A hierarchical order of factors in the generation of FLK1- and SCL-expressing hematopoietic and endothelial progenitors from embryonic stem cells

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Summary

The receptor tyrosine kinase FLK1 and the transcription factor SCL play crucial roles in the establishment of hematopoietic and endothelial cell lineages in mice. We have previously used an in vitro differentiation model of embryonic stem (ES) cells and demonstrated that hematopoietic and endothelial cells develop via sequentially generated FLK1$^+$ and SCL$^+$ cells. To gain a better understanding of cellular and molecular events leading to hematopoietic specification, we examined factors necessary for FLK1$^+$ and SCL$^+$ cell induction in serum-free conditions. We demonstrate that bone morphogenetic protein (BMP) 4 was required for the generation of FLK1$^+$ and SCL$^+$ cells, and that vascular endothelial growth factor (VEGF) was necessary for the expansion and differentiation of SCL-expressing hematopoietic progenitors. Consistently, *Flk1*-deficient ES cells responded to BMP4 and generated TER119$^+$ and CD31$^+$ cells, but they failed to expand in response to VEGF. The Smad1/5 and map kinase pathways were activated by BMP4 and VEGF, respectively. The overexpression of SMAD6 in ES cells resulted in a reduction of FLK1$^+$ cells. In addition, a MAP kinase kinase 1 specific inhibitor blocked the expansion of SCL$^+$ cells in response to VEGF. Finally, VEGF mediated expansion of hematopoietic and endothelial cell progenitors was inhibited by TGFβ1, but was augmented by activin A. Our studies suggest that hematopoietic and endothelial commitment from the mesoderm occurs via BMP4-mediated signals and that expansion and/or differentiation of such progenitors is achieved by an interplay of VEGF, TGFβ1 and activin A signaling.

Key words: Hematopoiesis, Vasculogenesis, FLK1, SCL, BMP4, VEGF, TGFβ1

Introduction

There has been great interest in identifying factors that control hematopoietic commitment, as they can provide molecular mechanisms involved in hematopoietic establishment. Previous studies implicate that endoderm-derived factors and mesoderm-inducing factors could play a role in hematopoietic and endothelial cell differentiation. For example, in *Xenopus*, the formation of erythroid cells from the animal cap is induced by BMP4 and basic fibroblast growth factor (bFGF) or by BMP4 and activin A, and the generation of erythroid cells by exogenously expressed GATA1 can be potentiated by bFGF (Huber et al., 1998). Studies of quail-chick chimeras have shown that the endoderm can induce the formation of hematopoietic cells from the somatopleural mesoderm, which normally does not have such potential (Pardanaud et al., 1996). In addition, the formation of blood islands from quail epiblasts is dependent on bFGF (Flamme and Risau, 1992). In this system, bFGF-mediated blood island formation correlates with the induction of the *Flk1* gene (*Kdr* – Mouse Genome Informatics) (Flamme et al., 1995), suggesting that bFGF is crucial for the emergence of the hemangioblast, the common progenitor of hematopoietic and endothelial cells (Sabin, 1920; Murray, 1932; Wagner, 1980). Furthermore, in quail embryos, bFGF, vascular endothelial growth factor (VEGF) and transforming growth factor (TGF) β1 can induce hematopoietic differentiation from the somatopleural mesoderm (Pardanaud et al., 1996). Similarly, in mice, the primitive endoderm can induce hematopoietic differentiation from the anterior epiblast, a tissue that cannot generate hematopoietic cells (Belaoussoff et al., 1998). Specifically, both activin A and BMP4 can induce hematopoietic differentiation from the anterior headfold region (Kanatsu and Nishikawa, 1996), and Indian hedgehog can promote hematopoietic differentiation from the anterior epiblast (Dyer et al., 2001).

Although these studies support the notion that TGFβ family members and bFGF can induce hematopoietic differentiation,

they do not provide mechanisms involved in specifying hematopoietic cell lineages. In addition, these studies are limited to erythroid cell induction. Thus, it is still unclear how the generation of the earliest cell population, which is committed to become hematopoietic and endothelial cells, is regulated. Gene targeting studies indicate that FLK1, a receptor tyrosine kinase, and SCL (TAL1 – Mouse Genome Informatics), a basic helix-loop-helix transcription factor, are required at the initial stages of the establishment of hematopoietic and endothelial cell development. In mice, *Flk1* expression can be detected in the presumptive mesodermal yolk sac blood island progenitors as early as embryonic day (E) 7 (Yamaguchi et al., 1993; Dumont et al., 1995). Consistent with its expression pattern, *Flk1*-deficient mice display defects in blood vessels and yolk sac blood island formation and die between E8.5 and E9.5 (Shalaby et al., 1995). Furthermore, *Flk1*$^{-/-}$ ES cells fail to participate in vessel formation or contribute to primitive or definitive hematopoiesis in chimeras generated with wild-type embryos, suggesting a cell autonomous requirement of FLK1 in hematopoietic and endothelial cell development (Shalaby et al., 1997). *Scl*-deficient mice also exhibit defects in hematopoietic and endothelial cell lineages and die around E10.5 (Robb et al., 1995; Shivdasani et al., 1995). The endothelial cell defects in these mice are in the remodeling of the primary vascular plexus in the yolk sac (Visvader et al., 1998).

In an effort to analyze hematopoietic and endothelial cell differentiation more systematically, we recently examined the developmental kinetics of the expression of FLK1 and SCL by using in vitro differentiated knock-in ES cells that express a non-functional human CD4 at the *Scl* locus (Chung et al., 2002). We demonstrated that CD4-expressing cells from in vitro differentiated *Scl*$^{+/CD4}$ ES cells (embryoid bodies, EBs) correlated with that of *Scl* and reported that hematopoietic and endothelial cells developed via sequentially generated FLK1 and SCL-expressing cells. FLK1^{+}SCL^{-} cells first emerged in differentiating ES cells followed by FLK1^{+}SCL^{+} cells, which developed from FLK1^{+} cells. FLK1^{-}SCL^{+} cells ultimately developed from FLK1^{+}SCL^{+} cells by downregulating FLK1. Thus, the formation of FLK1- and SCL-expressing cells marks the onset of hematopoietic and endothelial cell differentiation. In this study, we used an in vitro serum-free differentiation model of ES cells (Adelman et al., 2002) to identify factors regulating the onset of hematopoietic and endothelial cell lineage differentiation. We show that BMP4 was required for the generation of the FLK1^{+} and SCL^{+} cells. We also show that VEGF, via FLK1-mediated signals, is required for the expansion of hematopoietic and endothelial cell progenitors. Finally, we demonstrate that the generation of SCL^{+} cells by BMP4 and VEGF was inhibited by TGFβ1, but augmented by activin A. Collectively, our studies reveal a temporal, hierarchical order of factors that function to establish hematopoietic and endothelial cell lineages.

In this paper, CD4 refers to the non-functional human CD4 gene/protein.

Materials and methods

Cell culture

R1 ES cells (kindly provided by Dr Andras Nagy at Mount Sinai Hospital, Samuel Lunenfeld Research Institute, Toronto), *Scl*$^{+/CD4}$ knock-in ES cells (Chung et al., 2002), *Flk1*$^{-/-}$ (kindly provided by A. Schuh, University of Toronto) and *Flt1*$^{-/-}$ ES clones (kindly provided by G.-H. Fong, University of Connecticut School of Medicine) were maintained on STO feeder cells in the presence of leukemia inhibitory factor (LIF). EBs were generated as described (Choi et al., 1998). Serum-free differentiation was achieved by differentiating ES cells in serum replacement (SR, Gibco/BRL) instead of fetal calf serum (FCS) (Adelman et al., 2002). All factors were added at the initiation of EB differentiation, unless otherwise indicated. BMP4, VEGF$_{165}$, VEGF$_{121}$, TGFβ1, activin A, SHH and WNT3A were purchased from the R&D Systems, and used at concentrations as follows: BMP4 (5 ng/ml); VEGF (10 ng/ml), unless otherwise indicated; BMP2 (5 ng/m), TGFβ1 (1 ng/ml); activin A (2 ng/ml), SHH (0.6 μg/ml) and WNT3A (5 ng/ml). Basic fibroblast growth factor (bFGF) was purchased from the Upstate Biotechnology (Lake Placid, NY) and used at 10 ng/ml. Noggin purchased from R&D Systems was added at the initiation of EB differentiation. U0124 and U0126 were purchased from Calbiochem. U0124 (5, 10μM) or U0126 (5, 10 μM) was added at day 3 of ES cell differentiation.

Hematopoietic colonies were generated as described previously (Faloon et al., 2000). Briefly, cells obtained from day 5-6 EBs were replated in methyl cellulose containing 10% plasma-derived serum (PDS, Antech; Texas), 5% protein-free hybridoma medium (PFHM2, Gibco/BRL), L-glutamine (2 mM), transferrin (300 μg/ml; Boehringer Mannheim) and MTG (4.5×10^{-4} M), together with the following cytokines: kit ligand (KL, 1% conditioned medium), IL3 (1% conditioned medium), IL1 (5 ng/ml), IL6 (5 ng/ml), IL11 (5 ng/ml), Epo (2 units/ml), MCSF (5 ng/ml), GSCF (2 ng/ml) and GMCSF (3 ng/ml). Hematopoietic colonies were counted 5-7 days later. IL1, IL6, IL11, GCSF and MCSF were purchased from R&D Systems. KL was obtained from medium conditioned by CHO cells transfected with a KL expression vector (kindly provided by Genetics Institute). EPO was purchased from Amgen (Thousand Oaks, CA) and IL3 was obtained from medium conditioned by X63 Ag8-653 myeloma cells transfected with a vector expressing IL3 (Karasuyama and Melchers, 1988).

ES clones expressing SMAD6 were generated by electroporating ES cells with a linearized Flag-mouse SMAD6 expressing construct (kindly provided by Dr Miyazono at University of Tokyo, Japan). ES cells were selected with G418 (500 μg/ml). Clones were verified by a western blot analysis with an anti-Flag-tag antibody (Sigma).

