs; Fig. 1Bii). The low numbers of BL-CFCs also coincided with the presence of an unusually high number of FLK1−cells at day 3 (~75% versus ~25%). Taken together, these data suggest that the culture conditions might not have been ideal to assess functional differences between cell fractions.

CD117 expression on day 3 FLK1+ cells has been reported as an early marker of hematopoietic commitment (Kataoka et al., 1997). It is noteworthy that CD117 expression in day 3 FLK1+ cells is limited to a fraction of FLK1+/ENG− cells and is not expressed on FLK1+/ENG+ cells (Fig. 2A). This again is consistent with ENG as an early marker of hematopoietic commitment within a pool of FLK1+ cells in day 3 EBs. ENG overexpression in bulk populations of day 4–6 EBs has been reported to have no effect on definitive hematopoietic colony formation (Baik et al., 2012). This again contrasts with the observation that ENG overexpression in relevant cell populations, i.e. FLK1+ cells from day 3 EBs that are enriched in hematopoietic progenitors or in TIE2+/CD117+ cells in day 2 liquid cultures that are enriched in haemogenic endothelial precursors, there is both an increase in definitive hematopoietic colony formation and CD45+ cell emergence. Only a proportion of Tie2+/CD117+ intermediates in liquid cultures possess functional HE potential (Lancrin et al., 2009). ENG overexpression in TIE2+/CD117+ cells in day 2 liquid cultures led to a decrease in CD117+ and 41+ expressing Tie2+ cells albeit with increased functional HE cells. Whether this is due to perturbation of surface marker expression or selection of cells that progress in culture is not known. Bearing in mind that FLK1− mesoderm is closer to the apex of embryonic haematopoesis than TIE2+/CD117+ cells, it is also interesting to note that forced expression of ENG in the former have a greater impact (~3 fold) probably by increasing numbers of HE precursors than perturbation in the latter (~2 fold), which have a more restricted differentiation potential. Taken together, this study underscores the importance of using refined rather than bulk cell populations when using ES/EB differentiation cultures to interpret the role of a gene during early blood development.

Overall, our data supports a role for ENG in priming the hemogenic program at a very early stage of embryonic development i.e. as early as in FLK1+ mesodermal progenitors. The observation that this is at least in part mediated by increased NO synthesis is particularly relevant given the role of NO in mediating sheared induced HSC emergence from the aorta-gonad-mesonephros during embryonic development (Adamo et al., 2009; North et al., 2009). ENG expression in the AGM is regulated by the ENG promoter in conjunction with an up-stream ~8 kb and two intronic +7 kb and +9 kb enhancers that collectively assemble components of the Ets/Gata hemogenic code (Pimanda et al., 2006; Pimanda et al., 2008). These cis-regulatory elements of ENG are bound by FLI1, GATA2 and SCL in E11.5 AGM which in turn constitute a recursively wired triad of hematopoietic transcription factors that maintain each other’s expression at this site by binding their own enhancer elements in the AGM (Pimanda et al., 2007b). Constituents of the FLI1/ GATA2/SCL network that regulates ENG also regulates BMP signaling and RUNX1 activity in the AGM (Narula et al., 2010; Narula et al., 2013; Pimanda et al., 2007a). RUNX1 is required for the in vivo generation of definitive hematopoietic cells (Okuda et al., 1996) and its expression is also shear induced (Adamo et al., 2009). Taken together with its role in potentiating NO synthesis in endothelial cells, the transcriptional connectivity of ENG expression in the AGM to a highly integrated regulatory network that governs blood emergence (Marks-Bluth and Pimanda, 2012; Pimanda and Göttgens, 2010), raises the prospect for a role for ENG in promoting the emergence of definitive hematopoietic cells from hemogenic precursors in the AGM.

