Negative regulation of primitive hematopoiesis by the FGF signaling pathway

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Hematopoiesis is controlled by multiple signaling molecules during embryonic and postnatal development. The function of the fibroblast growth factor (FGF) pathway in this process is unclear. Here we show that FGF plays a key role in the regulation of primitive hematopoiesis in chicks. Using hemoglobin mRNA expression as a sensitive marker, we demonstrate that timing of blood differentiation can be separated from that of initial mesoderm patterning and subsequent migration. High FGF activity inhibits primitive blood differentiation and promotes endothelial cell fate. Conversely, inhibition of FGFR activity leads to ectopic blood formation and down-regulation of endothelial markers. Expression and functional analyses indicate that FGFR2 is the key receptor mediating these effects. The FGF pathway regulates primitive hematopoiesis by modulating Gata1 expression level and activity. We propose that the FGF pathway mediates repression of globin gene expression and that its removal is essential before terminal differentiation can occur. (Blood. 2006;108:3335-3343)

Introduction

During the early development of amniotes, blood cells are generated exclusively from the extraembryonic yolk sac mesoderm.[1-3] Intraembryonic hematopoietic niches such as aorta-gonad-mesonephros and liver mature later and initiate the second wave of hematopoiesis.[4-6] Located in the extreme lateral regions of the developing embryo, primitive blood cells are derived from ventral mesoderm precursors ingressing through the posterior part of the primitive streak during gastrulation. In birds, postingression precursor cells undergo extensive migration before the establishment of the circulation to populate the entire hemogenic region called area vasculosa.[3,7-9] Endothelial cells have a similar origin and gastrulate through the posterior streak. Molecular, genetic, and cell biologic analyses have demonstrated that precursor cells in the streak and during early migration still have the potential to differentiate into either the blood or the endothelial lineage.[10-15] Extraembryonic mesoderm (EEM) contains at least 2 additional tissue types, somatic and splanchnic EEM, which differentiate into smooth muscle and connective tissue and constitute dorsal and ventral linings of the extraembryonic coelom.[16,17] Blood and endothelial cells separate early from the splanchnic mesoderm as aggregates on its ventral side, variously called blood islands, angioblasts, or hemangioblasts.[18-20] These aggregates are referred to as blood islands in this work. In lateral EEM, blood islands (BIs) give rise to both blood and endothelial cells, whereas in medial EEM and the lateral plate, only endothelial cells are generated.[18,21,22] It is not well understood why some BIs give rise to both, whereas others give rise to only endothelial cells.

Chicken hemoglobins were used for initial descriptive and biochemical studies on primitive hematopoiesis[21,23] and for later investigations on transcriptional control.[24-26] More recent studies using different model organisms revealed a complex genetic network controlling primitive hematopoiesis.[3,10,13,15,27-29] Analyses focusing on signaling molecules indicated the involvement of the BMP,[30-32] VEGF,[33-36] WNT,[37,38] and Notch[39,40] pathways in various aspects of primitive hematopoiesis. The role of the fibroblast growth factor (FGF) pathway in this process is unclear. It is known to be important for mesoderm induction during gastrulation,[41-43] for dorsoventral patterning of the mesoderm,[44,45] and for mesoderm cell movement during and after gastrulation.[46,47] In addition, FGF can act as a potent inducer of neovascularization and neoangiogenesis[48,49] and plays a role in promoting proliferation and maintenance of hematopoietic progenitors and stem cells.[50] Its precise function in primitive hematopoiesis has been debated, with some evidence suggesting a positive regulatory role[51-53] and other evidence indicating a negative one.[54,55]

In this study, we investigate the function of the FGF pathway in chick primitive hematopoiesis. Globin mRNA expression is used as a sensitive readout for terminal differentiation. We focus our study on the differentiation step only, separating it from potential roles of the FGF pathway in earlier (induction, patterning, and migration) or later (proliferation and maturation) steps. We demonstrate that FGF signals can elicit strong inhibition of hemoglobin expression and up-regulate endothelial markers. Inhibition of the FGF signaling pathway results in ectopic blood formation and completely inhibits endothelial marker expression. Among 4 known FGFRs, we show that FGFR2 is the key receptor functioning in this process. These effects are achieved through crosstalk with the VEGF pathway yet can be attributed primarily to the FGF pathway. We also show that it acts in primitive hematopoiesis through Gata1 by modulating Gata1 expression level and activity.

