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The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF

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The differentiation of embryonic stem (ES) cells offers a powerful approach to study mechanisms implicated in cell fate decision. A major hurdle, however, is to promote the directed and efficient differentiation of ES cells toward a specific lineage. Here, we define in serum-free media the minimal factor requirement controlling each step of the differentiation process, resulting in the production of highly enriched hematopoietic progenitors. Four factors – Bmp4, activin A, bFGF (Fgf2) and VEGF (VegfA) – are sufficient to drive the selective and efficient differentiation of mouse ES cells to hematopoiesis. Each of these factors appears to regulate a step of the process: Bmp4 promotes the very efficient formation of mesoderm; bFGF and activin A induce the differentiation of these mesodermal precursors to the hemangioblast fate; and VEGF is required for the production of fully committed hematopoietic progenitors. The stimulation of mesodermal precursors by bFGF and activin A switches on very rapidly the hematopoietic program, allowing us to dissect the molecular events leading to the formation of the hemangioblast. *Runx1*, *Scl* (*Tal1*) and *Hhex* expression is upregulated within 3 hours of stimulation, whereas upregulation of *Lmo2* and *Fli1* is observed later. Interestingly, increased expression levels of genes such as *cMyb*, *Pu.1* (*Sfpi1*), *Gata1* and *Gata2* are not observed at the onset of hemangioblast commitment. This stepwise control of differentiation is extremely efficient, giving rise to a very high frequency of hematopoietic precursors, and provides an optimal system for understanding the molecular machineries involved in blood progenitor commitment.

KEY WORDS: Bmp4, Embryonic stem cell, Hemangioblast, Hematopoiesis, VEGF, Mouse

INTRODUCTION

Upon differentiation, mouse embryonic stem (ES) cells can give rise to primitive and definitive hematopoietic precursors, an in vitro process that has been shown to recapitulate the in vivo development of yolk sac hematopoiesis (Keller et al., 1993; Palis et al., 1999). This progressive differentiation can be monitored by measurement of gene expression and analysis of biological potential by secondary replating, which enables the quantification of all blood progenitors via their colony-forming ability. As they lose their self-renewal and pluripotent characteristics, ES cells form epiblast-like cells that differentiate further to give rise to mesodermal precursors (Kouskoff et al., 2005; Pelton et al., 2002). The first blood precursor, the hemangioblast, derives from the mesoderm and gives rise to primitive and definitive hematopoiesis, smooth muscle and endothelium (Ema et al., 2003; Huber et al., 2004; Yamashita et al., 2000). The existence of this precursor has been established in vitro in mouse and human ES differentiation models (Kennedy et al., 2007; Lu et al., 2007; Wang et al., 2004), but also in vivo in mouse (Huber et al., 2004) and zebrafish (Vogeli et al., 2006). However, Ueno and Weissman recently challenged the concept of such a precursor through the direct clonal analysis of yolk sac blood islands (Ueno and Weissman, 2006). The carefully orchestrated process that generates the hemangioblast can be mimicked in vitro upon the differentiation of ES cells in well-defined conditions; fetal calf serum being one of the key component of these culture conditions (Choi et al., 1998; Nishikawa et al., 1998). Although the inherent characteristics of ES cell lines are likely to be important for efficient differentiation, the batch of serum used appears critical in obtaining the most effective differentiation. This fact illustrates the complexity of serum composition and the many parameters that may modulate, positively or negatively, the differentiation process. To better dissect and understand the molecular mechanisms of cell fate specification, refined culture conditions are needed to allow the specific and efficient differentiation of ES cells toward hematopoiesis. Several serum-free culture conditions have been described for the in vitro derivation of hematopoietic cells from mouse ES cells (Adelman et al., 2002; Ng et al., 2005; Park et al., 2004; Wiles and Johansson, 1997) or human ES cells (Tian et al., 2004; Zambidis et al., 2006). However, none of these previously published studies described serum-free culture conditions that allowed a selective differentiation of ES cells toward hematopoietic precursors, and that induced the robust production of these progenitors.

To optimize the culture conditions allowing the formation of blood progenitors upon ES cell differentiation in vitro, we tested several serum-free media and many growth factors. We found that only four factors were required for the very efficient and highly selective production of both primitive and definitive hematopoietic precursors. Our data show that the differentiation process can be separated into discrete steps that are each dependant on only one or two factors. Moreover, the stepwise nature of these culture conditions allowed us to further dissect the molecular program that regulates mesoderm specification at the onset of hematopoiesis.

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MATERIALS AND METHODS

ES cell growth and differentiation

ES cells were maintained on irradiated mouse embryonic feeder cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% fetal calf serum (FCS; PAA, Yeovil, UK), penicillin, streptomycin, LIF (1%

conditioned medium from CHO cells) and 1.5×10^{-4} M monothioglycerol (MTG; Sigma). Prior to the onset of differentiation, cells were transferred on gelatinized plates, once in the same medium and a second time in a similar medium but using Iscove's Modified Eagle Medium (IMDM) instead of DMEM. For the generation of embryoid bodies (EBs), ES cells were trypsinized and plated at various densities in differentiation cultures. For stimulation with Bmp4, activin A, bFGF and VEGF, cells were plated at 25,000 cells/ml. For serum-supplemented culture, cells were plated with 15% serum (Harlan SERA-LAB, Loughborough, UK) at 25,000 cells/ml for early time points (up to day 3.5) and at 10,000 cells/ml for later time points. Differentiation of EBs was carried out in 60 mm Petri grade dishes in StemPro (Gibco) reconstituted according to the manufacturer's instructions and supplemented with 2 mM L-glutamine (Gibco), 200 μg/ml transferrin (Roche), 0.5 mM ascorbic acid (Sigma) and 4.5×10^{-4} M MTG. Bmp4, bFGF, activin A and VEGF (all from R&D Systems) were reconstituted according to the manufacturer's recommendations and used routinely at 5 ng/ml unless otherwise stated. We tested several other commercially available serum-free media with less success than StemPro: Knockout Serum Replacement, DMEM/F12 and IMDM/F12 (all from Gibco).

Secondary colony assays

For the generation of blast cell colonies (BL-CFC assay), after trypsinization (TrypLE Express, Gibco) EB cells were plated at a density of 2×10^4 cells/ml in 1% methylcellulose supplemented with 10% FCS, VEGF (5 ng/ml), IL6 (5 ng/ml) and 25% D4T endothelial cell conditioned medium (Choi et al., 1998). For the growth of hematopoietic precursors, cells were plated at 2×10^4 cells/ml in 1% methylcellulose containing 10% plasma-derived serum (PDS; Antech), 5% protein-free hybridoma medium (PFHM-II; Gibco-BRL) and the following cytokines: KL (1% conditioned medium), TPO (5 ng/ml), erythropoietin (2 U/ml), IL11 (25 ng/ml), IL3 (1% conditioned medium), GM-CSF (3 ng/ml), G-CSF (30 ng/ml), M-CSF (5 ng/ml) and IL6 (5 ng/ml). Cultures were maintained at 37°C, 5% CO_2. LIF, IL3 and c-Kit ligand were derived from media as previously described (Fehling et al., 2003). VEGF, GM-CSF, M-CSF, G-CSF, TPO, IL6 and IL11 were purchased from R&D Systems.

Matrigel plug assay

Individual vascular smooth muscle (VSM) or blast colonies were transferred to 50 μl Matrigel plugs in 96-well plates. Matrigel (BD Biosciences) was diluted 1:1 with IMDM supplemented with 10% FCS, 50 ng/ml VEGF, 5 ng/ml bFGF and allowed to solidify at 37°C before the addition of colonies. Cultures were maintained at 37°C, 5% CO_2, 5% O_2 for 4-8 days. CD31 staining was performed on tubule-like structures after removal of most Matrigel by vigorous washing in PBS (only possible for large structures). After paraformaldehyde fixation for 30 minutes, tubule structures were incubated in 10% serum in PBS for 30 minutes, followed by 4 hours incubation with CD31-bio or non-specific CD3-bio control (BD Biosciences). After four 15-minute washes in PBS, bound antibody was revealed using Vectastain ABC-AP and Blue-AP Substrate Kit III (Vector Laboratories) according to the manufacturer's instructions.