FACS analysis

EB cells were dissociated with 7.5 mM EDTA/PBS (pH 7.4) for 2 minutes. Cells were centrifuged, resuspended in staining/wash buffer (4% FCS in PBS), passed through a 20-gauge needle four or five times to generate a single cell suspension, and the cell number was counted. After centrifugation, cells were resuspended at a density of 5×10^{6} cells/ml in staining/wash buffer. Cells were placed into each well of V-shaped 96-well plate at 5×10^{5} cells/well. For a single color staining for FLK1, biotinylated anti-FLK1 antibody, freshly diluted (1:1000) in staining/wash buffer, was added and incubated for 15 minutes on ice. Subsequently, cells were washed three times with staining/wash buffer. Streptavidin-phycoerythrin (secondary reagent) (Pharmingen), freshly diluted in staining/wash buffer, was added and incubated on ice for 15 minutes in the dark. Cells were washed three times, re-suspended in staining/wash buffer, and transferred to 5 ml polypropylene tubes for analysis. A double-color staining for human CD4 and FLK1 was carried out by staining the cells with biotinylated mouse anti-human CD4 monoclonal antibody (CALTAG), followed by streptavidin-allophycocyanin (Sav-APC; Pharmingen) and phycoerythrin (PE)-conjugated anti-FLK1 monoclonal antibody (Pharmingen). A three-color FACS analysis of FLK1, human CD4 and TER119 was carried out by staining the cells first with biotinylated mouse anti-human CD4 monoclonal antibody and anti-mouse TER119 antibody (Pharmingen), followed by FITC-

conjugated goat anti-rat IgG_{2b} (Pharmingen). Finally, Sav-APC and PE-conjugated anti-FLK1 monoclonal antibody were added. A three-color FACS analysis of FLK1, CD4 and CD31 was carried out by staining the cells first with biotinylated mouse anti-CD4 monoclonal antibody and FITC-conjugated anti-mouse CD31 (Pharmingen), followed by Sav-APC and PE-conjugated anti-FLK1 monoclonal antibody. Cells were analyzed on a FACS Caliber (Becton-Dickinson), and FACS data were analyzed with CellQuest software (Becton-Dickinson).

Biochemical analysis

For detecting SMAD1/5 phosphorylation, ES cells were differentiated in serum-free conditions. Basic FGF (10 ng/ml) or BMP4 (5ng/ml) was added at the onset of differentiation (day 0). EBs were harvested on day 1.5 or 2.75 and lysed in 1× RIPA-B buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% NP-40; 0.5% deoxycholate) containing protease inhibitor cocktail (Roche), NaF, NaOVa and phosphatase inhibitor cocktail I (Sigma). Alternatively, EBs were generated in serum-free conditions in the absence of exogenously added factors, collected on day 1.5, stimulated with bFGF or BMP4 for 30 to 60 minutes at 37°C, and lysed as described above. For Erk and AKT phosphorylation detection, ES cells were differentiated in serum-free conditions with BMP4. Three days after, EBs were harvested, washed off three times, and then cultured for overnight in IMDM. The following day, EBs were harvested and stimulated with VEGF for 30 minutes.

Cleared cell lysates after centrifugation were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), followed by immunoblotting. Blots were blocked in TBS containing 5% nonfat milk and 0.5% Tween 20 for 1 hour at room temperature and incubated with indicated antibodies overnight. Antibodies used were as follows: rabbit anti-pSMAD1/5 (Rosendahl et al., 2002), rabbit anti-SMAD1 (Upstate Biotechnique), mouse anti-phospho Erk1/2 (Santa Cruz), rabbit anti-Erk1/2 (Santa Cruz), rabbit anti-pAKT1/2/3 (Santa Cruz) and rabbit anti-AKT1/2/3 (Cell Signaling). One percent BSA was used for blocking and antibody incubation instead of 5% skim milk for detecting phospho-SMAD1/5 bands. Blots were then washed and incubated with horseradish-peroxidase conjugated anti-mouse (Sigma) or anti-rabbit (Santa Cruz) IgG antibodies for 1 hour at room temperature. Immunodetection was achieved by using an ECL-plus detection system (Amersham).

Gene expression analysis

ES cells were differentiated in the presence of fetal calf serum (FCS) or serum replacement (SR) media with or without BMP4. EBs were collected at different time points as noted and RNA was purified following the Triazol protocol (Gibco-BRL). All RNA samples were treated with DNaseI (amplification grade from Gibco-BRL) before cDNA synthesis to eliminate any contaminating genomic DNA. Semi-Quantitative RT-PCR was performed as described (Choi et al., 1998; Faloon et al., 2000). cDNA normalization was carried out with β-actin gene by using Phosphorimager Storm 840 and ImageQuant software. Specific primers used are as follows (Choi et al., 1998; Faloon et al., 2000).

β-actin: sense, 5′ATGAAGATCCTGACCGAGCG3′; antisense 5′TACTTGCGCTCAGGAGGAGC3′.

Rex1: sense, 5′CGTGTAACATACACCATCCG3′; antisense, 5′GAAATCCTCTTCCAGAATGG3′.

T: sense, 5′CATGTACTCTTTCTTGCTGG3′; antisense, 5′GGTCTCGGGAAAGCAGTGGC3′.

Flk1: sense, 5′CACCTGGCACTCTCCACCTTC3′; antisense, 5′GATTTCATCCCACTACCGAAAG3′.

Fgf5: sense, 5′GGCAGAAGTAGCGCGACG3′; antisense, 5′CCGTAAATTTGGCACTTG3′.

Gata4: sense, 5′CTAAGCTGTCCCCACAAGGC3′; antisense, 5′CAGAGCTCCACCTGGAAAGG3′.

Scl: sense, 5′ATTGCACACACGGGATTCTG3′; antisense, 5′GAATTCAGGGTCTTCCTTAG.

Bmp4: sense, 5′TGTGAGGAGTTTCCATCACG3′; antisense, 5′CAGCGAAGGACTGCAGGGCT3′.

Vegf: sense, 5′TCAGAGAGCAACATCACCAT3′; antisense, 5′ACCGCCTTGGCTTGTCACAT3′.

Results

The generation of FLK1 expressing cells by BMP4 in serum-free conditions

In order to better understand molecular and cellular mechanisms involved in $FLK1^+$ and SCL^+ cell development, we initially examined $FLK1^+$ cell generation from ES cells using serum or serum-free differentiation conditions. As shown in Fig. 1A, the generation of $FLK1^+$ cells was readily detectable in the presence of serum, but greatly reduced in serum-free conditions, which were achieved by differentiating ES cells in serum replacement (Adelman et al., 2002). As hematopoietic and endothelial cells develop from the mesodermal precursor, it was possible that the failure of $FLK1^+$ cell generation in the absence of serum was due to a block in mesoderm formation. Therefore, we determined the status of the brachyury (*T*) gene expression, a marker for the mesoderm (Herrmann, 1991). When ES cells were differentiated in the presence of serum, *Rex1* (*Zfp42* – Mouse Genome Informatics), which is expressed in undifferentiated ES cells (Rogers et al., 1991), was downregulated by day 2-3 (Fig. 2A) (Faloon et al., 2000). As cells downregulated *Rex1*, they progressed successfully to $FLK1^+$ cells via the *T*-expressing mesodermal stage (Fig. 2A). Interestingly, *Rex1* was also rapidly downregulated and the expression of *T* was also induced when ES cells were differentiated in serum-free conditions. However, *T* was still expressed at high levels in day 5.5 embryoid bodies (EBs, in vitro differentiated progenies of ES cells). The expression of *Flk1* gene was not detectable until day 5 when ES cells were differentiated in serum-free conditions. Collectively, these results indicate that ES cells can initiate differentiation and reach the mesodermal stage in a serum-independent manner. However, the successful generation of $FLK1^+$ cells required serum factors.

To identify factor(s) that mediate $FLK1^+$ cell generation, we examined factors known to affect mesoderm differentiation. Of the molecules tested [BMP4, BMP2, bFGF, activin A, TGFβ1, WNT3A, sonic hedgehog (SHH) and VEGF], only BMP4 was able to significantly induce the generation of $FLK1^+$ cells in serum-free conditions (Fig. 1A, data not shown). BMP2 could also induce $FLK1^+$ cells, but it was much less efficient. BMP4 could induce $FLK1^+$ cells in the range of 5-30 ng/ml (lot dependent, data not shown). The BMP4 effect on $FLK1^+$ cell generation could be blocked by noggin, an antagonist of BMPs (Zimmerman et al., 1996), showing a specificity for BMP4 in this process. More importantly, Noggin could also inhibit $FLK1^+$ cells in serum, although not completely, suggesting that the BMP4 effect we observed in serum-free conditions was relevant and that BMP4 is a serum factor involved in $FLK1^+$ cell generation (Fig. 1B).

To investigate the mechanism of how BMP4 induces $FLK1^+$ cells, ES cells were differentiated in serum-free conditions, with or without BMP4, and subjected to gene expression analyses. As shown in Fig. 2B, both *T* and *Gata4* genes,