MATERIALS AND METHODS

Murine ES cell culture and differentiation

The Bry/GFP (Fehling et al., 2003) and ENG−/− and ENG+/− (Cho et al., 2001) murine ES cells have been previously described. Murine ES cells were seeded on feeder coated plates in media composed of 84% Dulbecco’s Modified Eagle Medium (DMEM, PAA Laboratories), 15% Fetal Bovine serum (FBS, Invitrogen) pre-tested for maintenance of ES cells, 0.1% of 0.15 M dilution of monothioglycerol (MTG, Sigma Aldrich) and 1000 units/ml mouse recombinant Lekuaemia inhibitory factor (Esgro, Millipore). To generate EBs, cells were cultured in media consisting of IMDM supplemented with 15% FBS (PAA laboratories) pretested for efficient embryonic stem cell differentiation, 1% L-Glutamine (Invitrogen), 1% penicillin-streptomycin, 0.3% of 0.15 M MTG, 0.6% of 30 mg/ml transferrin (Life Technologies) and 1% of 5 mg/ml ascorbic acid (Sigma Aldrich). Cells were then seeded on ultra-low attachment 60 mm plates (Sterilin) and placed in a humidified incubator at 37°C and 5% CO2. These culture conditions were optimal for growth of day 1–5 EBs. Cells isolated...
from EB cultures were re-aggregated in EB media at a density of 5×10^4 cells/ml (Sroczyńska et al., 2009).

**Generating iEng ES cell line**

Eng cDNA (Open Systems, clone MM1013-202764815) was amplified from the pCMV-SPORT6 plasmid using 5'-GAGAGATATCT-TCGCTGGTGGAGC-3' and 5'-GAGACTCGAGCGCCATG-CTGCTGGTGAGC-3' (EcoRV and XhoI restriction sites respectively) primers. The fragment was digested with EcoRV/XhoI restriction enzymes and ligated into a 2A-eGFp-pLox backbone (Kyba et al., 2002). Plasmids were sequenced using a BigDye Terminator Sequencing Kit (Life Technologies). The expression plasmid was electrotransfected with Cre-recombinase plasmid into Ainv18 ES cells (0.4 cm, Life Technologies) at 250 V and 450 μF/araday (201 Ω). Two days post electrotransfection the cells were cultured in the presence of Neomycin (480 μg/ml final; Sigma Aldrich), until the clones became visible. Primers used for sequencing were: 2A-eGFp F: ctggatctcaacctaaattt, T: tattctgaagctctgtagc; PA-SV40 R: ttgtgtctcaactcata, pGKnEO F: ctgatcggataagttcag.

**Liquid BL-CFC assay**

Day 3 EBs were dissociated into single cells using trypsin (Gibco), and stained with a Flk1-PE or Flk1-bio antibody (eBioscience). Sorted FLK1+ cells were seeded on gelatin coated plates at a density of 7.5–8.5×10^4 cells/9.6 cm² (in a humidified incubator 37°C, 5% CO₂). The culture medium consisted of IMDM supplemented with 10% FBS pre-tested for differentiation, 1% L-glutamine, 1% L-glutamine, 0.6% 30 mg/ml transferrin, 0.3% 0.15 M MTG, 0.5% 5 mg/ml ascorbic acid, 15% D4T conditioned medium, 0.1% 5 μg/ml VEGF, and 0.1% 10 μg/ml IL-6 (R&D systems).

**Methylcellulose blast colony forming (BL-CFC) assay**

2×10⁴ FLK1+ cells collected from day 3.5–3 EBs were seeded in methylcellulose mix which consisted of IMDM supplemented with 55% methylcellulose (Biological), 10% FBS pretested for efficient EB differentiation, 1% L-glutamine, 1% L-glutamine, 0.6% 30 mg/ml transferrin, 0.3% 0.15 M MTG, 0.5% 5 mg/ml ascorbic acid, 15% D4T conditioned medium, 0.1% 5 μg/ml VEGF, and 0.1% 10 μg/ml IL-6 (R&D systems).

**Flow cytometry and cell sorting**

Cells were stained on ice for 30 minutes with either a biotinylated or fluochrome-conjugated primary antibody. Dead cells were excluded from the analysis on the basis of Hoechst 33258 uptake (1 μg/ml final concentration; Invitrogen). A list of all primary and secondary antibodies can be found in supplementary material Table S1. Flow cytometry analysis was performed using flow cytometers; LSR or BD Canto II. Sorting was performed on an Aria II, Influx or Jazz.