Gene expression analysis

For gene-specific PCR, total RNA was extracted from each sample using the RNeasy Plus Mini Kit (Qiagen). Two micrograms of RNA was reverse-transcribed into cDNA using random hexamers and the Omniscript RT Kit (Qiagen). The PCR reactions were performed using Biomix Taq (Bioline) and 0.2 μM each primer. Cycling conditions were: 94°C for 5 minutes, followed by 30 cycles of amplification (94°C denaturation for 30 seconds, 60°C annealing for 30 seconds, 72°C elongation for 60 seconds), then a final incubation at 72°C for 10 minutes. Sequences of the gene-specific PCR primers are available upon request. Real-time PCR was performed on an ABI 7900 system (Applied Biosystems) using Universal ProbeLibrary (Exiqon) and Primer Designer (Roche). All expression data were calculated relative to actin controls as $2^{-\Delta ct}$.

Flow cytometry

EBs were harvested, trypsinized, and the single-cell suspension was directly analyzed for GFP expression or further stained. Cells were blocked with FcRγII/III antibody (24G2 supernatant) prior to staining with various combinations of antibodies CD45-FITC, Flk1-bio, CD41-PE, CD34-bio, CD31-bio, Tie2-PE (all Pharmingen) or Ve-cad-647 (eBioscience) for 20 minutes on ice in PBS containing 10% FCS, followed by strep-PECy5 or strep-PECy7 (Pharmingen) for 20 minutes on ice in PBS containing 10% FCS. Non-specific antibody binding was always controlled for by staining cells with the appropriate isotype controls. After two washes, cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences). For all analysis, flow cytometry data were initially gated on the forward scatter versus side scatter profile, enabling the separation of live cells from debris and dead cells.

Immunofluorescence

Single VSM colonies were picked, transferred on gelatin-coated coverslips and cultured in IMDM supplemented with 10% FCS, 2 mM L-glutamine, 4.5×10^{-4} M MTG, VEGF (25 ng/ml), bFGF (20 ng/ml) and 25% D4T endothelial cell conditioned medium. After 6-10 days, grown colonies were fixed in 2% paraformaldehyde for 20 minutes, washed twice in PBS, permeabilized in 0.2% Triton X-100 in PBS, washed in 10% FCS, 0.2% Tween 20 in PBS, and then blocked with 10% FCS in PBS for 10 minutes. Colonies were incubated for 1 hour with anti-CD31-bio and anti-smooth muscle actin-FITC (Sigma). Bound anti-CD31 antibodies were visualized using a secondary streptavidin-Cy3 (Caltag).

RESULTS

Identification of optimum serum-free media and best combination of cytokines for hematopoietic differentiation

To define the minimal requirement for the generation of blood progenitors from mouse ES cells in absence of serum, we assessed several serum-free media and soluble factors previously shown to induce some degree of mesoderm or hematopoiesis specification (Cerdan et al., 2004; Chadwick et al., 2003; Faloon et al., 2000; Kennedy et al., 2007; Park et al., 2004; Wiles and Johansson, 1997). We used a mouse ES line (GFP-Bry) carrying the cDNA for green fluorescent protein targeted to the brachyury locus to monitor mesodermal differentiation (Fehling et al., 2003). We have shown previously using this ES line that GFP expression recapitulated effectively brachyury expression and could be used to track mesoderm formation in vitro and in vivo (Fehling et al., 2003; Huber et al., 2004). Using embryoid body (EB) formation to differentiate ES cells, we screened several serum-free media as a base for the addition of growth factors, including StemPro, Knockout Serum Replacement, IMDM/F12 and DMEM/F12 (all from Gibco). Selection for a suitable serum-free basal medium was determined on the basis of ES cell relative survival without induction of mesodermal differentiation and without inhibition of differentiation upon serum addition. These criteria were most effectively fulfilled with StemPro. Cell survival in StemPro medium was comparable to that in serum-supplemented culture at day 1, but declined progressively over the next 2 days of culture (see Fig. S1A in the supplementary material). In contrast to serum-supplemented culture, no cell expansion was observed (see Fig. S1A, right panel, in the supplementary material) and no mesodermal differentiation was detected as illustrated by the absence of GFP^+ cells (Fig. 1A). Addition of serum to this serum-free medium at the start of the EB culture induced the generation of mesodermal GFP-Bry^+ cells as well as the formation of committed blood progenitors co-expressing GFP-Bry and VEGF receptor 2 (Flk1; also known as Kdr – Mouse Genome Informatics) (Fig. 1A) as previously described (Fehling et al., 2003). Using this StemPro basal medium, we tested soluble factors alone or in combinations according to the published literature (Faloon et al., 2000; Park et al., 2004; Wiles and Johansson, 1997). A mix of Bmp4, activin A, bFGF (Fgf2) and VEGF (VegfA) was best able to recapitulate the effect of serum, although to a lesser