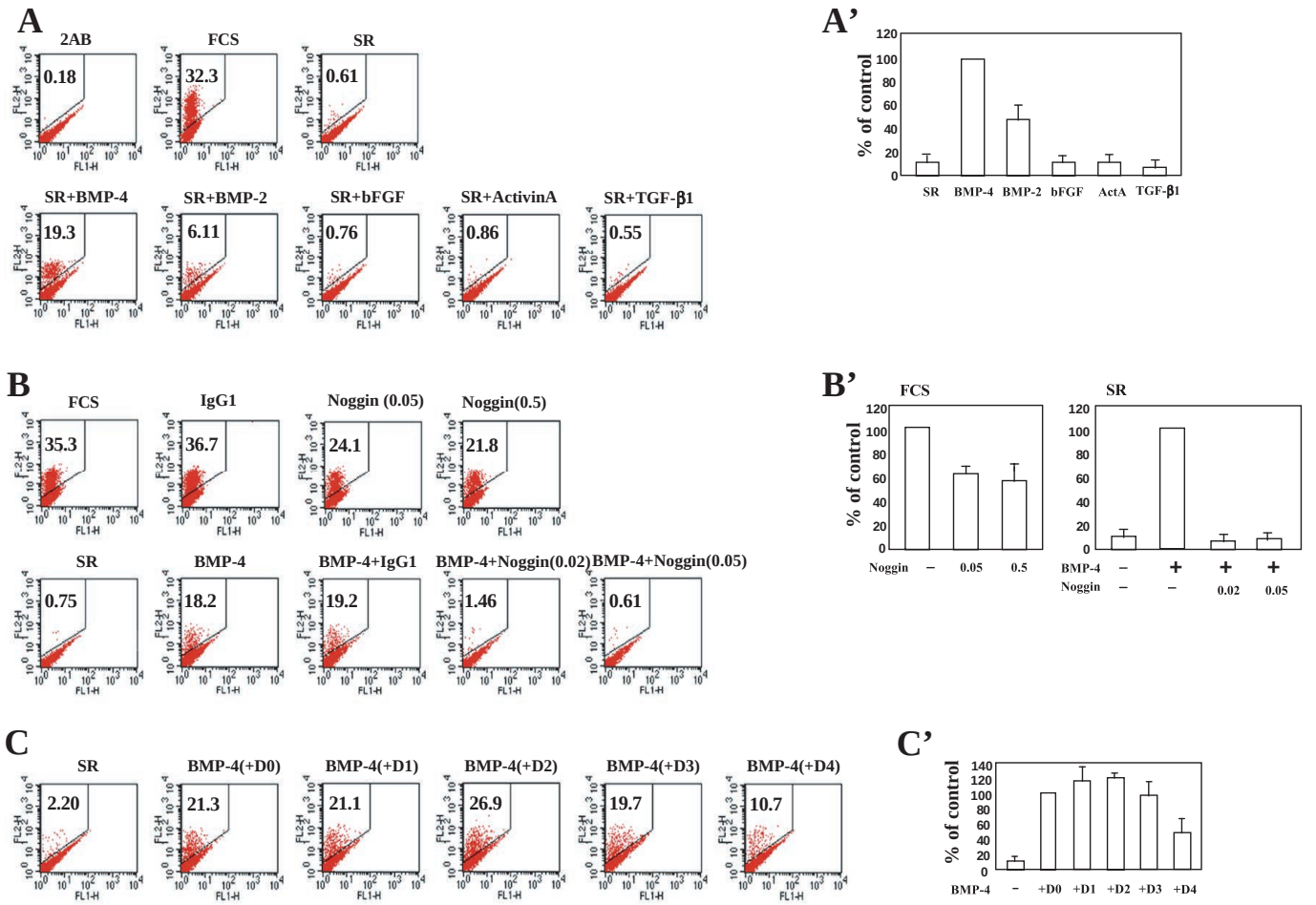


Fig. 1. BMP4 induces FLK1$^+$ cells. (A) ES cells were differentiated in serum (FCS) (upper panel) or in serum-free conditions (SR) with BMP4 (5 ng/ml), BMP2 (5 ng/ml), bFGF (10 ng/ml), activin A (2 ng/ml) or TGFβ1 (1 ng/ml). At day 2.75-3 of differentiation, EB cells were FACS analyzed for FLK1 expression. Numbers in insets indicate the percentage of FLK1$^+$ cells. 2AB indicates cells stained with secondary antibody alone. (A′) Results from four independent experiments are shown as a percentage of BMP4 control. Error bars indicate s.e.m. (B) ES cells were differentiated in serum (FCS) alone or with Noggin (upper panel). Alternatively, ES cells were differentiated in serum-free conditions (SR) with BMP4 alone or with BMP4 and Noggin (lower panel). At day 2.75-3 of differentiation, EB cells were FACS analyzed for FLK1 expression. Numbers in parenthesis indicate Noggin concentration (μg/ml). IgG1 isotype antibodies were used as control. Numbers in insets indicate the percentage of FLK1$^+$ cells. (B′) Results from three (FCS) or five (serum-free conditions) independent experiments are shown as a percentage of serum alone (FCS) or BMP4 (SR) control. Error bars indicate s.e.m. (C) ES cells were differentiated in serum-free conditions, and then BMP4 was added at different days (D0, D1, D2, D3 or D4). At day 6 of differentiation, EB cells were FACS analyzed for FLK1 expression. Numbers in insets indicate the percentage of FLK1$^+$ cells. (C′) Results from three independent experiments are shown as a percentage of BMP4 (D0) control. Error bars indicate s.e.m.

expressed in mesoderm and visceral endoderm (Arceci et al., 1993), respectively, were expressed at low levels in EB cells differentiated in serum-free conditions. However, *Fgf5*, a marker for ectoderm (Haub and Goldfarb, 1991; Hebert et al., 1991), was expressed at high levels in these cells indicating that ES cells readily gave rise to ectoderm in serum-free conditions. When ES cells were differentiated in the presence of BMP4, both *T* and *Gata4* genes were upregulated. However, *Fgf5* expression was downregulated. This suggested that BMP4 functions to induce mesoderm at the expense of ectoderm. Moreover, the kinetics of *T* and *Flk1* expression in BMP4 were similar to that of serum, such that the downregulation of *T* coincided with the up regulation of *Flk1* gene expression. This suggested that BMP4 could also induce FLK1$^+$ cells from the mesoderm. Thus, we determined if *T* expressing EB cells from days 2-3 were still able to respond to BMP4 to generate FLK1$^+$ cells. *Rex1* is downregulated at these time points. As shown in Fig. 1C, we found similar percentage of FLK1$^+$ cells as long as BMP4 was added up to day 3. The percentage of FLK1$^+$ cells was lower when BMP4 was added on day 4 or 5 compared with earlier time points. This could be interpreted that *T*-expressing mesoderm lost the ability to respond to BMP4. Alternatively, this could be due to a decrease in the number of mesodermal cells, which respond to BMP4. The latter view is supported by greater level of cell death of EB cells at later time points in the absence of any added factors (not shown). Together, this suggests that BMP4 functions at two distinct steps. First, BMP4 can induce mesoderm. Second, BMP4 can also induce FLK1$^+$ cells from mesoderm.

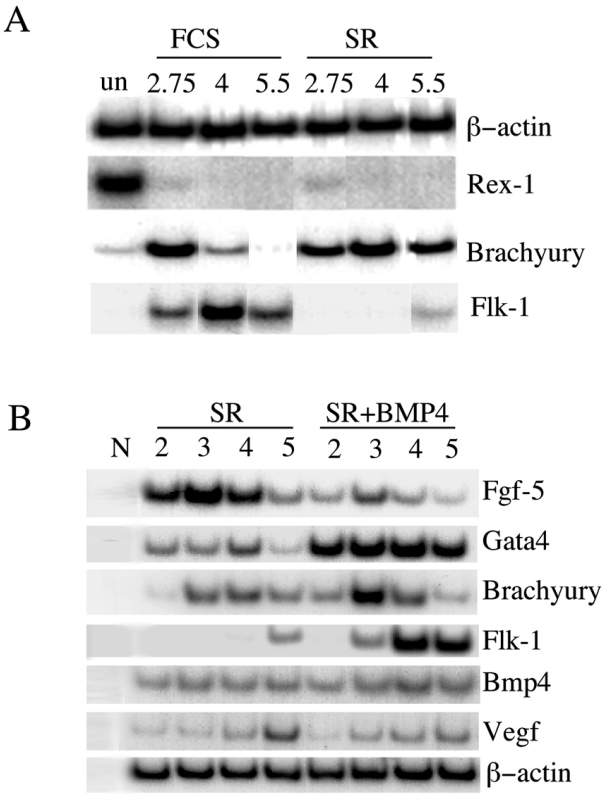


Fig. 2. Gene expression analysis. (A) RNA from EBs differentiated either in serum (FCS) or in serum-free conditions (SR), was subjected to semi-quantitative RT-PCR. Numbers on top indicate days of EB differentiation. un, undifferentiated ES cells. (B) EBs were generated in serum-free conditions (SR) or SR+BMP4, collected at different time points, and RNA was subjected to semi-quantitative RT-PCR. Numbers on top indicate days of differentiation. N, negative H_2O control.

VEGF is required for expansion of hematopoietic and endothelial progenitors

Our studies indicate that BMP4 was required for FLK1$^+$ cell generation. To determine if BMP4 was also necessary for SCL-expressing cell development, we examined CD4-expressing cells from *in vitro* differentiated *Scl*$^{+/CD4}$ ES cells (Chung et al., 2002). As shown in Fig. 3A, very few FLK1$^+$ or CD4 (i.e. SCL)-expressing cells were detectable when ES cells were differentiated in serum-free conditions. When ES cells were differentiated in the presence of BMP4, cells expressing CD4 were detectable at low levels in day 5-6 Ebs (5-6%, Fig. 3A,B) and increased at day 7 (11-12%, Fig. 3B,C). This suggested that BMP4 could induce the generation of SCL-expressing cells. However, the percentage of cells expressing CD4 in the presence of BMP4 was much lower compared with that from the serum control (~6% versus ~40%, respectively, Fig. 3A). Thus, we searched for additional factor(s) that could cooperate with BMP4 to generate CD4-expressing cells. As shown in Fig. 3A, the addition of BMP2,