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Materials and methods

Chick embryology

Fertilized hens' eggs were purchased from Shiroyama Farm (Kanagawa, Japan) and incubated at 38.5°C to the desired stages. For tissue graft, bead graft, chemical treatment, and electroporation experiments, we used the modified New culture method.[68] Differentiation of isolated streak tissue pieces was performed on vitelline membrane as in New culture but without a host embryo. For in situ hybridization and immunohistochemistry, standard published protocols were followed.[56] All in situ analyses were carried out with either digoxigenin- or fluorescein-labeled RNA probes and developed with alkaline phosphatase staining. All tissue sections shown were 10-μm paraffin sections. Electroporation was performed with Intracel electroporator (Intracel, Hertfordshire, United Kingdom) at the following setting: 5 volts, 3 pulses (50 ms with 250-ms interval). All image-acquisition equipment was purchased from Olympus (Tokyo, Japan). Whole-mount embryos were visualized in PBS solution with an Olympus SZX12 microscope using a DF PLAPO 1.2×/0.133 PF2 objective lens. Embryo sections were mounted with canada balsam and visualized with an Olympus BX51 microscope using Olympus UPlanSApo objective lenses (20×/0.75; 40×/0.9; 100×/1.40 oil-immersion). For both whole-mount embryos and embryo sections, an Olympus DP70 camera was used to capture images, and DP Controller 2.1 image software was used to acquire them. Final images were assembled with Photoshop 8.0 (Adobe Systems, San Jose, CA).

Chemicals

Proteins and chemical inhibitors were purchased from commercial sources. The following concentrations were used for bead soaking prior to graft: BMP2 (20 μg/mL, 355-BEC); BMP4 (20 μg/mL, 314-BP); Chordin (100 μg/mL, 758-CN); Noggin (12 μg/mL, 1967-NG); FGF4 (50 μg/mL, 235-F4); Nodal (12 μg/mL, 1315-ND); Dkk1 (25 μg/mL, 1096-DK); VEGF165 (10 μg/mL, 93-VE); Wnt3a (10 μg/mL, 1324-WN); Epo (25 μg/mL, 959-ME) (all from R&D Systems, Minneapolis, MN); FGF8 (50 μg/mL; Sigma, St Louis, MO; no. F6926); SU5402 (42 mM; Calbiochem, San Diego, CA; no. 572630). For chemical treatment, embryos were cultured with SU5402 (85 μM, 6 × 50% inhibitory concentration [IC_{50}]) or SU5416 (10 μM, 10 × IC_{50}, Calbiochem; no. 676487) dissolved in albumen, with less than 0.2% of final DMSO presence.