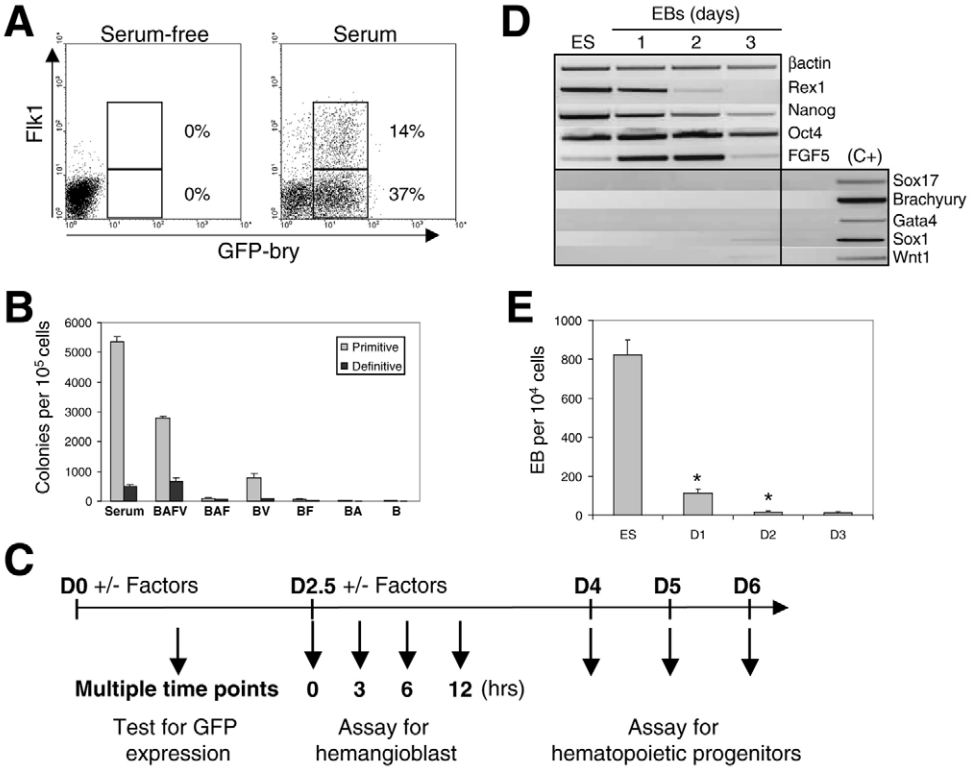

Fig. 1. Transition from ES cells to epiblast-like cells. (**A**) Flk1 and GFP expression analysis by flow cytometry on mouse day-3 embryoid bodies (EBs) grown in serum-free (left) or serum-supplemented (right) culture. (**B**) GFP-Bry ES cells were differentiated in serum-free media supplemented with serum or with the indicated combination of factors added at the start of the culture (B, Bmp4; A, activin A; F, bFGF; V, VEGF). At day 5, EB-derived cells were tested for the presence of hematopoietic progenitors in a secondary replating assay. Primitive, primitive erythrocytes; definitive, all definitive colonies (macrophage, macrophage/erythrocyte, mixed colonies and granulo-macrophage colonies). (**C**) Outline of the experimental design employed in subsequent experiments. (**D**) Gene expression analysis in ES cells and day 1, 2 or 3 serum-free EB-derived cells by RT-PCR. β-actin expression levels were used for cDNA quantity control. cDNA from day-4 EBs grown in serum was used as positive control for *Sox17*, brachyury, *Gata4*; cDNA from day-6 EBs grown in neuronal condition (Li et al., 1998) was used as positive control for *Sox1* and *Wnt1* PCR. (**E**) ES cells grown in serum-free media rapidly lose their ability to form EBs. ES cells and day 1, 2 or 3 EB-derived cells were replated in semi-solid conditions allowing the formation and quantification of EBs. *A statistically significant difference ($P<0.05$) compared with ES cell control. All data shown are representative of at least three experiments. For B and E, data are presented as the mean number of colonies from three dishes. Error bars represent s.e.m.

extent, allowing the generation of primitive and definitive hematopoietic precursors (Fig. 1B). The relative fraction of each type of definitive colony was not significantly different whether EBs were grown in serum or with cytokine mixes (not shown).

The removal of leukemia inhibitor factor and feeder cells triggers the transition from ES to epiblast-like cells

To improve the efficiency of differentiation, we analyzed the effect of individual factors on the progressive differentiation from ES cells to fully committed blood progenitors as depicted in Fig. 1C. The first step in this differentiation process is characterized by a loss of pluripotency and acquisition of an epiblast-like phenotype (Pelton et al., 2002). Analysis of EB-derived cells cultured for up to 3 days in serum-free conditions without added factors showed that already by day 1, the expression of *Rex1* (*Zfp42*), a transcription factor specifically expressed in ES cells, was strongly downregulated. In parallel, the expression of *Fgf5*, which marks epiblast-like cells, was transiently upregulated on day 1 and 2 (Fig. 1D). Real-time PCR quantification confirmed that *Rex1* was downregulated 5-fold by day 1 and more than 100-fold by day 3, whereas *Fgf5* was transiently upregulated up to 7-fold on day 1 and 2 (see Fig. S2 in the supplementary material). The expression of both *Oct4* (*Pou5f1*) and *Nanog* was maintained up to day 2 of differentiation, but by day 3 the expression of these two genes was also downregulated (Fig. 1D). These changes in gene expression were accompanied by a strong decrease in the potential to form EBs upon secondary replating when compared with the potential of ES cells for generating EBs under similar conditions (Fig. 1E). By the second day of EB culture in serum-free media without added soluble factors, the transition from ES cell to epiblast cell appeared complete: *Rex1* expression was barely detectable, *Oct4* and *Nanog* were still expressed, *Fgf5* expression was strongly upregulated, and very few EBs were generated upon secondary replating. The removal of leukemia inhibitor factor (LIF) and feeder cells, which together keep ES cells undifferentiated, appears sufficient to trigger the progression from ES cell to epiblast-like cell stage. Analysis of the expression level of genes indicative of neuronal (*Sox1*, *Wnt1*) or mesoderm/endoderm (*Gata4*, brachyury, *Sox17*) differentiation, did not reveal any overt sign of random differentiation toward any of these lineages (Fig. 1D). Our data indicate that no exogenously added factors are necessary for the transition from ES cells to epiblast-like cells. Although ES cells were able to progress to epiblast-like cells, we never detected GFP expression driven from the brachyury locus.

Bmp4 promotes the efficient formation of mesoderm but a poor specification to hemangioblast fate

The next step of differentiation, the progression from epiblast-like cells to mesoderm, was monitored by assessing the percentage of GFP^+ cells during a time-course of GFP-Bry ES cell differentiation. Various cytokines were tested for their potential to induce mesoderm when added at the start of the EB culture. Bmp4 induced robust mesoderm commitment, whereas VEFG and bFGF did not induce significant GFP expression, and activin A induced only limited GFP upregulation (Fig. 2A,C and see Fig. S3 in the supplementary material). When added at 4 ng/ml, Bmp4 induced a pattern of GFP^+ cell formation highly similar to that seen in the serum condition. By day 2.5 of differentiation, the first GFP^+ cells were detected. This percentage of GFP^+ cells increased steadily to reach nearly 100% by

day 3.75, when the cells started to downregulate GFP expression, as previously observed (Fehling et al., 2003). A dose titration of Bmp4 showed that although a wide range of concentrations induced GFP$^+$ cell formation, both low (0.8 ng/ml) and high (100 ng/ml) concentrations were suboptimal for mesoderm induction (Fig. 2A). The level of GFP expression was not influenced by the Bmp4 concentration (see Fig. S4A in the supplementary material). Interestingly, at day 4 of differentiation, all Bmp4 concentrations tested led to a slight increase in GFP level relative to the serum control. A dramatic increase in GFP$^+$ cell recovery was observed between day 2 and 3 of differentiation, which correlates with the onset of mesoderm formation and supports the inductive rather than survival nature of Bmp4 action (see Fig. S4B in the supplementary material). In support of a lack of direct correlation between survival and induction, Bcl2 overexpression in ES cells led to a 5-fold increase in survival upon EB formation in serum-free conditions, but this enhanced survival did not replace the requirement for Bmp4 to promote mesoderm specification (see Fig. S1B,C in the supplementary material). In addition to brachyury upregulation, Bmp4 stimulation induced the upregulation of several genes indicative of paraxial mesoderm, including *Fgf8*, follistatin, *Mesp2* and *Tbx6* (Fig. 2B). These data suggest that Bmp4 might be able to stimulate the formation of a broad range of mesoderm subpopulations. Our data do not fully address the direct action of Bmp4 on epiblast-like cells. However, it is unlikely that Bmp4 acts through an intermediate visceral endoderm-like layer. Indeed, we failed to detect the presence of a visceral endoderm-like layer in EBs, either by morphology or by DBA (*Dolichos biflorus* agglutinin) staining, whereas this layer was clearly stained with DBA in E7.25 embryos (see Fig. S5 in the supplementary material).