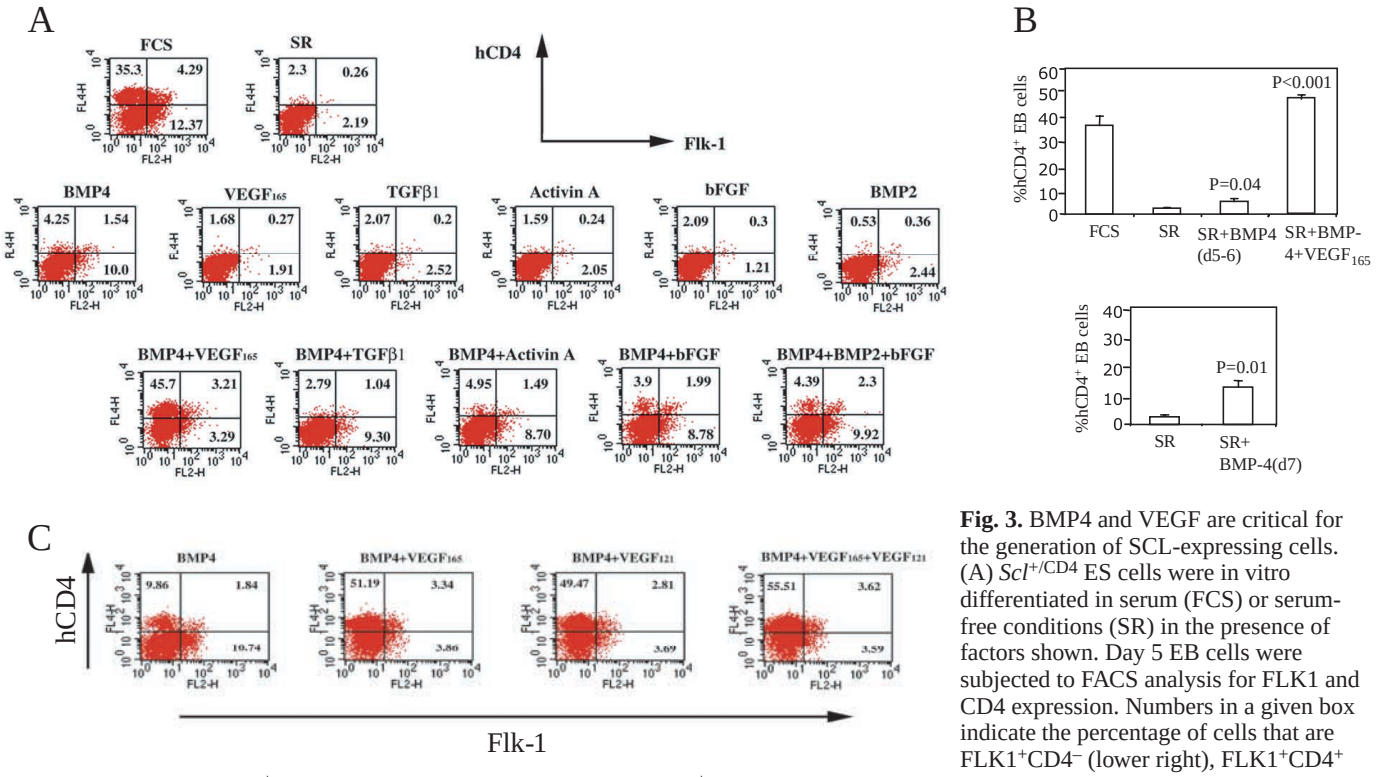


Fig. 3. BMP4 and VEGF are critical for the generation of SCL-expressing cells. (A) *Scl*$^{+/CD4}$ ES cells were in vitro differentiated in serum (FCS) or serum-free conditions (SR) in the presence of factors shown. Day 5 EB cells were subjected to FACS analysis for FLK1 and CD4 expression. Numbers in a given box indicate the percentage of cells that are FLK1$^+$CD4$^-$ (lower right), FLK1$^+$CD4$^+$ (upper right), or FLK1$^-$CD4$^+$ (upper left). (B) Statistical analyses of CD4$^+$ cells developing in BMP4 (day 5-6 and 7) or BMP4+VEGF (day 5-6). (C) Both $VEGF_{121}$ and $VEGF_{165}$ are effective in SCL-expressing cell induction. Day 7 *Scl*$^{+/CD4}$ EB cells, differentiated in serum-free conditions in the presence of BMP4, BMP4+$VEGF_{121}$, BMP4+$VEGF_{165}$ or BMP4+$VEGF_{121}$+$VEGF_{165}$ were subjected to FACS analysis for FLK1 and CD4 expression. Numbers in a given box indicate the percentage of cells that are FLK1$^+$CD4$^-$ (lower right), FLK1$^+$CD4$^+$ (upper right), or FLK1$^-$CD4$^+$ (upper left).

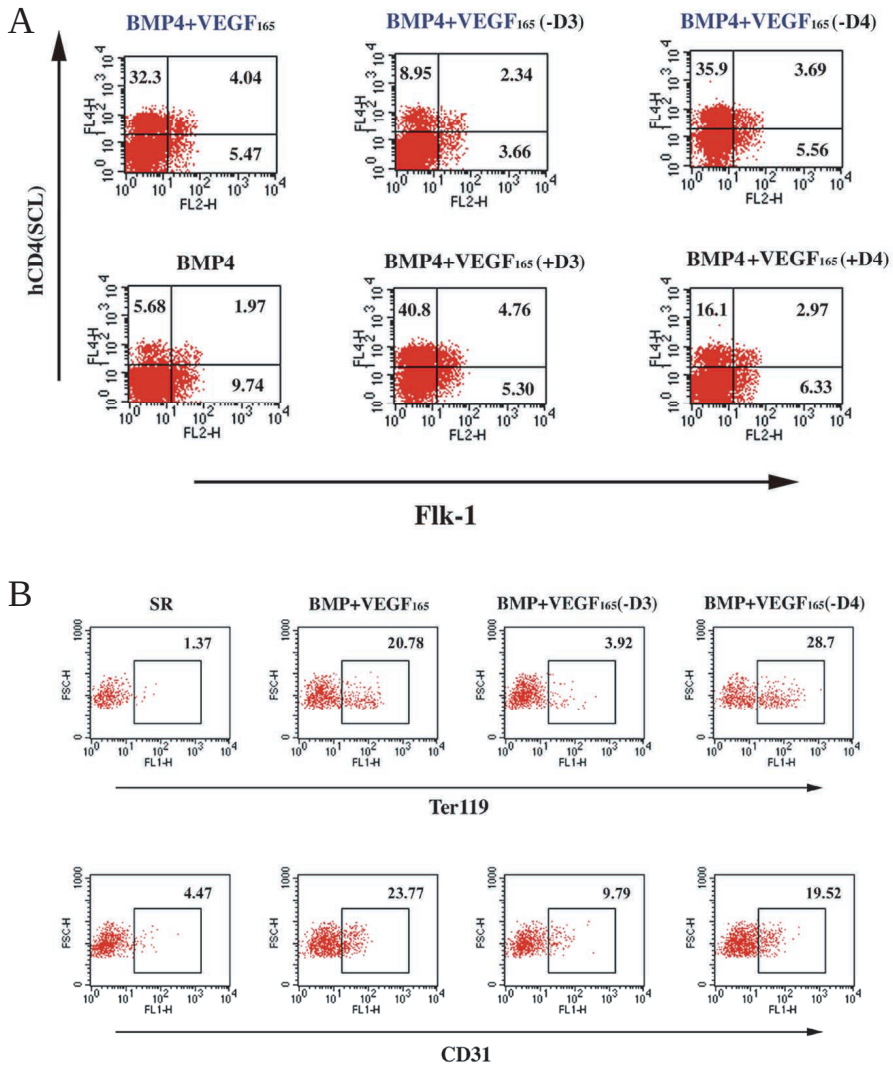


Fig. 4. Kinetic analyses of VEGF function. (A) *Scl*$^{+/CD4}$ ES cells were differentiated in serum-free conditions in the presence of BMP4 and VEGF (upper panel) or BMP4 alone (lower panel). When ES cells were differentiated in BMP4 and VEGF, the factors were removed on day 3 or 4 from the medium by washing the EBs several times and returning them to fresh EB differentiation medium without BMP4 and VEGF. When ES cells were differentiated with BMP4, VEGF was added to the differentiation medium on day 3 or 4 of differentiation at 10 ng/ml. At day 5-6, the cells were subjected to FACS analysis for FLK1 and CD4 expression. Numbers in a given box indicate the percentage of cells that are FLK1$^+$CD4$^-$ (lower right), FLK1$^+$CD4$^+$ (upper right) or FLK1$^-$CD4$^+$ (upper left). (B) *Scl*$^{+/CD4}$ ES cells were differentiated in serum-free conditions in the presence of BMP4 and VEGF. The factors were washed off on day 3 or day 4, and cells expressing Ter119 or CD31 were measured on day 6.

bFGF, activin A or TGFβ1 did not affect the generation of CD4-expressing cells. However, the level of cells expressing CD4 dramatically increased when both BMP4 and VEGF were added to serum-free differentiation conditions. Importantly, the CD4$^+$ levels approximated the level of serum differentiation (~49%). The percentage of CD4$^+$ cells increased up to 10 ng/ml of VEGF in the presence of BMP4. VEGF at higher concentrations (50 ng/ml) did not further increase CD4$^+$ cells (data not shown).

There are two major soluble isoforms of VEGF present in the mouse: VEGF$_{121}$ and VEGF$_{165}$. As endothelial differentiation and hematopoiesis were strongly stimulated in the presence of both VEGF$_{121}$ and VEGF$_{165}$ in zebrafish (Liang et al., 2001), we tested whether VEGF$_{121}$ and VEGF$_{165}$ synergized. As shown in Fig. 3C, VEGF$_{121}$ was as effective as VEGF$_{165}$ in generating CD4$^+$ cells. The percentage of CD4$^+$ cells further increased, although slightly, in the presence of both VEGF$_{121}$ and VEGF$_{165}$. These results suggest that VEGF$_{121}$ and VEGF$_{165}$ do not synergistically increase SCL-expressing cells in the EB system.

In an effort to determine if VEGF is constitutively required for the generation of SCL-expressing cells, we determined the window of time in which VEGF can function to generate SCL-expressing cells. Our experimental strategy was to differentiate ES cells in serum-free conditions in the presence of BMP4 and VEGF up to day 3 or day 4. Factors were then washed out, and CD4-expressing cells were analyzed on day 5.5 (Fig. 4A, upper panel). Alternatively, ES cells were differentiated with BMP4 alone, VEGF was added on day 3 or day 4, and CD4-expressing cells were analyzed on day 5.5 (Fig. 4A, lower panel). This scheme is based on our previous studies that FLK1-expressing cells emerged between days 1.5 and 2 of EB differentiation, expanded up to day 4, and then decreased (Chung et al., 2002). When BMP4 and VEGF were removed on day 3 (Fig. 4A, upper middle panel), there was a remarkable decrease in CD4$^+$ cells. However, the removal of BMP4 and VEGF on day 4 (Fig. 4A, upper right panel) did not affect the percentage of CD4$^+$ cells generated compared with the control (Fig. 4A, upper left panel). Conversely, when VEGF was added on day 3, CD4$^+$ cells developed as expected (Fig. 4A, lower middle panel), while the addition of VEGF on day 4 did not augment CD4$^+$ cell numbers significantly (Fig. 4A, lower right panel). We next determined if VEGF induced SCL-expressing cells between days 3 and 4 were sufficient to generate mature hematopoietic and endothelial cells. Similar to previous experiments, BMP4 and VEGF were added at the time of differentiation and removed on day 3 or day 4, and EB cells were subjected to FACS analyses for TER119 (erythroid) or CD31 (endothelial) expression on day 6. As shown in Fig. 4B, both TER119 and CD31-expressing cells decreased greatly when BMP4 and VEGF were removed on day 3. However, the percentage of both TER119$^+$ and CD31$^+$ cells was largely unchanged when BMP4 and VEGF were removed on day 4. Collectively, our findings suggested that VEGF is only required for a