**Western blot**

Protein extractions were performed using RIPA lysis buffer. Protein concentrations were quantified using Bradford assay (BioRad). For Western blotting, 20–40 μg of protein per sample were loaded together with SDS Sample Buffer (Invitrogen) and antioxidant (Invitrogen) onto a 10% polyacrylamide gel (Invitrogen) and electrophoresed for 20 minutes at 80 V, followed by 60 minutes at 180 V in MOPS SDS Running buffer (Novex). The SeeBluePlus2 (Invitrogen) marker was used as a molecular weight indicator. Subsequently, proteins were transferred to nitrocellulose membrane using the iBlot dry transfer system (Invitrogen). Transferred proteins were visualized by reversibly staining membranes with Ponceau S solution (Sigma) for 15 minutes. Membrane were washed with PBS supplemented with 0.1% Tween20 (PBS/T), blocked in buffer containing PBS/T and 5% fat-free powdered milk (Marvel) or 5% BSA and blotted overnight with primary antibodies in blocking buffer. The eNos (BD Transduction Laboratories) was used at a 1:5,000 dilution. The pSmad2 and pSmad3/5 (Cell Signaling Technology) were used at 1:1,000 dilution. The anti-rabbit-HRP and anti-mouse-HRP (Cell Signaling Technology) were used at 1:10,000 dilution. Following three washing steps, each for 5 minutes at room temperature in PBS/T, secondary antibodies were applied. After a final set of washes, the membranes were developed with ECL Plus Western Blotting detection reagents (Amersham), according to manufacturer’s protocol and the membranes were transferred to an X-ray film cassette. Films were exposed and developed in a MAS automated developing machine. Antibodies were removed from the membrane by 15 minutes incubation in stripping buffer (200 mM glycine, 0.1% SDS, 1% Tween-20, pH 2.2). After three washing steps, the membranes were blocked, as described previously, and primary antibody anti β-actin (Cell Signaling Technology; 1:10,000), was applied followed by the secondary antibody.

**RT-PCR**

Prior to RNA isolation from samples, all equipment was treated with RNaseZap (Ambion) to remove any contaminating RNases. RNA was extracted using and RNeasy extraction kit (Qiagen) following manufacturer’s instructions. Typically RNA was eluted with 30 μl RNase-free water. cDNA was then synthesised using the GoScript Reverse Transcriptase (Promega) following manufacturer’s guidelines. Briefly, up to 5 μg of RNA was incubated with random primers and nuclease-free water at 70°C for 5 minutes, and then placed immediately on ice. A reverse transcription mix (5 μl GoScript reaction buffer, MgCl₂, 10 mM dNTP mix, recombinant RNasin, GoScript reverse transcriptase and water) was added to the denatured RNA for amplification. The cDNA was analysed for expression using the Express SYBR Green QPCR Supermix Universal (Life Technologies), following manufacturer’s guidelines.

The primers used for qPCR were: Eng F: aaattcgtctgtagcttt; Eng R: gctgtagctgtagcttt; Gata2 F: cactgtagctgtagcttt; Gata2 R: acgtagctgtagctt; Flk1 F: ccccgacgctcctttcc; Flk1 R: cctggatctcactgccttt; Nos3 F: atcagtctgtagc; Nos3 R: ccagggctgtagc; B-actin F: tggagctgtagc; B-actin R: cgctcggagggtagc.

**NO measurement**

NO levels were measured using the OXiSelect Intracellular Nitric Oxide Assay Kit (Cell Biolabs, Inc.) according to the manufacturer’s instructions. Briefly, on day 3 of liquid blast cultures, cells were harvested and replated in the presence or absence of Dox and L-NAME for 24 hours. Cells were then harvested and washed with PBS, followed by incubation with 1× NO Fluorometric probe for 2 hours at 37°C and 5% CO₂. 1× lysis buffer was added to the cultures and incubated for 5 minutes. 150 μl of the sample suspension was transferred to black polystyrene half-area 96-well plates (Costar) and fluorescence was measured at 485 nm/520 nm. The levels of NO were calculated relative to fluorescence from the corresponding untreated cells (without probe).
Statistical analysis
For each experiment statistical analysis was performed by calculating the standard error of the mean (SEM) across three independent experiments each performed in triplicate for colony assays, and three independent experiments for analyses of change in cell frequency over time between different treatment conditions. Statistical significance of the results was determined using unpaired t-tests. All P values were two-tailed and <0.05 and <0.001 were considered significant (*) and highly significant (**) respectively.

Acknowledgements
The authors thank Dr Züliga-Pflücker (University of Toronto) for the ENG-/- and ENG +/− murine ES cells.

Competing interests
The authors declare no competing or financial interests.

Author contributions
R.N and K.K performed research and analysed data. T.T, S.T and B.G contributed to study design and data interpretation. R.N., G.L, V.K and J.E.P contributed to study design, data interpretation and manuscript preparation. All authors have read and approved the manuscript.