During the course of differentiation with serum, mesodermal precursor formation is shortly followed by the appearance of hemangioblast precursors (Fehling et al., 2003). These hemangioblast precursors can be followed by the upregulation of VEGF receptor 2 (Flk1) and by their ability to form blast colonies in secondary replating assays (Choi et al., 1998; Faloon et al., 2000). These blast colonies are able to generate hematopoiesis, endothelium and smooth muscle lineages when further cultured under appropriate conditions (Ema et al., 2003; Huber et al., 2004; Yamashita et al., 2000). Upon stimulation with Bmp4, we observed the upregulation of Flk1 expression in around 30% of EB-derived cells (Fig. 2C). However, despite Flk1 expression, very few blast

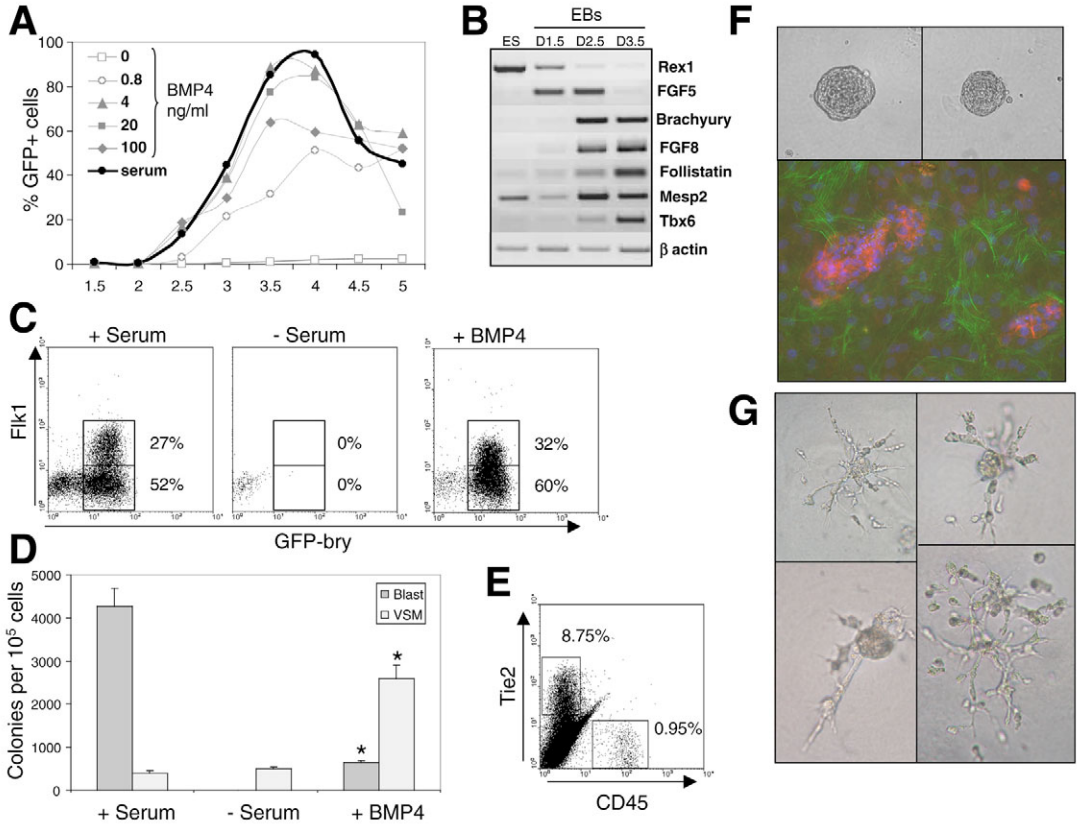

Fig. 2. Bmp4 induces mesodermal specification. (**A**) GFP-Bry ES cells were differentiated in serum-free media either with serum or with various concentrations of Bmp4 added at the start of the culture (day 0). At the indicated time point in days, the percentage of live cells positive for GFP was determined by flow cytometry. (**B**) Gene expression analysis performed on ES cells and day 1.5, 2.5 or 3.5 serum-free plus Bmp4 EB-derived cells by RT-PCR. β-actin expression levels were used for cDNA quantity control. (**C**) Cells derived from day-3.5 EBs were assessed for their relative percentage of GFP-Bry and Flk1 expression. (**D**) Cells derived from day-3.5 EBs were assessed for their ability to form blast and VSM (vascular smooth muscle) colonies upon secondary replating. Colonies were scored 4 days after replating. *A statistically significant difference (P<0.05) compared with the serum control. Data are presented as the mean number of colonies from three dishes. Error bars represent s.e.m. (**E**) Tie2 and CD45 expression levels were analyzed on pools of replated colonies derived from day-3.5 EBs stimulated with Bmp4. (**F**) Morphology of VSM colonies in secondary replating at day 4 of culture (20× magnification). Individual colonies were expanded on glass coverslips for 8 days and stained for the expression of CD31 (red) and smooth muscle actin (green); nuclei are stained in blue with DAPI (20× magnification). (**G**) Individual VSM colonies were cultured for 4 days in Matrigel plugs (10× magnification). All data shown are representative of at least three experiments.

colonies were generated in secondary replating (Fig. 2D); this was observed at all Bmp4 concentrations tested (not shown) and the main type of colonies generated had a smooth and compact aspect (Fig. 2F, top panel). Staining of pooled colonies revealed the presence of a $Tie2^{+}CD45^{-}$ subpopulation (Fig. 2E) that also expressed VE-cadherin (cadherin 5) and CD31 (Pecam1) at low level (not shown) and is likely to represent endothelial cells. These colonies gave rise upon further culture to endothelial and smooth muscle cells as defined by cell morphology and by their respective expression of CD31 and smooth muscle actin (Fig. 2F, lower panel). When cultured in Matrigel plugs, individual colonies gave rise to a tubule-like structure, further indication of their endothelial nature (Fig. 2G). These colonies, termed vascular smooth muscle (VSM) colonies, have been described previously (D'Souza et al., 2005). Overall, these data demonstrate that in a dose-dependant manner, Bmp4 is able to induce the very efficient formation of mesodermal precursors, as nearly 100% of the cells become GFP^{+}. Furthermore, Bmp4 stimulation results in Flk1 upregulation, but Bmp4 alone is not sufficient to promote the robust generation of hemangioblast precursors. The main type of precursors generated in the presence of Bmp4 appears to be restricted to the endothelium and smooth muscle fate.

Stimulation of mesodermal cell with activin A and bFGF induces the formation of hemangioblast precursors

To define the factors required for the efficient progression of mesoderm to hemangioblast, we generated EBs in the presence of Bmp4 for 2.5 days then added various combinations of factors and analyzed the presence of hemangioblast by secondary replating assay. Addition of VEGF induced a low level of hemangioblast commitment (Fig. 3A). The best induction of hemangioblast was observed when bFGF and activin A were added together, with as little as 5 ng/ml of each factor sufficient to trigger a strong induction