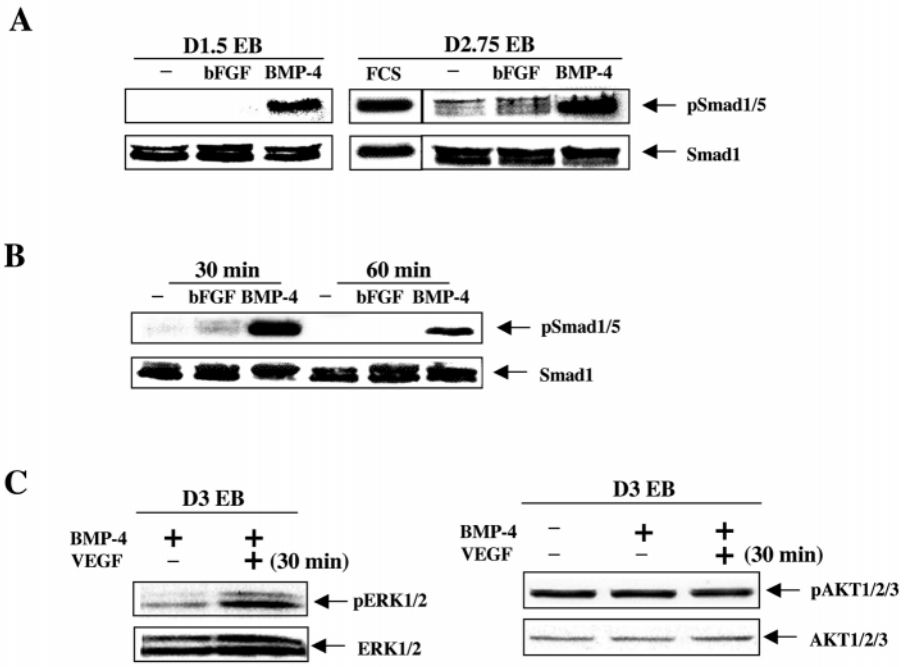


Fig. 5. BMP4 induces phosphorylation of SMAD1/5, while VEGF induces phosphorylation of Erk1/2 during EB differentiation. (A) ES cells were differentiated up to day 1.5 (left) or day 2.75 (right) in serum-free conditions in the presence of either bFGF or BMP4. (B) EBs harvested from day 1.5 differentiated in serum-free conditions were treated with either bFGF or BMP4 for 30 and 60 minutes. (C) ES cells were differentiated in serum-free conditions with BMP4. Three days after, BMP4 was washed off overnight and EBs were treated with VEGF for 30 minutes. EB cell lysates were subjected to SDS-PAGE, followed by immunoblotting with pSMAD1/5, SMAD1 (A,B), pERK1/2, ERK1/2, pAKT1/2/3 or AKT1/2/3 (C). One out of three experiments is shown.

short time to induce SCL, TER119 and CD31-expressing hematopoietic and endothelial cells.

SMAD1/5 and ERK1/2 activation by BMP4 and VEGF

BMP4 activates the SMAD pathway, and VEGF the MAPK, PI3K, PLCγ and PKC pathways (Itoh et al., 2000; Giles, 2001). To define the signals that control FLK1$^+$ and SCL$^+$ cell generation, we further examined signaling events downstream of BMP4 and VEGF during ES cell differentiation. First, ES cells were differentiated in serum-free conditions with BMP4 added at the onset of differentiation. Subsequently, SMAD1/5 phosphorylation was determined on days 1.5 and 2.75 of ES cell differentiation. As shown in Fig. 5A, SMAD1/5 phosphorylation was detected in EB cells differentiated in BMP4 as well as in serum. Importantly, SMAD1/5 phosphorylation was not detected (or was considerably reduced) in the absence of serum. Basic FGF, which activates the MAP kinase pathway, was included as a negative control.

To determine the kinetics of SMAD1/5 phosphorylation, ES cells were differentiated in serum-free conditions. EBs were then collected on day 1.5, treated with BMP4 or bFGF for 30-60 minutes, and the phosphorylation status of SMAD1/5 was analyzed (Fig. 5B). The phosphorylation of SMAD1/5 occurred in EBs treated with BMP4, but not with bFGF. Collectively, these studies demonstrate that the SMAD1/5 pathway was activated in EB cells differentiated with BMP4. SMAD1/5 phosphorylation by BMP4 occurred rapidly as shown by the kinetic studies.

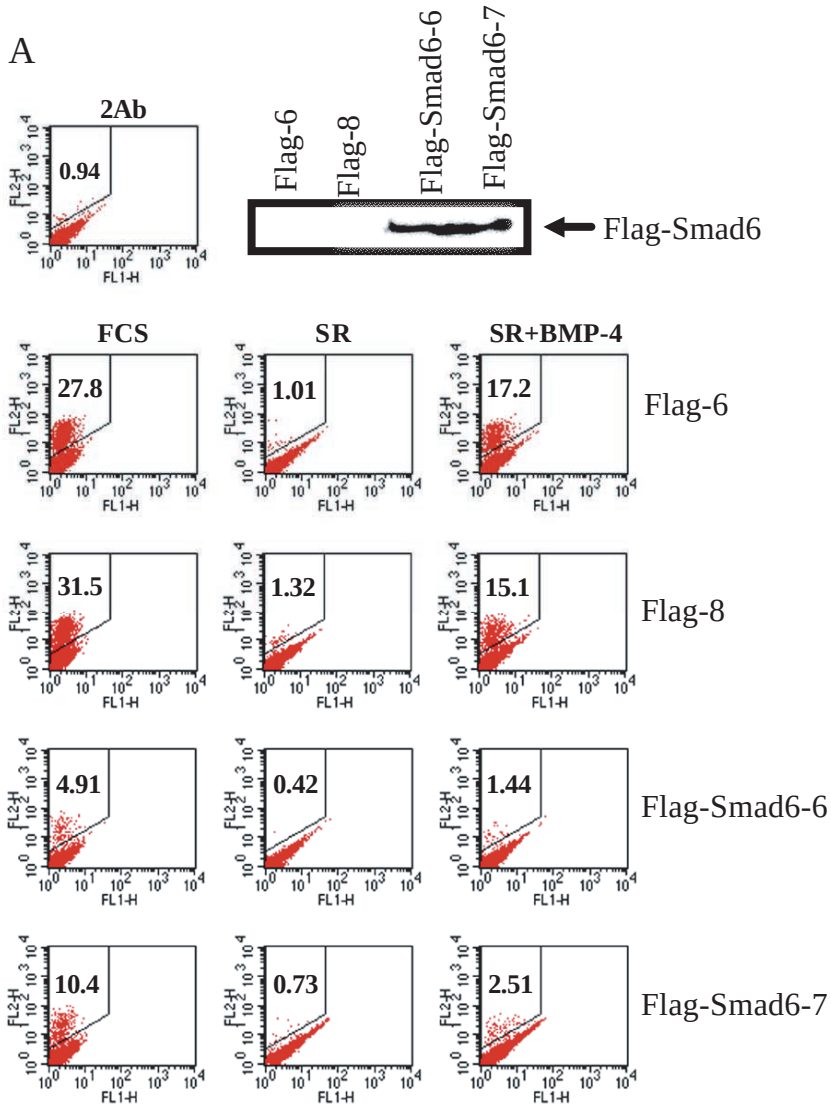
The cell analyses suggested that VEGF is required for the expansion of SCL-expressing cells. As the MAPK pathway and AKT activation via PI3 kinase have been implicated in cell proliferation and cell survival, we examined ERK1/2 and AKT phosphorylation by VEGF. To achieve this, ES cells were differentiated in serum-free conditions with BMP4. Three days later, BMP4 was washed out overnight and EBs were treated with VEGF for 30 minutes. Cells were then collected and analyzed for ERK1/2 phosphorylation. The AKT phosphorylation was also measured to determine if the PI3-kinase pathway was activated. As shown in Fig. 5C, ERK1/2 phosphorylation was induced when EBs were stimulated with VEGF, while AKT phosphorylation was not affected. Collectively, our studies indicate that the generation of FLK1$^+$ and SCL$^+$ cells by BMP4 and VEGF involves the activation of the SMAD1/5 and MAP kinase pathways, respectively.

ES cells over expressing SMAD6 display defects in FLK1$^+$ cell generation

The finding that the SMAD1/5 pathway was activated at the time of FLK1$^+$ cell formation suggested that it plays a functional role in this process. To determine whether the SMAD1/5 activation by BMP4 signaling is crucial for FLK1$^+$ cell development, we generated ES clones over expressing SMAD6, which inhibits either the recruitment of SMAD1 to the receptor or the heterodimer formation between phosphorylated SMAD1 and SMAD4 (Imamura et al., 1997; Hata et al., 1998). If activation of the SMAD1/5 pathway is crucial for BMP4-mediated FLK1$^+$ cell generation, the overexpression of SMAD6 should block the formation of FLK1$^+$ cells. As shown in Fig. 6, FLK1$^+$ cells developed as expected from control clones (Flag 6 and Flag 8). However, SMAD6 overexpressing clones (Flag SMAD6-6 and Flag SMAD7, and data not shown) were not able to respond to BMP4 and failed to generate FLK1$^+$ cells in serum-free conditions. Importantly, SMAD6 overexpressing clones generated much lower FLK1$^+$ cells even in the full complement of serum compared with controls (Fig. 6). Therefore, activation of the SMAD1/5 pathway is crucial for FLK1$^+$ cell formation.

MAP kinase kinase 1 (MKK1) specific inhibitor can block the generation of SCL$^+$ cells

To examine further the significance of the activation of the map kinase pathway in SCL$^+$ cell development, we tested if U0126,



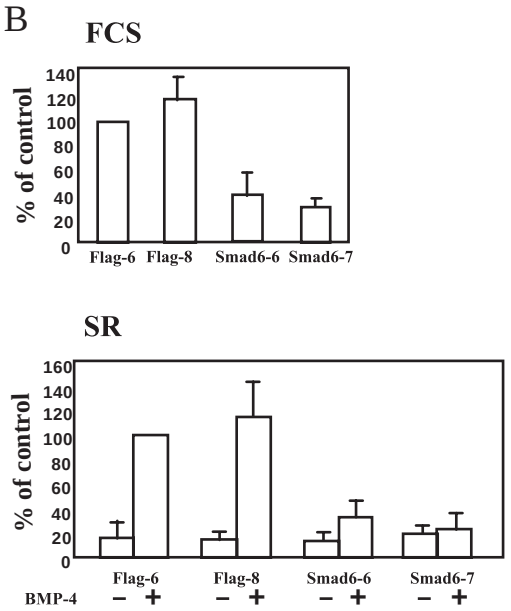


Fig. 6. Overexpression of SMAD6 in ES cells inhibits the generation of FLK1$^+$ cells. (A) ES cells stably expressing SMAD6 were differentiated and subjected to FACS analysis for FLK1 expression. Numbers in insets indicate the percentage of FLK1$^+$ cells. 2Ab indicates cells stained with secondary antibody alone. Flag-6 and Flag-8 indicate control ES cell clones. Flag SMAD6-6 and SMAD6-7 represent two independent ES cell clones stably expressing Flag-SMAD6. Expression of exogenous Flag-SMAD6 was verified with anti-FLAG antibody (arrow). (B) Results from four independent experiments are shown as a percentage of Flag-6 control. Error bars indicate s.e.m.

a specific inhibitor of map kinase kinase 1 (MKK1) (Favata et al., 1998), could inhibit SCL$^+$ cell generation. To achieve this, we differentiated ES cells in serum-free conditions with BMP4 to ensure that FLK1$^+$ cells were generated. After three days, BMP4 was washed out. VEGF and U0126 or VEGF and U0124 (a control for U0126) were added, and SCL$^+$ cells were analyzed on day 5. VEGF and DMSO, the solvent used to dissolve U0124 and U0126, was also included as an additional control. As shown in Fig. 7, CD4$^+$ cells decreased in the presence of U0126 in a dose-dependent manner. This suggests that SCL$^+$ cell generation by VEGF required the map kinase activation.