Molecular biology

DNA fragments for gene-specific in situ probes were generated by polymerase chain reaction (PCR), cloned in pGEM-T vectors, and confirmed by sequencing (ρ: nucleotides [nt's] 31-465 of CHEST324a16; ϵ nt's 55-475 of CHEST26i6; α^{π}: nt's 39-488 of CHEST23l23; α^{A} nt's 40-485 of CHEST973g24; *gata1*: nt's 577-1061 of NM_205464 with full-length cDNA kindly given by T. Evans (Albert Einstein College of Medicine, New York, NY); *ets1*: nt's 41-782 of 052594.1; *lmo2*: nt's 7-962 of NM204271; *vegfr2*: nt's 3477-5149 of AY382882; *Vecad*: nt's 642-2368 of AF522067; *fgfr1-4*: Shin et al[57]). Constitutively active FGFR2 (CA-FGFR2) was constructed based on studies by Webster and Donoghue.[58] A chick FGFR2 fragment lacking the extracellular and transmembrane domains was created by PCR (forward: 5′-ATCGATATGGGGAGCAGCAAGCCCAAGGACCCCAGCCAGCGCCC-TGACTTCAGCAGCCAGCCCGCTGTC-3′, and reverse: 5′-ACTAGTTCAT-GTTTTAACGCTCCCATTC-3′, with myristoylation sequence from c-Src: MGSSKSKPKDPSQR tagged to the 5′ end of the forward primer). An additional Lys661Glu mutation was introduced to create ligand-independent kinase activity. Final CA-FGFR2 was cloned into pCAGGS vector for efficient expression in vivo.[59] The activity was confirmed by double-phosphorylated ERK (dp-ERK) antibody staining. FGFR2-specific fluorescein-tagged morpholino (5′-CTAGAATGATTTACCTTCGGGT-TCC-3′) was designed against exon1-intron1 boundary and purchased from Gene Tools (Philomath, OR) together with standard control morpholino. Chick FGFR2 contains a 14.3-kb first intron, and blocking the splicing of first exon led to severe reduction of the mature transcript as revealed by in situ analysis with c-terminal–specific probe (Figure 5R-S). Morpholinos were electroporated at 3 mM and DNA constructs at 1 μg/μL.

Results

Expression of hemoglobin genes during chick primitive hematopoiesis

Based on pseudoperoxidase activity of the heme group, chemicals such as benzidine and diaminofluorene have traditionally been used for staining differentiated red blood cells.[8,60,61] As an alternative and more sensitive and specific method, we investigated mRNA expression patterns of hemoglobin genes during early chick development. Chick hemoglobin loci contain 4 beta-type (β^{H}, β^{A}, ρ, and ϵ) and 3 alpha-type (α^{π}, α^{A}, and α^{D}) globin genes. Five of them (α^{π}, α^{A}, α^{D}, ρ, and ϵ) are expressed in primitive blood cells.[21,23] We generated probes for α^{A}, α^{π}, ρ, and ϵ and performed in situ analysis with precirculation chick embryos (before Hamburger and Hamilton Stage 13 [HH13]). All 4 genes were strongly expressed in area vasculosa. Figure 1 shows the expression patterns at HH10 of α^{π} (A), α^{A} (B), ρ (C), and ϵ (D), respectively. As revealed in sections (Figure 1E-H), each gene was expressed in all differentiated blood cells. Since all 4 genes were detected at the early somite stage, we performed detailed analysis on hemoglobin ρ (ρ) expression (Figure 1I-M). The earliest expression was seen at HH6-7. At HH7, ρ can be readily detected in differentiating BIs (Figure 1J,N-P). Within individual BIs, a few inner cells were