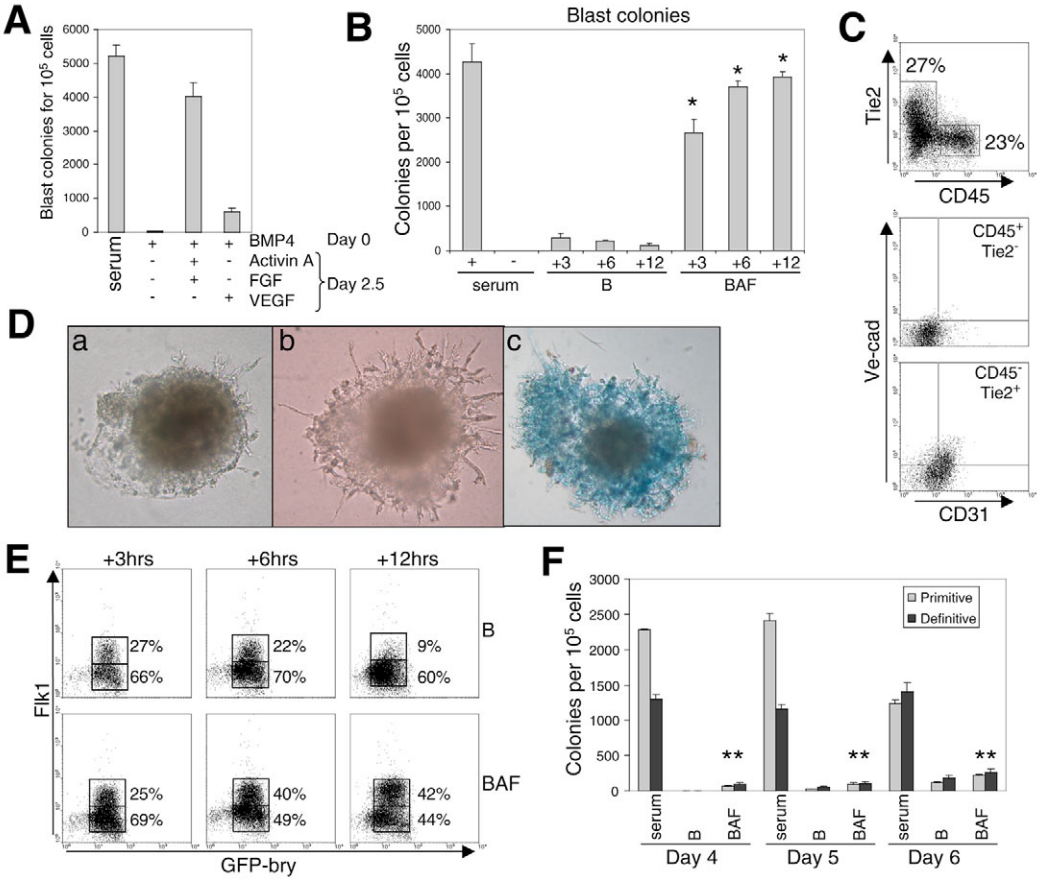

Fig. 3. Activin A and bFGF induce hemangioblast specification. (**A**) VEGF, activin A or bFGF were added at day 2.5 of serum-free culture supplemented with Bmp4 at 4 ng/ml from day 0. EBs were harvested at day 3 and assessed for their ability to form blast colonies by secondary replating. (**B**) activin A and bFGF were added at day 2.5 of serum-free culture supplemented with Bmp4 at 4 ng/ml from day 0. EBs were harvested 3, 6 or 12 hours after the induction with activin A and bFGF and assessed for their ability to form blast colonies by secondary replating. *A statistically significant difference ($P<0.05$) compared with Bmp4-induced cultures. (**C**) Tie2, CD45, CD31 and VE-cadherin expression levels were analyzed on pools of blast colonies. Top panel presents CD45 and Tie2 staining; the level of CD31 and VE-cadherin expression was then assessed on $CD45^{+}Tie2^{-}$ (middle) and $CD45^{-}Tie2^{+}$ (bottom) cells. (**Da-c**) Individual blast colonies (examples a, b and c) were tested for their potential to generate endothelium tubule-like structures in Matrigel plugs. Colony in c is stained with CD31 antibody. 10× magnification, bright field. (**E**) At 3, 6 and 12 hours after activin A and bFGF stimulation, cells were tested for their relative expression of GFP-Bry and Flk1 by flow cytometry. (**F**) EBs were grown for 2.5 days in serum-free culture with 4 ng/ml Bmp4 and then activin A and bFGF were added. At days 4, 5 and 6 of differentiation, EB-derived cells were tested for the presence of hematopoietic progenitors by colony-forming ability in secondary replating assay. Primitive, primitive erythrocytes; definitive, all definitive colonies (see Fig. 1). Primitive colonies were scored 5 days after replating, definitive colonies after 8 days. **A statistically significant difference ($P<0.05$) compared with the serum control. All data shown are representative of at least three experiments. For A, B and D, data are presented as the mean number of colonies from three dishes. Error bars represent s.e.m.

(Fig. 3A). The kinetics of hemangioblast specification was very fast as already by 3 hours after induction, a strong increase in hemangioblast generation could be observed, gradually reaching a plateau by 12 hours post-induction (Fig. 3B). Further analysis of the blast colonies derived from these serum-free-generated hemangioblasts showed that they gave rise to primitive and definitive hematopoietic precursors upon expansion followed by replating (see Fig. S6 in the supplementary material). The presence of endothelial cells within these blast colonies was assessed by staining of pooled colonies. These data revealed the presence of a $CD45^-$ subpopulation of cells expressing high levels of the Tie2 (Tek) endothelial marker and low levels of both CD31 and VE-cadherin (Fig. 3C), indicating the presence of endothelial cells. In addition, individual blast colonies were shown to form $CD31^+$ endothelium tubule-like structures in Matrigel plugs (Fig. 3D). In parallel to the formation of hemangioblast precursors upon activin A and bFGF stimulation, we observed an accumulation of cells expressing Flk1 (Fig. 3E). In cultures stimulated with Bmp4 only, few cells still expressed Flk1 by 12 hours, whereas in the presence of activin A and bFGF, 42% of the cells still expressed high levels of Flk1. Hemangioblast cells represent the first committed hematopoietic precursors derived from mesoderm, but they are not yet fully restricted to a hematopoietic fate as they can also give rise to smooth muscle and endothelium (Ema et al., 2003; Huber et al., 2004). Upon further differentiation, they will give rise to restricted precursors for both primitive and definitive hematopoietic lineages (Choi et al., 1998; Yamashita et al., 2000). The formation of these more-committed blood precursors upon sequential induction by Bmp4, activin A and bFGF, was assessed at later time points by secondary replating allowing the formation of primitive and definitive colonies. As shown in Fig. 3F, when compared with the serum condition at all time points analyzed, few primitive or definitive colonies were derived from EBs grown in serum-free media supplemented either with Bmp4 alone or with Bmp4, activin A and bFGF. Altogether, these data show that mesodermal precursors were able to efficiently and rapidly form hemangioblast upon stimulation with bFGF and activin A. However, although hemangioblast formation was very robust, these hemangioblasts failed to further commit to more-mature hematopoietic precursors without the addition of growth factors other than Bmp4, activin A and bFGF in the serum-free culture conditions.

VEGF is required for the progression from hemangioblast to committed hematopoietic precursors

To investigate the requirement for the formation of these more-mature hematopoietic precursors, we generated EBs in the presence of Bmp4, stimulated them with activin A and bFGF at day 2.5, then tested the effect of VEGF and/or interleukin 6 (IL6), both factors routinely used to grow blast colonies in secondary replating assay. IL6 was unable to trigger the formation of hematopoietic precursors (not shown). By contrast, VEGF was very efficient at inducing the generation of primitive and definitive precursors (Fig. 4A). At all time points analyzed, EBs stimulated with the sequential addition of Bmp4, activin A, bFGF and VEGF (collectively BAFV) contained more precursors of primitive and definitive lineages than did EBs grown with serum. Variation in VEGF concentration did not significantly affect the frequency of progenitors produced; high VEGF concentrations (above 40 ng/ml) gave rise to fewer progenitors (not shown). The timing of VEGF addition appeared critical for the optimum formation of these hematopoietic precursors; best results were achieved when VEGF was added along