FLK1, but not FLT1, is responsible for VEGF-mediated expansion of hematopoietic and endothelial cell progenitors

VEGF signals mainly through FLK1, FLT1 and neuropilin receptors (Neufeld et al., 1999; Robinson and Stringer, 2001). As FLK1 and FLT1 are implicated in both hematopoietic and endothelial cells differentiation, and VEGF$_{121}$, which does not bind neuropilin receptors (Soker et al., 1998), could induce CD4-expressing cells (Fig. 3C), we examined *Flk1*$^{-/-}$ and *Flt1*$^{-/-}$ ES clones to determine which of these two receptors was responsible for the VEGF-mediated generation of *Scl*-expressing cells. Our experimental strategy was to differentiate *Flk1*$^{-/-}$ and *Flt-1*$^{-/-}$ ES cells in serum-free conditions in the presence of BMP4 alone or BMP4 and VEGF, followed by an examination of TER119- and CD31-expressing cells as an indirect reference for SCL induction. If FLK1 is the major receptor that mediates VEGF signals that lead to the generation of SCL-expressing cells, it was expected that *Flk1*$^{-/-}$ ES cells would give rise to a similar percentage of TER119$^+$ or CD31$^+$ cells in response to BMP4 versus BMP4 and VEGF. However, *Flt1*$^{-/-}$ ES cells would give rise to an increase in TER119$^+$ and CD31$^+$ cells. As shown in Fig. 8A, VEGF failed to increase TER119$^+$ and CD31$^+$ cells from *Flk1*$^{-/-}$ ES cells, while TER119$^+$ and CD31$^+$ cells expanded in response to VEGF in *Flt1*$^{-/-}$ ES cells. *Flt1*$^{-/-}$ EB cells gave rise to higher levels of TER119$^+$ and CD31$^+$ cells even with BMP4 alone, consistent with studies by Fong et al. (Fong et al., 1999), which showed that *Flt1*$^{-/-}$ embryos contained higher levels of hemangioblasts. Hematopoietic replating data also showed similar results. As shown in Fig. 8B, the number of erythroid, macrophage and

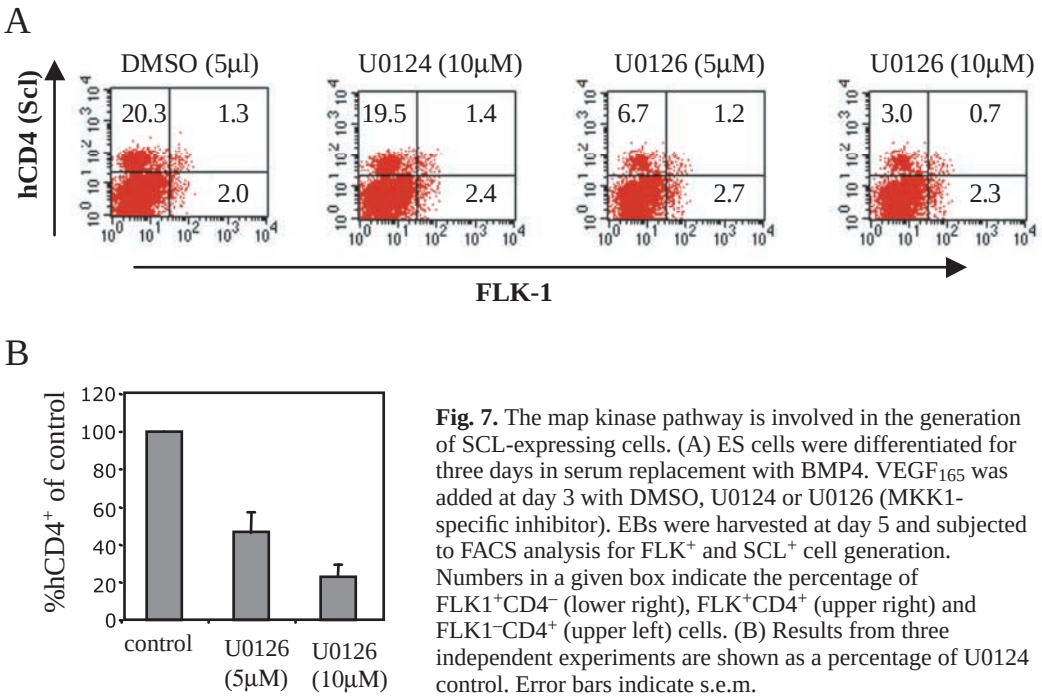


Fig. 7. The map kinase pathway is involved in the generation of SCL-expressing cells. (A) ES cells were differentiated for three days in serum replacement with BMP4. $VEGF_{165}$ was added at day 3 with DMSO, U0124 or U0126 (MKK1-specific inhibitor). EBs were harvested at day 5 and subjected to FACS analysis for FLK^+ and SCL^+ cell generation. Numbers in a given box indicate the percentage of $FLK1^+CD4^-$ (lower right), FLK^+CD4^+ (upper right) and $FLK1^-CD4^+$ (upper left) cells. (B) Results from three independent experiments are shown as a percentage of U0124 control. Error bars indicate s.e.m.

erythroid/macrophage colonies was similar when *Flk1*$^{-/-}$ ES cells were differentiated in BMP4 or BMP4 and VEGF. Control cells (*Scl*$^{+/CD4}$, Fig. 9C and data not shown) and *Flt-1*$^{-/-}$ ES cells gave rise to higher number of erythroid, macrophage and erythroid/macrophage colonies in response to BMP4 and VEGF compared with BMP4 alone. Collectively, our results indicate that the expansion of hematopoietic progenitors requires VEGF signaling through the FLK1 receptor.

Hematopoietic and endothelial cell differentiation in response to BMP4, VEGF, and TGFβ1

We initially observed that $CD4^+$ cells decreased somewhat when ES cells were differentiated with BMP4 and TGFβ1 compared to BMP4 alone (Fig. 3A). As it was possible that TGFβ1 inhibited $CD4^+$ cell generation, we examined *Scl*$^{+/CD4}$ ES cells differentiated in BMP4+VEGF+TGFβ1. We also tested activin A in combination with BMP4 and VEGF, as we previously showed that activin A could augment hematopoietic differentiation (Faloon et al., 2000). As shown in Fig. 9A, the generation of $CD4^+$ cells greatly decreased when TGFβ1 was added to the culture containing BMP4 and VEGF. However, the percentage of $CD4^+$ cells increased, although slightly, when activin A was added to the culture of BMP4 and VEGF.

To compare the generation of SCL-expressing cells to hematopoietic and endothelial cell differentiation, we examined day 6.5 EB cells differentiated with BMP4+VEGF for TER119 and CD31 as a measure of hematopoietic (erythroid) and endothelial cell differentiation, respectively (Fig. 9B). As seen before in Fig. 4, TER119-expressing cells increased significantly in the presence of BMP4+VEGF compared with BMP4 alone. $CD31^+$ cells also increased in the presence of BMP4+VEGF compared with BMP4 alone. Importantly, when TGFβ1 was added to BMP4 + VEGF, the generation of $TER119^+$ cells decreased considerably. $CD31^+$ cells also decreased in response to TGFβ1, although the decrease in $CD31^+$ cells by TGFβ1 was not as evident as in the $TER119^+$ cells.

To verify the FACS data, EB cells generated in the presence of BMP4, BMP4+VEGF, BMP4+VEGF+TGFβ1 or BMP4+VEGF+activin A were subjected to hematopoietic replating (Fig. 9C). As shown, erythroid, macrophage and erythroid/macrophage bipotential colonies all developed in BMP4 alone. Importantly, the number of these colonies increased when VEGF was added to the BMP4 culture. Again, TGFβ1 inhibited the generation of hematopoietic progenitors. We again saw a slight increase of hematopoietic differentiation by activin A. There did not seem to be a qualitative difference in the generation of primitive and definitive erythroid progenitors by BMP4 or BMP4+VEGF, as both types of colonies were present in EBs generated in BMP4 or BMP4+VEGF (data not shown). Collectively, our data demonstrate that efficient hematopoietic and endothelial cell generation requires an ordered function of BMP4, VEGF, TGFβ1 and activin A. BMP4 is required initially, followed by VEGF. TGFβ1 and activin A then modulate the function of VEGF by inhibiting or enhancing the generation of SCL-expressing cells.