Funding
This work was supported by grants from the National Health and Medical Research Council of Australia, Australian Research Council and the Dr Tom Bee Stem Cell Research Fund to JEP. Cancer Research UK to V.K. and G.L. and the Biotechnology and Biological Sciences Research Council, Leukaemia and Lymphoma Research, The Leukaemia and Lymphoma Society, Cancer Research UK, and core support grants by the Wellcome Trust to the Cambridge Biotechnology and Biological Sciences Research Council, Leukaemia and Stem Cell Research Fund to JEP, Cancer Research UK to V.K. and G.L. and the Research Council of Australia, Australian Research Council and the Dr Tom Bee Stem Cell Research Fund to JEP. The authors thank Dr Zu (2013). Mathematical model of a gene regulatory network reconciles effects of genetic perturbations on hematopoietic stem cell emergence. Dev. Biol. 379, 258-269.


Fig. S1. Representative flow cytometry of FLK1 and ENG expression on single cell suspensions obtained from day 1-day 5 EBs.
**Fig. S2.** (A) Representative flow cytometry plot showing the expression of ENG and Bry-GFP in day 2 EBs (left panel). $5 \times 10^4$ GFP-/ENG$^-$ (Green), GFP-/ENG$^+$ (Red) and GFP$^+$ (Blue) sorted cells were seeded in re-aggregation media for 24 hours. Representative flow cytometry graphs showing the expression of Bry-GFP/FLK1 for each sorted cell fraction (right panels). (B) Representative flow cytometry showing expression of TIE2 and CD41 in FLK1$^-$/ENG$^+$ and FLK1$^+$/ENG$^-$ cells collected from day 3 EBs.
**Fig. S3.** (A) (i) Flow cytometry graph showing the expression of ENG and FLK1 in day 2.5 EBs. (ii) Bar chart showing the number of BL-CFCs in cell fractions sorted from day 2.5 EBs. 20,000 cells were plated in BL-CFC methylcellulose media, and colonies were counted after four days of culture. (B) An image showing the typical morphology of a BL-CFC at 10× magnification. *P<0.05.*

**Fig. S4.** (A) RT-PCR performed on RNA isolated from FLK1+/ENG- and FLK1+/ENG+ samples collected at day 3 of liquid blast cultures. mRNA expression was tested for genes involved in hemangioblast specification, Gata1, Gata2, Pecam1 and Fli1. Gene expression was quantified relative to β-actin.
Fig. S5. Ratios of definitive hematopoietic colonies generated by the FLK1\(^{+}\)/ENG\(^{-}\) and FLK1\(^{+}\)/ENG\(^{+}\) cells.

Fig. S6. FLK1\(^{-}\)/ENG\(^{-}\) cells upregulate ENG expression in liquid culture. (A) Flow plot showing the expression of FLK1 and ENG in day 3 EBs. (B) Flow plots showing the expression of FLK1 and ENG in FLK1\(^{-}\)/ENG\(^{-}\) and FLK1\(^{-}\)/ENG\(^{+}\) sorted cells pre-culture (Upper panel) and 24 hours post-culture (lower panel).
**Fig. S7. Schematic diagram of the strategy used to generate iEng::GFP embryonic stem cell line.** The construct containing Eng cDNA::2A-eGFP fragment was electroporated with a cre-recombinase expression plasmid into Ainv18 ES cells. Cre mediated recombination generated and ES cell line containing Eng cDNA::2A-eGFP fragment downstream of a tet responsive element (TRE). The reconstitution also generated a functional Neomycin resistance gene under PGKneo promoter, promoting clone selection using G418.
Fig. S8. Dox induced expression of ENG in EBs. (A) iEng:eGFP ES cells were cultured in the presence or absence of Dox for four days. Dox was added at the beginning of differentiation. (i) Histogram showing the percentage of ENG positive cells. (ii) Flow cytometry graphs showing the expression of GFP over 4 days of EB differentiation. (iii) RT-PCR showing the levels of *Eng* expression in FLK1⁺/ENG⁺ cells (Cells cultured without Dox) and FLK1⁺/GFP⁺ cells (Cells cultured in the presence of Dox) sorted from day 3 EBs. (B) Histograms showing the percentage of FLK1 positive cells and transcript abundance of Bry during days 1–4 of EB differentiation with and without Dox.*P*<0.05.
**Fig. S9. Endoglin overexpression early in ES/EB differentiation reduced BL-CFC numbers.** (A) (i) Bar chart showing the number of blast and secondary EB colonies. Cells were treated with or without Dox at the ES cell stage. Bulk day 2 EBs were dissociated and 20,000 cells were plated in 1 ml of BL-CFC mix. Blast colonies and secondary EBs were counted after 4 days of culture. The error bars represent standard deviation of the mean. The experiment was performed in technical triplicates. (ii) Phase contrast images showing a typical blast colony and secondary EB. Images were taken at 10× magnification. *P<0.05.