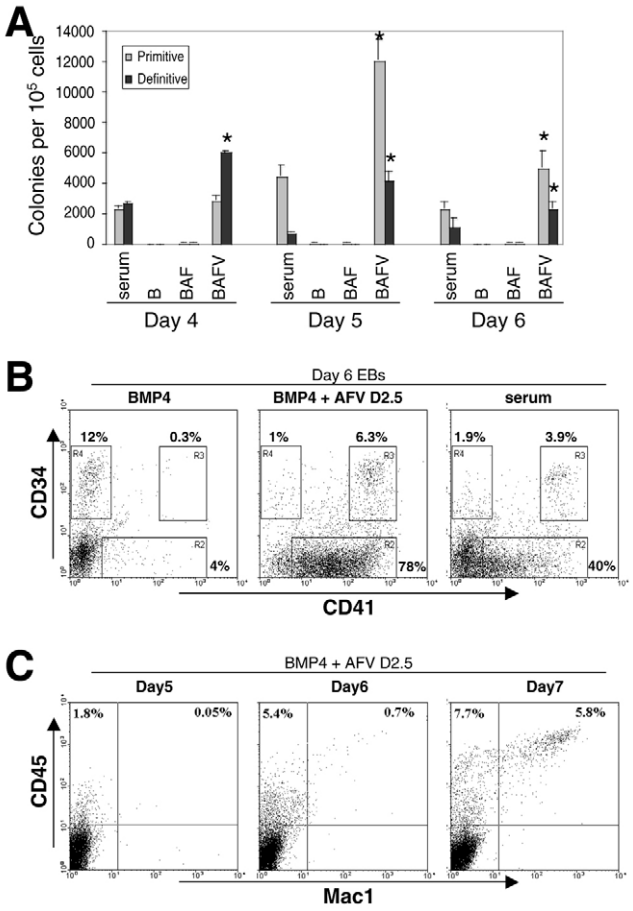

Fig. 4. VEGF induces the formation of committed blood progenitors. (**A**) EBs grown for 2.5 days in serum-free culture with 4 ng/ml Bmp4 were stimulated with activin A and bFGF or with activin A, bFGF and VEGF and assayed at days 4, 5 and 6 for the presence of hematopoietic progenitors by colony-forming ability in secondary replating assay. *A statistically significant difference ($P<0.05$) compared with the serum control at the same time point. Data are presented as the mean number of colonies from three dishes. Error bars represent s.e.m. (**B**) CD41 and CD34 expression was determined by flow cytometric analysis of cells derived from day-6 EBs stimulated as indicated. (**C**) CD45 and Mac1 expression was determined by flow cytometric analysis of cells derived from EBs stimulated with Bmp4 followed by activin A, bFGF and VEGF. Data are shown for EBs harvested at days 5, 6 and 7. All data shown are representative of at least three experiments.

with activin A and bFGF. Expression of the αIIb integrin subunit (CD41; Itga2b) has been shown to define the onset of primitive and definitive hematopoiesis both in vivo and in vitro (Emambokus and Frampton, 2003; Ferkowicz et al., 2003; Mikkola et al., 2003; Mitjavila-Garcia et al., 2002). By day 6 of differentiation, most cells within the BAFV-stimulated EBs belonged to the hematopoietic lineage as defined by their expression of CD41 (Fig. 4B). At days 5 and 6, few cells within the BAFV-stimulated EBs expressed markers of more-mature hematopoiesis such as CD45 (Ptprc) and Mac1 (Itgam), but by day 7 $CD45^+Mac1^-$ and $CD45^+Mac1^+$ subpopulations were clearly detected (Fig. 4C). All hematopoietic progenitors were found in the $CD41^+$ fractions (Fig. 5A), both primitive and definitive precursors were present in the $CD41^+CD34^-$ subpopulation, and $CD41^+CD34^+$ potential was restricted to

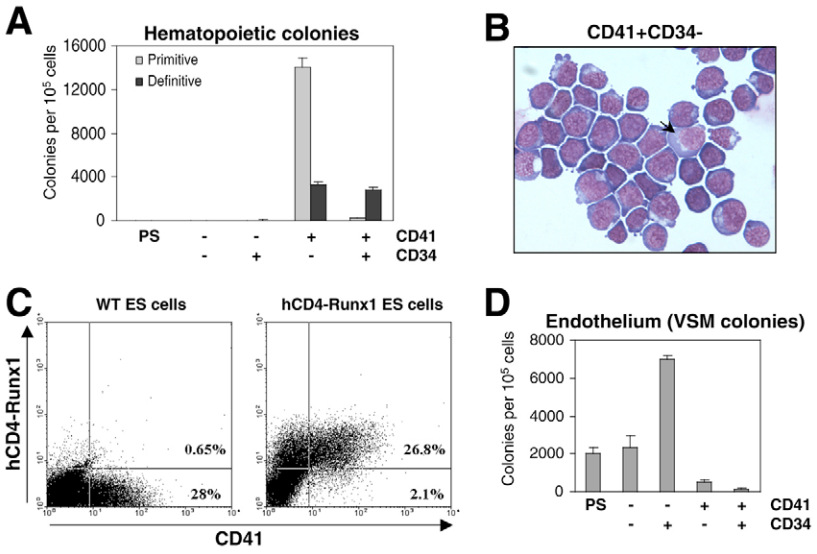

Fig. 5. Hematopoietic and endothelium precursors are defined by CD41 and CD34 relative expression. (**A**) Cells expressing CD41 and CD34 were sorted from day-5 EBs grown in serum-free conditions supplemented with Bmp4, followed by activin A, bFGF and VEGF at day 2.5. Each fraction was tested for the presence of hematopoietic progenitors by colony-forming ability in secondary replating assay. (**B**) May-Grunwald Giemsa staining of $CD41^+CD34^-$ sorted cells; arrow indicates more-mature cells (40× magnification). (**C**) Day-5 serum-supplemented EBs derived from wild-type ES cells or ES cells carrying a truncated human (h) *CD4* cDNA knocked into the *Runx1* locus, were stained for CD41 and hCD4 coexpression. (**D**) The CD41 and CD34 fractions were tested for their ability to form VSM (vascular smooth muscle) colonies upon secondary replating. Data are presented as the mean number of colonies from three dishes. Error bars represent s.e.m. All data shown are representative of at least three experiments. PS, presort.

definitive progenitors. The main population, $CD41^+CD34^-$, was mostly composed of immature blastic cells with a few more-mature cells but no mature megakaryocytes (Fig. 5B). Attempts to generate B or T lymphocytes on OP9 or OP9-Dll1 stroma cell lines were unsuccessful for all fractions (not shown). To further characterize the $CD41^+$ population, we analyzed Runx1 expression within this population using an ES cell line in which the truncated human CD4 has been knocked into the *Runx1* locus (P.S., V.K. and G.L., unpublished). Runx1 expression was detected in most $CD41^+$ cells (Fig. 5C), another indication that most $CD41^+$ cells are committed to the hematopoietic program. In Bmp4-stimulated EBs, the main positively stained population was $CD34^+CD41^-$. When tested in replating assays after purification, this population only gave rise to VSM endothelium colonies (Fig. 5A,D). Upon expansion in three-dimensional Matrigel plugs, these VSM colonies gave rise to tubule-like structures (not shown). Altogether, these data demonstrate that VEGF is sufficient to trigger the commitment and/or expansion of more-mature blood precursors for both primitive and definitive lineages. It is very unlikely that VEGF acts as a mere survival factor, as Bcl2 overexpression was not able to substitute for VEGF for the efficient production of hematopoietic precursors (not shown). The very high proportion of $CD41^+$ hematopoietic cells within the EBs further highlights the efficiency with which this combination of factors specifically promotes hematopoietic commitment at the expense of other lineages.

To address whether these serum-free culture conditions could be used with other murine ES lines, we tested two further lines: RI (129sv) and F1 (129sv/C57Bl/6). Both lines were differentiated in the serum-free conditions, stimulated with Bmp4 at day 0, then with activin A, bFGF and VEGF at day 2.5. Both sets of EBs derived from RI and F1 ES cells gave rise to a very robust hematopoiesis in the serum-free supplemented condition, producing a significantly higher frequency of both primitive and definitive precursors than was obtained in the serum condition (see Fig. S7 in the supplementary material). Similarly to the GFP-Bry line, a very high proportion of $CD41^+$ cells was detected in EBs at days 4 and 5 of differentiation. These results demonstrate that the sequential addition of soluble factor Bmp4, followed by activin A, bFGF and VEGF, can be used very reproducibly and successfully for different ES cell lines.