Discussion

BMP4 in hematopoietic and vascular commitment

Here, we employed ES-derived $FLK1^+$ and SCL^+ cells as means to further understand inductive signals leading to the generation of hematopoietic and endothelial cells. We demonstrated that ES cells developed only to the mesodermal stage in serum-free conditions and the progression from the *Brachyury* expressing mesoderm to $FLK1^+$ cells required serum factor(s). Among the factors tested in these EBs, only BMP4 was able to induce the generation of $FLK1^+$ cells. In EBs generated in serum replacement alone, low levels of

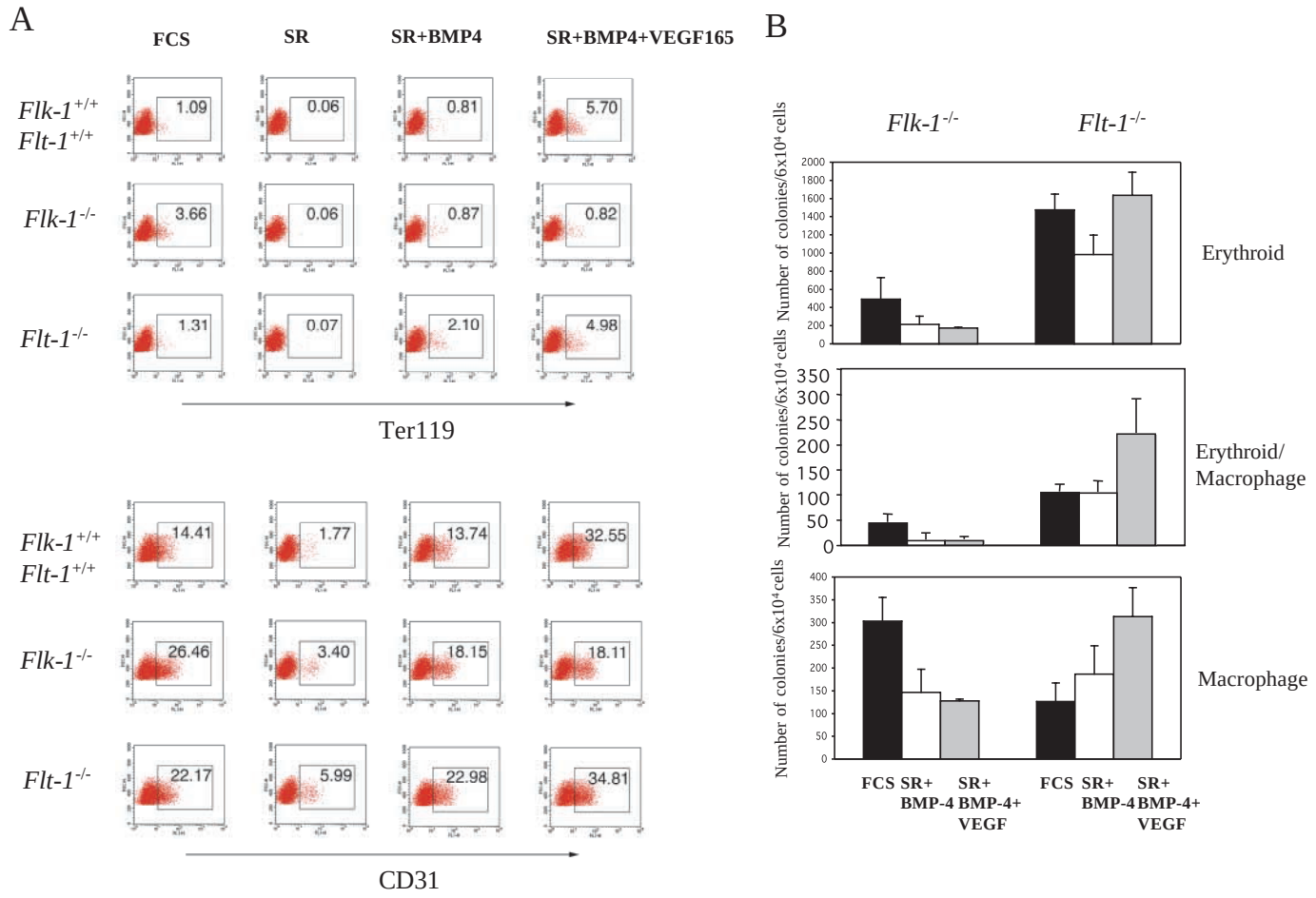
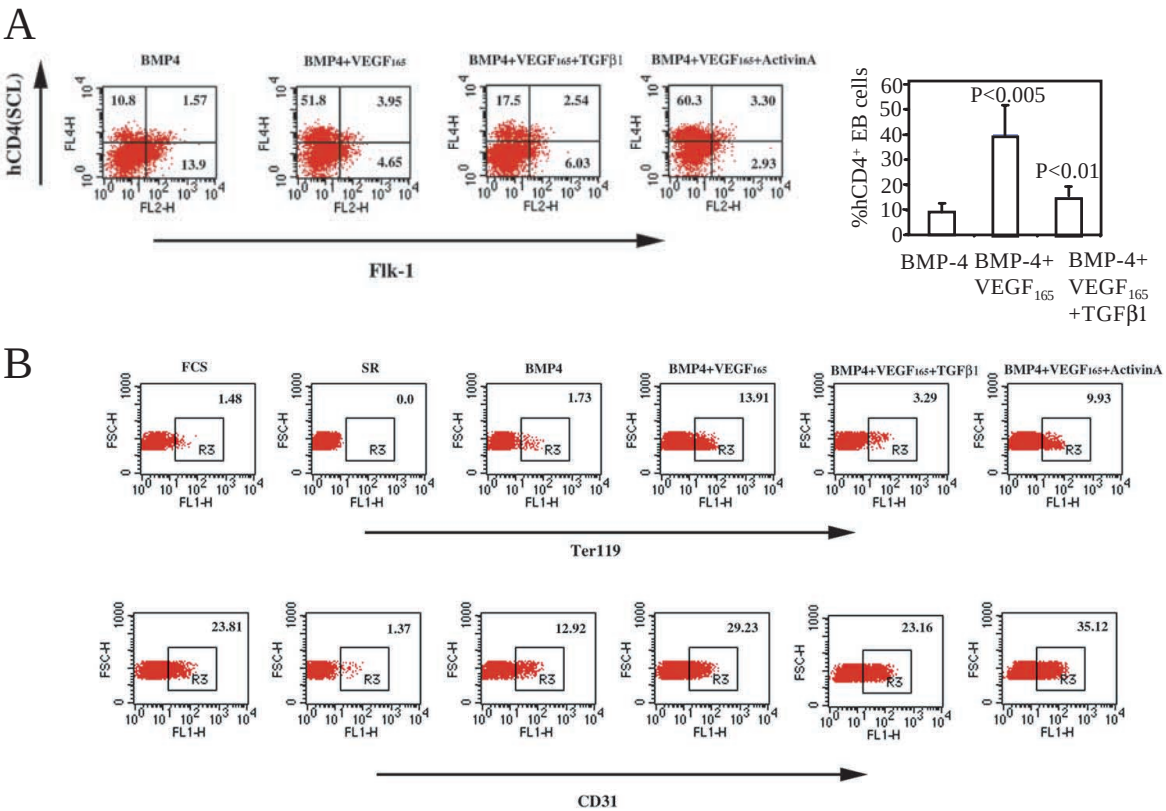


Fig. 8. VEGF induces SCL-expressing cells mainly through the FLK1 receptor. (A) Wild-type, *Flk1*$^{-/-}$ and *Flt1*$^{-/-}$ ES clones were differentiated in serum or serum-free conditions, in the presence of BMP4 and VEGF as shown. Day 5-6 EBs were analyzed for CD31 and TER119 expression. The percentage of TER119 and CD31-positive cells are shown. One representative experiment out of three is shown. (B) *Flk1*$^{-/-}$ and *Flt1*$^{-/-}$ EB cells generated in the presence of FCS (black bar), SR+BMP4 (white bar), or SR+BMP4+VEGF (grey bar) were re-plated in a semi-solid medium as described in the Materials and methods. Hematopoietic colonies were counted 5-7 days after re-plating. No hematopoietic colonies formed when EB cells from SR were re-plated, thus not shown. Error bars indicate standard deviations from triplicate plating. One representative experiment out of three is shown.

Flk1 were detected at later time points. Importantly, we also observed that BMP4 was expressed in serum-free conditions (Fig. 2B). Therefore, it is possible that the endogenous BMP4 expression alone is sufficient for ES cells to induce mesoderm and limited number of FLK1-expressing cells. However, robust FLK1 induction was achieved by the addition of BMP4. Importantly, it is thought that BMP4 expression is under the autologous feedback (Jones et al., 1992; Adelman et al., 2002). However, we did not observe *Bmp4* gene induction by the addition of BMP4 in ES cell differentiation (Fig. 2B).

Our kinetic studies show that *T* was induced in EBs differentiated in BMP4. Furthermore, we also observed that FLK1^{+} cells still developed when BMP4 was added to EB cells expressing *T* but not *Rex1*. This suggests that BMP4 functions at two distinct steps. First, BMP4 can induce mesoderm. Second, BMP4 can also induce FLK1^{+} cells from mesoderm. It is not clear whether these FLK1-expressing cells still represent the mesoderm. Fehling et al. (Fehling et al., 2003) recently showed that T-expressing, but FLK1^{-}, cells progressed to give rise to T- and FLK1-expressing cells. Therefore, it is possible that FLK1 expression can specify a subset of mesoderm that ultimately generates the hematopoietic and vascular systems.

Our studies have demonstrated that ES cells differentiated in serum-free conditions in the presence of BMP4 alone could still give rise to SCL-expressing cells and hematopoietic progenitors. In addition, we observed SCL gene induction in *Flk1*$^{-/-}$ EBs differentiated in BMP4 (data not shown). Previous studies also support the idea that BMP4-mediated signals are required for hematopoietic specific gene expression. For example, Johansson and Wiles (Johansson and Wiles, 1995) reported that BMP4 could induce *T* and β-H1globin from ES cells differentiated in chemically defined medium. Furthermore, Adelman et al. (Adelman et al., 2002) reported that BMP4 could induce expression of *Eklf* and *Gata1* erythroid-specific genes. Whether BMP4 can induce hematopoietic cells directly from mesoderm is not clear. All of these studies, including our own, used an ES/EB system, in which hematopoietic differentiation occurs via the mesoderm followed by generation of FLK1 expressing cells. Thus, future