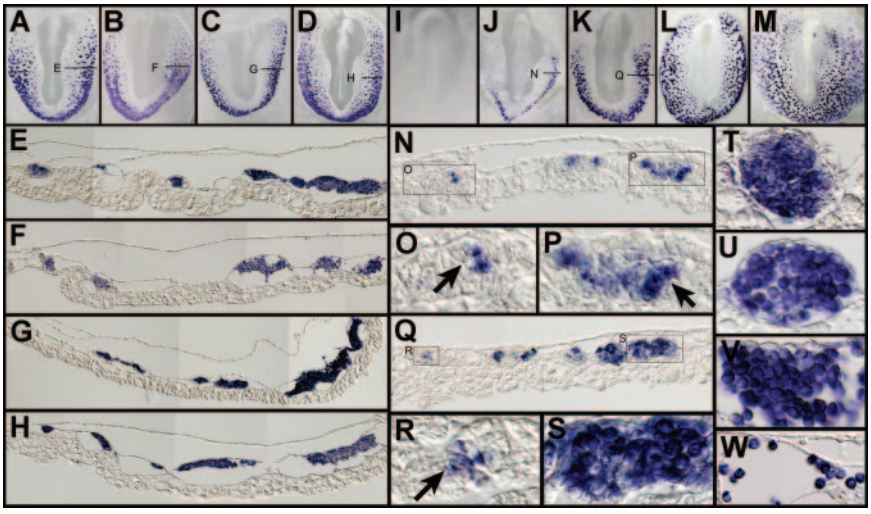

Figure 1. Hemoglobin expression during early development. (A-H) Expression at HH10 of α^{π} (A), α^{A} (B), ρ (C), and ϵ (D) globin transcripts and sections through indicated level of α^{π} (E), α^{A} (F), ρ (G), and ϵ (H). (I-W) Expression of ρ at HH5 (I), 7 (J), 8 (K), 10 (L), and 12 (M). Sections of HH7 (N-P) and HH8 (Q-S) embryos are shown as indicated. Arrows point to cells initiating expression within a BI. Representative BI morphology is shown for HH9 (T), 10 (U), 11 (V), and 12 (W).

observed to initiate ρ expression (Figure 1O), followed by more cells within a cluster (Figure 1P-S). Mature and laterally located BIs from HH8 onward contained all inner cells positive for ρ (ρ^+) surrounded by negative cells (Figure 1S-W). Morphologically distinct blood cells were visible from HH10 (Figure 1L-M,U-W). At the onset of circulation (HH12-13; Figure 1M,W), the majority of blood cells had lost contact with each other and with the endothelial wall, which was a prominent feature for ρ^+ cells up to HH10-11 (Figure 1U-V). The detection sensitivity with globin mRNA probes (presomite stage) is greater than with heme group–reacting compounds (4-5 somite stage), in agreement with biochemical studies on chicken globin proteins[23,62,63] and mouse globin genes.[64] Using ρ as a sensitive marker, we performed further experiments to investigate the regulation of primitive hematopoiesis.

Posterior streak mesoderm can differentiate into ρ-expressing blood cells in the absence of cell migration

Explant culture showed streak tissue, and dissociated streak cells are able to differentiate into blood cells in mice.[14] In chicks, dissociated posterior streak and early migratory mesoderm cells can form blood colonies in culture.[35] This was confirmed by streak tissue graft using ρ as a marker (Figure 2A-D). We separated HH4 streak into 3 pieces (anterior, middle, and posterior; Figure 2A), transplanted each piece to the extreme lateral region of the area opaca of same-stage host embryos, and cultured to HH10. The posterior streak piece can differentiate into ρ^+ cells with developmental timing similar to that of the host embryo (Figure 2D). Both the intensity of ρ expression and the spatial distribution of BIs were comparable to the host (Figure 2D). The middle piece can also differentiate, but to a lesser degree as predicted from fate map studies, into ρ^+ cells (Figure 2C). The anterior piece failed to generate any ρ^+ cell (Figure 2B) and instead differentiated into more axial structures with notochord and somites (data not shown). These data suggested that posterior streak mesoderm precursors contain intrinsic ability to undergo proper migration and differentiation. Since the host area opaca can provide a substrate for migration and possible inducing signals from the extraembryonic ectoderm and endoderm, we cultured posterior pieces directly onto the vitelline membrane (Figure 2E). The posterior half of the HH4 streak was further divided into anterior (third quarter) and posterior (fourth quarter) pieces. Explants were cultured until control embryo reached HH10 (Figure 2F). The ρ^+ cells can be seen in both the third and the fourth quarters (Figure 2G-H), with more ρ^+ cells and stronger expression in the fourth quarter (Figure 2G). In vitelline membrane culture, the cells failed to migrate and as a consequence the characteristic spatial distribution of BIs was lost.