Analysis of the molecular program at the onset of hemangioblast specification

The serum-free culture conditions described above represent a perfect system with which to further dissect the molecular program established at the onset of blood specification. To analyze the expression pattern of genes indicative of the differentiation process, we first performed an expression survey over the time-course of the EB culture from day 0 to 6 (Fig. 6A), with Bmp4 added at time 0 only (lanes B), or with Bmp4 followed by activin A and bFGF addition at day 2.5 (lanes BAF) or by activin A, bFGF and VEGF addition at day 2.5 (lanes BAFV). By day 1 of differentiation, *Rex1* expression was markedly decreased, followed by the progressive upregulation of *Fgf5*, indicating a transition from ES cells to epiblast-like cells. Expression of brachyury was detected after 2 days, a mark of commitment to the mesodermal germ layer. Interestingly, brachyury expression seemed to be downregulated faster when cells were stimulated with BAF or BAFV than with Bmp4 only. *Flk1* expression was detected by day 3 in all conditions and was shortly followed by the expression of genes implicated in hematopoietic commitment such as *Scl* (*Tal1*), *Runx1* and *Gata1*, and by βH1, the embryonic hemoglobin expressed only in primitive erythrocytes. All genes indicative of hematopoietic commitment were expressed at a low level in EBs stimulated with Bmp4 only or under BAF conditions. However, the expression of this set of genes was strongly upregulated when VEGF was added at day 2.5. This expression study nicely complements the hematopoietic potential data (Fig. 3D, Fig. 4A) showing that few precursors were generated within EBs in the absence of VEGF induction. To further refine our study of the molecular program that is established at the onset of hematopoiesis commitment, we analyzed gene expression following the stimulation of mesodermal precursors by activin A and bFGF. After 2.5 days of culture with Bmp4, EBs were stimulated with activin A and bFGF and harvested after 1, 2, 3 or 6 hours of induction to assess the dynamic expression of some genes implicated as being crucial in hematopoietic development (Fig. 6B,C). Within 2 hours of induction, the expression of *Runx1* was significantly upregulated, and increased expression of *Hhex* was detected 3 hours following induction. Expression of *Scl*, *Fli1* and *Lmo2* became significantly upregulated by 6 hours, although a small increase in *Scl* expression level could already be observed 1 hour

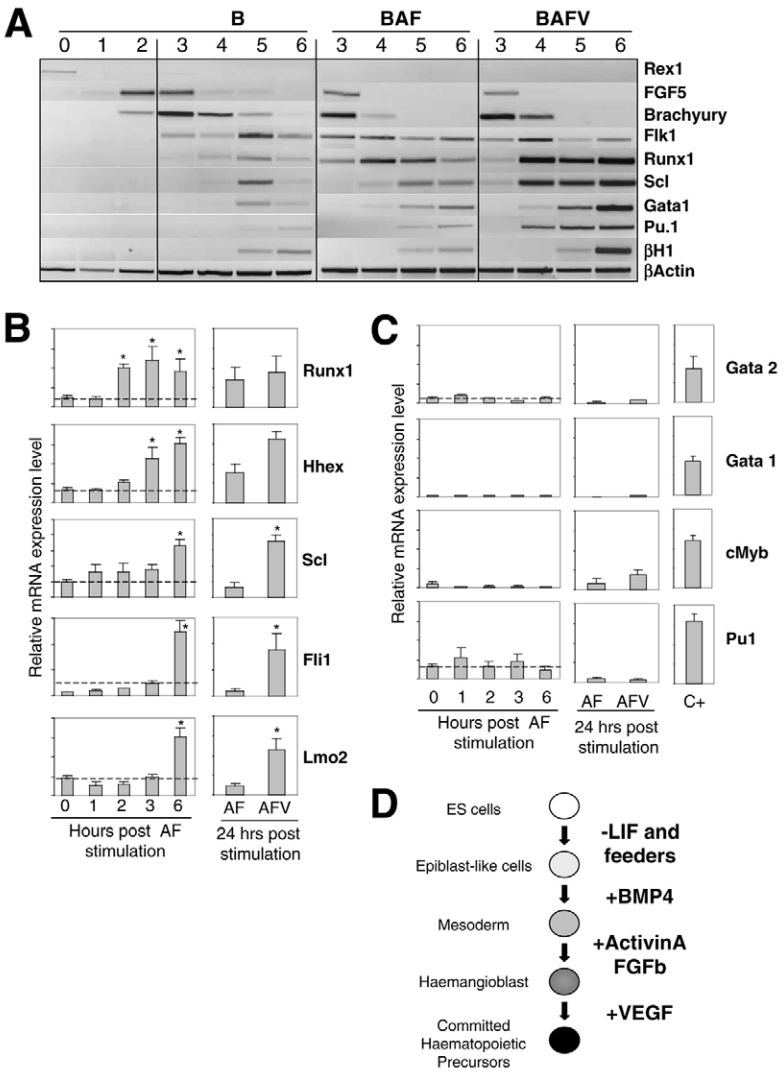

Fig. 6. Molecular program at the onset of hemangioblast specification. (**A**) ES cells were induced to differentiate in serum-free culture conditions with Bmp4 for 6 days (lanes B), Bmp4 followed by activin A and bFGF at day 2.5 (lanes BAF) or Bmp4 followed by activin A, bFGF and VEGF at day 2.5 (lanes BAFV). Day 0, 1 and 2: Bmp stimulation only for the three conditions. Equal amounts of RNA from ES cells or from EB-derived cells were reverse-transcribed and analyzed by RT-PCR for the expression of the indicated genes. (**B**,**C**) EBs grown for 2.5 days in serum-free culture with 4 ng/ml Bmp4 were further stimulated with either activin A and bFGF (AF) or with activin A, bFGF and VEGF (AFV) and harvested 1, 2, 3, 6 or 24 hours after stimulation for gene expression analysis by real-time PCR. cDNA derived from EBs grown in serum condition for 4.5 days were used as positive control (C+). All real-time PCR data are presented as mRNA expression level relative to that of β-actin. *A statistically significant difference (P<0.05) compared with Bmp4-stimulated control at 0 hour. Data are mean±s.e.m. of triplicate values from three independent experiments. (**D**) Schematic representation of the differentiation steps leading to the formation of hematopoietic progenitors. All data shown are representative of at least three experiments.

after induction (Fig. 6B). By contrast, we did not observe any significant upregulation of *Gata1*, *Gata2*, *cMyb* or *Pu.1* (*Sfpi1*) (Fig. 6C): *Gata2* and *Pu.1* were expressed at a low and steady level, whereas *Gata1* and *cMyb* were below detection level – expression of these genes was successfully detected using cDNA derived from day 4.5 EBs grown with serum (Fig. 6C, the C+ panel).

Finally, to assess at the molecular level the crucial requirement for VEGF to sustain the hematopoietic program, we compared expression levels of all these genes on EBs stimulated with either activin A and bFGF or with activin A, bFGF and VEGF for 24 hours (Fig. 6B,C). Strikingly, the expression of *Scl*, *Fli1* and *Lmo2* was not maintained in EBs stimulated only with activin A and bFGF, demonstrating that the presence of VEGF was essential for persistent expression of these three genes. By contrast, expression of neither *Runx1* nor *Hhex* was significantly affected by the absence of VEGF. No significant changes were observed in the expression of *Gata1*, *Gata2*, *cMyb* and *Pu.1* in either culture condition. Overall, these data demonstrate that activin A and bFGF are indeed switching on the hematopoietic program in mesodermal precursors, as shown by the very rapidly upregulated expression of many crucial transcriptional regulators of that program. Importantly, these data lend further support to the crucial role of VEGF in sustaining rather than triggering this hematopoietic program. Indeed, neither the formation of hemangioblast precursors nor the onset of *Runx1*, *Hhex*, *Scl* or *Lmo2* expression requires the addition of VEGF. Subsequently, VEGF becomes crucial in driving the next steps of the hematopoietic program.

DISCUSSION

Altogether, our data show that commitment to the hematopoietic lineage can be driven very efficiently in the absence of serum by four growth factors and that each step of this differentiation can be controlled by only one or two factors (Fig. 6D). These culture conditions promote the differentiation of ES cells specifically toward mesoderm then hematopoiesis. This is reflected first by the very high proportion of GFP^+ cells (Fig. 2A) followed by a very high percentage of $CD41^+$ cells within the EBs at days 5 and 6 (Fig. 4B). Importantly, the sequential addition of factors is much more efficient at driving hematopoiesis than when all factors are added simultaneously at the onset of differentiation (compare Fig. 1B with Fig. 4A).