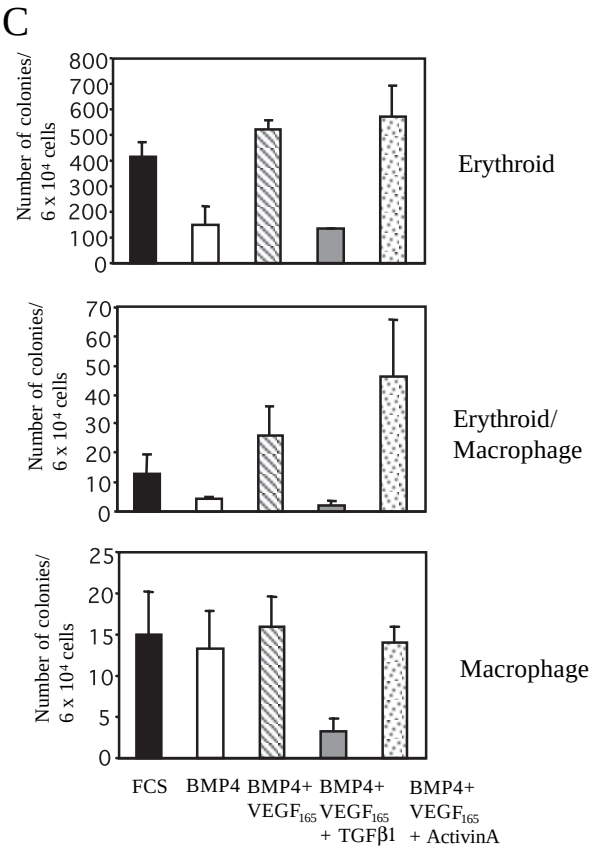


Fig. 9. TGFβ1 and activin A in VEGF-mediated expansion of SCL-expressing cells, hematopoietic and endothelial cell differentiation. Day 6 *Scl*$^{+/CD4}$ EB cells generated in serum-free conditions in the presence of BMP4; BMP4+VEGF; BMP4+VEGF+TGFβ1; or BMP4+VEGF+activin A were subjected to three-color analyses for FLK1, CD4 and hematopoietic or endothelial markers. (A) The percentage of FLK1$^+$CD4$^-$ (lower right), FLK1$^+$CD4$^+$ (upper right) or FLK1$^-$CD4$^+$ (upper left) cells is shown. Statistical values are shown on the right. (B) The percentage of TER119 and CD31-positive cells is shown. (C) EB cells generated in the presence of FCS; SR+BMP4; SR+BMP4+VEGF; SR+BMP4+VEGF+TGFβ1; or SR+BMP4+VEGF+activin A were subjected to hematopoietic re-plating. No hematopoietic colonies formed when EB cells from SR were re-plated, thus not shown. Hematopoietic colonies were counted 5-7 days after re-plating. Error bars indicate standard deviations from triplicate plating.

studies are required to examine if BMP4 can directly induce *Scl* within FLK1$^+$ cells.

Gene targeting studies largely support the notion that BMP4-mediated signals are important for hematopoietic and vascular development. For example, *Bmp4*-deficient mice die between E7.5 and E9.5 with defects in mesoderm formation and patterning. Those that survive up to E9.5 show severe defects in blood island formation (Winnier et al., 1995). Additionally, mice lacking the type I BMP receptor (*Alk3*) fail to complete gastrulation and die by E9.5 (Mishina et al., 1995). Mice deficient in *Smad1* or *Smad5*, downstream signaling molecules of TGFβ family members, display varying degrees of defects in hematopoietic and vascular development, perhaps owing to overlapping function between SMAD1, SMAD5 and SMAD8 (Tremblay et al., 2001). For example, *Smad1*-deficient mice display defects in chorioallantoic fusion and die between

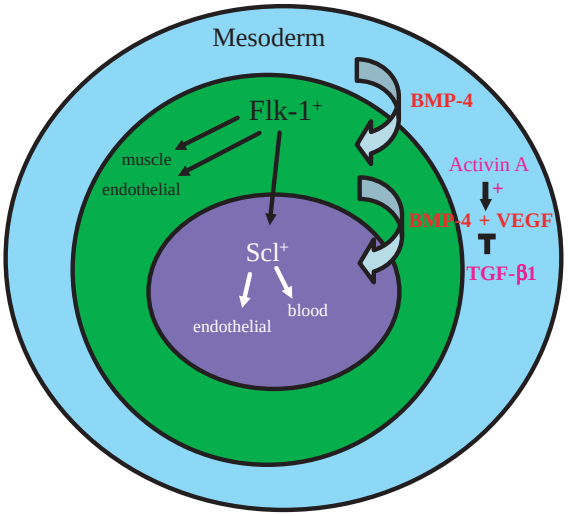


Fig. 10. A schematic diagram of BMP4, VEGF, activin A and TGFβ1 action in hematopoietic and endothelial cell development.

E9.5 and E10.5 (Tremblay et al., 2001; Lechleider et al., 2001). Although overall hematopoietic and vascular development appears to be normal, some *Smad1*-deficient embryos display defects in yolk sac angiogenesis (Lechleider et al., 2001). *Smad5*-deficient mice also show early embryonic lethality. The primitive plexus can be found in mutant embryos, but they fail to form organized vessels. There seems to be more primitive blood cells in E8.5 mutant yolk sacs, although E9.5 mutant yolk sacs contained almost none (Chang et al., 1999). Furthermore, *Smad5*-deficient yolk sacs contained higher frequency of high-proliferative potential colony forming cells (HPP-CFCs) and *Smad5*-deficient ES cells gave rise to an increased number of hematopoietic progenitors including blast colonies in vitro (Liu et al., 2003). Collectively, these studies demonstrate the importance of BMP4-mediated signals in early stages of mouse development including hematopoietic and endothelial cells.

VEGF, TGFβ1 and activin A collectively regulate hematopoietic differentiation

We showed herein that SCL-expressing cells developed when ES cells were differentiated in BMP4 alone. However, efficient expansion and differentiation of SCL-expressing cells required VEGF. Our studies indicate that VEGF function was required within a narrow window of time, such that the presence of VEGF between days 3 and 4 of EB differentiation readily generated SCL-expressing cells. The presence of VEGF between days 3 and 4 was sufficient for subsequent hematopoietic and endothelial cell differentiation (Fig. 4). Our studies are consistent with Endoh et al. (Endoh et al., 2002) who demonstrated that *Scl* gene reactivation from day 2 to day 4 after initiation of differentiation in *Scl*-null ES cells could rescue both primitive and definitive hematopoiesis.

Our studies indicate that VEGF signaling through FLK1 was responsible for augmenting SCL-expressing cells. First, *Flk1*$^{-/-}$ ES cells failed to respond to VEGF and gave rise to a similar percentage of TER119^{+} or CD31^{+} cells in the presence of BMP4 versus BMP4 and VEGF. Second, *Flt1*$^{-/-}$ ES cells responded to VEGF and generated higher levels of TER119^{+} or CD31^{+} cells as well as hematopoietic progenitors. Finally, our studies demonstrated that $VEGF_{121}$, which does not use neuropilin receptors, efficiently induces CD4-expressing cells. Collectively, our studies establish a hierarchical role of BMP4 and VEGF. BMP4 is required for the generation of FLK1- and SCL-expressing cells. VEGF function is to enhance the hematopoietic differentiation, as judged by the expansion of SCL expressing and hematopoietic progenitors. Our interpretation is consistent with studies by Nakayama et al. (Nakayama et al., 2000) that the sequential treatment of BMP4 followed by VEGF enhanced hematopoietic differentiation of ES cells and studies by Cerdan et al. (Cerdan et al., 2004), which showed that VEGF augmented erythroid development from human ES cells.

Gene targeting studies also support the notion that VEGF is crucial for proper hematopoietic and endothelial cell differentiation. For example, mice heterozygous for *Vegf* (*Vegf*$^{+/-}$) are embryonic lethal due to defects in vascular development (Ferrara et al., 1996; Carmeliet et al., 1996). The production of hematopoietic cells is significantly reduced in these mice. Conversely, mice with slightly higher levels of VEGF expression (two- to threefold) result in early embryonic lethality because of severe abnormalities in heart development (Miquerol et al., 2000). As for its mode of action, recent studies by Damert et al. (Damert et al., 2002) demonstrated that VEGF production in the yolk sac visceral endoderm was crucial for proper hematopoietic and endothelial cell development. In this study, the authors generated chimeras between *Vegf* wild-type tetraploid embryos and diploid *Vegf*$^{lo/lo}$ embryos and showed that defects in blood island formation and vascular development of Vegf$^{lo/lo}$ animals were rescued. Moreover, the hematopoietic cell population in the embryo proper of these chimeras increased as the contribution of *Vegf* wild-type tetraploid cells to the yolk sac visceral endoderm augmented. Importantly, chimeras generated between *Vegf*$^{lo/lo}$ tetraploid embryos with *Vegf*$^{+/+}$ ES cells showed defects in yolk sac vascular development. These studies indicate that tight regulation of VEGF expression is crucial for correct vascular and hematopoietic differentiation of the developing embryo.

Our studies suggest that coordinated VEGF, TGFβ1 and activin A function was important for efficient generation of hematopoietic progenitors. We observed that TGFβ1 inhibited BMP4+VEGF effect on hematopoietic and endothelial cell differentiation (Fig. 9). Activin A could slightly augment BMP4+VEGF effect. Consistently, mice with targeted mutations of TGFβ1 and TGFβ receptor II display abnormal yolk sac hematopoietic and endothelial cell development (Dickson et al., 1995; Oshima et al., 1996). The initial vasculogenesis occurs in these mice, but subsequent angiogenesis and capillary formation are defective. As for the hematopoiesis, Larsson et al. (Larsson et al., 2001) have shown that the number of erythroid progenitors was largely increased in TGFβ receptor I-deficient yolk sac compared with wild-type yolk sac, while CFU-GM and CFU-Mix appeared to be similar.

Role of the map kinase and SMAD pathways in hematopoietic and endothelial cell differentiation

We demonstrated that the SMAD1/5 and map kinase pathways were activated by BMP4 and VEGF, respectively, and that the activation of these pathways was crucial for the generation of FLK1^{+} and SCL^{+} cells (Figs 5-7). ES cells

overexpressing SMAD6 showed a decrease in FLK1$^+$ cells in response to BMP4. The MKK1-specific inhibitor U0126 was able to block the generation of SCL$^+$ cells. Furthermore, we did not observe ERK1/2 phosphorylation in *Flk1*$^{-/-}$ EBs when stimulated with VEGF (not shown). Consistent with our studies that the activation of map kinase pathways is crucial for hematopoietic and endothelial cell development, the yolk sac of *Mkk1*-deficient mice show diminished levels of blood cells and distended blood vessels (Giroux et al., 1999). Additionally, *Mkk1*-deficient embryos show defects in placental angiogenesis. Moreover, recent studies (Corson et al., 2003) demonstrate that there was a transient activation of ERK in nascent blood vessels. Collectively, these studies indicate that the activation of both the SMAD and MAP kinase pathways is crucial for blood and blood vessel formation.

In conclusion, we have positioned factors implicated in hematopoietic differentiation at each developmental stage of hematopoietic and endothelial cell formation (Fig. 10). Specifically, BMP4 is required sequentially from ES cells to mesoderm, from mesoderm to FLK1$^+$ cells, and from FLK1$^+$ to SCL$^+$ cells. VEGF then acts through FLK1 to expand SCL$^+$ cells. The activation of the SMAD and map kinase pathways by BMP4 and VEGF, respectively, is crucial in this process. TGFβ1 and activin A function to further modulate the expansion of hematopoietic and endothelial cells by BMP4 and VEGF. Future in vivo studies are required to verify the observations made in the ES/EB system.

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