**Fig. S10. Ratios of definitive hematopoietic colonies generated by cells with forced ENG expression.**
Fig. S11. Comparative HE and hematopoietic potential of ENG−/− and ENG+− ES cells. (A) (i,ii) Bar chart showing detailed analysis of day 3 EBs derived from Eng+− and Eng−− ES cells. 3×10^4 ES cells from each cell line were seeded in EB media for 3 days followed by the quantification of (i) cell number and (ii) FLK1+ cell percentage. (iii) Bar chart showing cell counts in day 2 liquid blast cultures. 8.5×10^4 FLK1+ cells were sorted from day 3 EBs derived from Eng+− and Eng−− ES cells, then seeded in liquid blast cultures. After 2 days, the cells were collected and counted. (B) 3×10^4 cells collected from Eng+− and Eng−− day 2 liquid blast cultures were seeded in methylcellulose hematopoietic assays. Primitive and definitive colonies were counted after four and nine days respectively. (C) FLK1+ cells collected from Eng+− and Eng−− day 3 EBs were seeded in LDA assays and wells were scored after four days. The data showed that the frequency of hematopoietic progenitors decreases from 1:119 (Eng+− ES cells) to 1:491 (Eng−−).
Table S1: Primary and secondary antibodies used for flow cytometry and cell sorting.

<table>
<thead>
<tr>
<th>Antigen name</th>
<th>Isotype/conjugate</th>
<th>Clone</th>
<th>Company/catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIE2 (CD202)</td>
<td>Rat/Biotin</td>
<td>TEK4</td>
<td>ebioscience/13-5987</td>
</tr>
<tr>
<td></td>
<td>Rat/PE</td>
<td>TEK4</td>
<td>ebioscience/12-5987</td>
</tr>
<tr>
<td>Endoglin (CD105)</td>
<td>Rat/Biotin</td>
<td>MJ7/18</td>
<td>ebioscience/13-1051</td>
</tr>
<tr>
<td></td>
<td>Rat/PE</td>
<td>MJ7/18</td>
<td>ebioscience/12-1051</td>
</tr>
<tr>
<td></td>
<td>Rat/Pacific blue</td>
<td>MJ7/18</td>
<td>Biolegend/120411</td>
</tr>
<tr>
<td>c-KIT (CD117)</td>
<td>Rat/ APC</td>
<td>2B8</td>
<td>ebioscience/17-1171</td>
</tr>
<tr>
<td></td>
<td>Rat/eFluor780</td>
<td>2B8</td>
<td>ebioscience/47-1171</td>
</tr>
<tr>
<td>Flk1 (CD309)</td>
<td>Rat/Biotin</td>
<td>Avas12α1</td>
<td>ebioscience/13-5821</td>
</tr>
<tr>
<td></td>
<td>Rat/PE</td>
<td>Avas12α1</td>
<td>ebioscience/12-5821</td>
</tr>
<tr>
<td></td>
<td>Rat/PE</td>
<td>Avas12α1</td>
<td>ebioscience/17-5821</td>
</tr>
<tr>
<td>CD41</td>
<td>Rat/Biotin</td>
<td>MWReg30</td>
<td>ebioscience/13-0411</td>
</tr>
<tr>
<td></td>
<td>Rat/Pe-Cy7</td>
<td>MWReg30</td>
<td>ebioscience/25-0411</td>
</tr>
<tr>
<td></td>
<td>Rat/APC</td>
<td>MWReg30</td>
<td>ebioscience/17-0411</td>
</tr>
<tr>
<td>CD45</td>
<td>Rat/percp-cy5.5</td>
<td>30-F11</td>
<td>Biolegend/103132</td>
</tr>
</tbody>
</table>