The strongly upregulated expression of known transcriptional regulators of hematopoiesis associated with a robust increase in the number of hemangioblast precursors can be detected within a few hours of stimulation with activin A and bFGF (Fig. 3B, Fig. 6B). This very rapid response suggests that activin A and bFGF act

together to promote the specification of mesodermal precursors to the hematopoietic program, rather than inducing the proliferation of a few hemangioblasts already present in the EBs. The strongest argument in favor of induction rather than proliferation relates to the kinetics of hemangioblast generation. Three hours after activin A and bFGF addition, we observe an average 8-fold increase in hemangioblast numbers (Fig. 3B). This increase cannot be exclusively the consequence of proliferation, as not even the fastest proliferating eukaryotic cell can divide two or three times in 3 hours. Furthermore, the hemangioblast assay is a clonal assay in semi-solid media and, consequently, if proliferation occurred after the replating this would still result in the formation and detection of only one blast colony.

Our results suggest that VEGF is not critically required for the formation of hemangioblast, but is essential for the formation and/or proliferation of both primitive and definitive hematopoietic precursors. In the replating assay, the formation of blast colonies requires the action of VEGF. However, the formation of blast colonies represents the further development of hematopoietic and endothelium progenitors from an already formed hemangioblast. Hemangioblast precursors express Flk1, but this does not imply that they require signaling through Flk1 or another VEGF receptor for their generation. To date, no published findings demonstrate the requirement for VEGF in the formation of hemangioblast precursors. In fact, data from Ema et al. suggest that without Flk1, hemangioblast and blast colonies are still generated, although to a lesser extent than in wild-type controls (Ema et al., 2003).

Our data demonstrate that Bmp4 alone, or a combination of Bmp4, activin A and bFGF, is able to stimulate the formation of some fully committed blood progenitors, but that the addition of VEGF dramatically enhances the frequency of these precursors (Fig. 4A). There are no statistical differences in the production of committed hematopoietic precursors (excluding the hemangioblast precursors) when ES cells are differentiated with Bmp4 alone or with Bmp4, activin A and bFGF (Fig. 3F, Fig. 4A). By contrast, the addition of VEGF resulted on average in a 250-fold increase for primitive and a 170-fold increase for definitive colonies when compared with Bmp4 induction alone (Fig. 4A). Even when compared with serum conditions, VEGF addition resulted in 3-fold and 6-fold increases for primitive and definitive colonies, respectively. Overall, the frequency of hematopoietic colonies generated by sequential stimulation by Bmp4 followed by activin A, bFGF and VEGF is higher than any other culture conditions tested in the present study. Two recent studies have explored the growth factor requirements for the derivation of hematopoietic precursors from human ES cells (Kennedy et al., 2007; Pick et al., 2007). Kennedy et al. showed that sequential addition of BMP4, followed by bFGF then by bFGF plus VEGF in StemPro medium triggered the formation of hemangioblast and more-mature hematopoietic precursors. In the second study, Pick et al. defined the combination of BMP4, VEGF, SCF (KITLG – Human Gene Nomenclature Database) and bFGF as being required to promote hematopoiesis as assessed by gene expression and colony-forming cell (CFC) formation. In both studies, however, the production of hematopoiesis was clearly not as efficient as in our culture conditions, and these studies did not address the precise role of each added factor. It is difficult, however, to directly compare the efficiency of culture conditions for human ES and mouse ES cells. In our culture conditions, as well as in these two human ES cell studies, Bmp4, bFGF and VEGF appear to be crucial for hematopoiesis. It would be very interesting to test whether the addition of activin A to EBs derived from human ES cells enhances the efficiency of progenitor generation.

The crucial role of Bmp4 during mesoderm development in vivo has already been clearly established using various knockout mice (Fujiwara et al., 2001; Gu et al., 1999; Mishina et al., 1995; Winnier et al., 1995). VEGF and its receptor Flk1 have also been shown to be crucial at the onset of blood and vasculature development during embryogenesis (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1995). By contrast, a role for activin A during hematopoietic development in vivo seems unlikely as activin $A^{-/-}$ mice have no reported blood defects (Matzuk et al., 1995). However, mice deficient for TGFβ1, a closely related member of TGF family, were shown to have vasculature and hematopoietic defects during development (Dickson et al., 1995). When assayed in the serum-free culture system described here, we observed that similarly to activin A, TGFβ1 in conjunction with bFGF was able to induce the very robust formation of hemangioblast (not shown).

These culture conditions represent a powerful system with which to dissect the molecular mechanisms implicated in hemangioblast commitment. The possibility to switch on the hematopoietic program with activin A and bFGF allowed us to perform a time-course of gene expression dynamics at the onset of hemangioblast commitment and to pinpoint genes that are crucial regulators of this commitment. Runx1, Scl and Hhex, which have been shown to regulate hemangioblast development (Chung et al., 2002; D'Souza et al., 2005; Ema and Rossant, 2003; Guo et al., 2003; Kubo et al., 2005; Lacaud et al., 2002), were progressively upregulated upon activin A plus bFGF induction. Six hours post-induction, increased expression was also observed for Fli1 and Lmo2, two transcription factors known to be important in the development of the hematopoietic program (Hart et al., 2000; Spyropoulos et al., 2000; Warren et al., 1994). Recent findings in zebrafish have highlighted the crucial role of Lmo2 in hemangioblast specification (Patterson et al., 2007), whereas a direct role for Fli1 in this process remains to be demonstrated. By contrast, we did not observe any significant changes in either Gata1 or Gata2 expression upon hemangioblast specification, despite reports of their involvement in this process (Lugus et al., 2007; Yokomizo et al., 2007). Gata2 function in hemangioblast development was assessed via an inducible overexpression. Rather than resulting from a direct effect of Gata2, increase in hemangioblast generation might have resulted from Gata2 expression impacting on Scl expression (Lugus et al., 2007). Gata2 is possibly not directly implicated in the establishment of the hemangioblast program, but its expression is essential for Scl expression (Gottgens et al., 2002). In our study, Gata1 expression was clearly not detected upon activin A plus bFGF induction, even after 24 hours (Fig. 6C). However, Yokomizo et al. have shown using a GFP mini-transgene that hemangioblastic cells are positive for Gata1 expression (Yokomizo et al., 2007). A possible explanation for this discrepancy is that the mini-transgene used to mimic Gata1 expression did not fully recapitulate the Gata1 expression pattern and that negative regulatory regions might be missing. Overall, our gene expression survey further highlights the key role of the Runx1, Scl, Hhex and Lmo2 transcription factors in regulating the onset of hematopoietic development. Importantly, these data underscore the power of our serum-free culture conditions, which allow us to catch mesodermal precursors as they become committed to hematopoiesis, and potentially to define new molecular players implicated in this process.

The true contribution of hemangioblast precursors to yolk sac hematopoiesis has been recently challenged (Ueno and Weissman, 2006). The apparent polyclonal origin of the blood island of yolk sac observed in this study might in fact have resulted from the very transient nature of this precursor. Indeed, hemangioblast progenitors

are mostly detected in the primitive streak at around 7.5 days post-coitum (Huber et al., 2004), and will have already progressed in their differentiation toward hematopoietic- and/or endothelium-restricted precursors when reaching the yolk sac, within which they are very likely to intermingle at this point.

In the present study, we have defined robust and reproducible culture conditions to specifically and efficiently drive hematopoietic differentiation from ES cells in the absence of serum. This system should help us to better dissect and understand early lineage commitment to hematopoiesis.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/8/1525/DC1

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