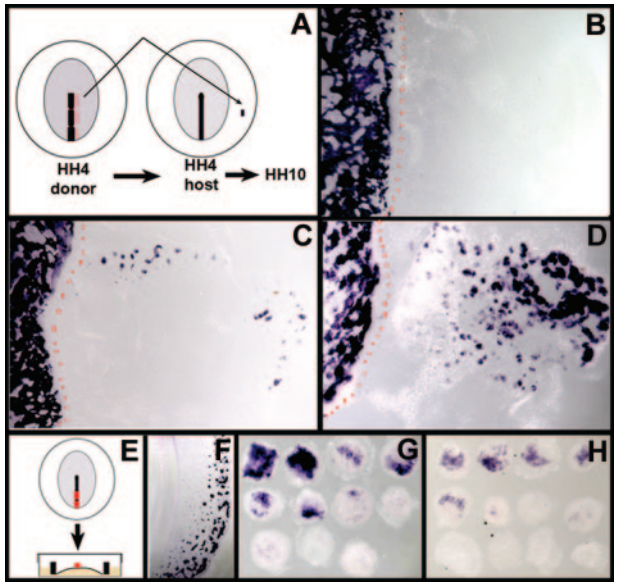

Figure 2. Differentiation potential of posterior streak mesoderm. (A-D) HH4 streak pieces are grafted to the lateral area opaca of host HH4 embryo and cultured to HH10 (A). Differentiation is assayed by ρ expression for anterior (B), middle (C), and posterior (D) pieces. Red dotted lines indicate the limit of host ρ expression. (E-H) HH4 streak pieces are cultured on vitelline membrane (E). Differentiation is assayed by ρ when control reaches HH10 (F) for posterior (G) and third (H) quarters.

Differentiation of primitive blood cells is inhibited by FGF signaling

The previous experiment suggested that signals regulating precursor cell migration can be separated from those regulating differentiation. In chicks, these 2 processes can also be separated temporally. Differentiation of primitive blood as marked by ρ expression is initiated at about HH7 (Figure 1J). Migration of EEM cells starts before HH4 and the majority of them reach their destinations by HH7. We focused on the step of hemoglobin gene transcription as a marker for the initiation of terminal differentiation. We first looked at the ability of molecules involved in various signaling pathways to affect the endogenous ρ expression. Beads soaked with secreted molecules were grafted either within or outside the future ρ domain (Figure 3A). HH6-7 embryos were used to minimize possible secondary effects due to their influence on cell migration. Results are summarized in Table 1. We found no single factor capable of inducing ρ ectopically, whereas only FGF beads (FGF4, 40/53, 75%; FGF8, 15/29, 52%) showed remarkable inhibition of endogenous ρ expression. As shown in Figure 3B and D, control beads had no effect on ρ expression, whereas FGF4 beads exerted strong inhibition (Figure 3C,E). A similar effect was seen with FGF8 beads (Figure 3G). The inhibitory effect of FGFs was not due to their ability to repel mesoderm cells, as sections revealed large numbers of ρ^- mesoderm cells near beads (Figure 3F). As summarized in Table 1 and shown in Figure 3H (BMP7) and Figure 3I (Noggin), we did not observe a similar prominent effect with other factors tested. We then asked whether the cells that failed to adopt blood fate can differentiate into endothelial cells. The effect of FGF bead graft was analyzed with 2 endothelial markers, Vegfr2 and Ets1. Both have been described to specifically mark endothelial cells at HH10.[65,66] We generated RNA probes for *Vegfr2* and *Ets1* and extended the expression study by focusing on EEM differentiation (Figure 3J-L, *Vegfr2*; Figure 3O-Q, *Ets1*). When FGF4 beads were grafted from HH6-7 and analyzed at HH10, strong upregulation of both transcripts was observed in cells adjacent to grafted beads (Figure 3M-N,R-S).

Inhibition of the FGF signaling pathway leads to ectopic blood cell differentiation

Our graft analysis suggested that FGF mediates the choice between blood and endothelial fates. Activation of FGF signaling inhibits blood differentiation and promotes endothelial formation. We then asked whether more blood cells can be generated when this pathway is inhibited. The ρ expression was analyzed in embryos treated with SU5402, a small molecule inhibitor specific for FGFR class RTK proteins.[67] We applied SU5402 starting from HH6-7, when the induction and extensive migration of mesoderm cells has largely been accomplished. Beads soaked in control DMSO had no effect (Figure 4A,C). Grafting of SU5402 beads resulted in large numbers of blood cells in their vicinity (Figure 4B,D-E). In particular, we saw blood cells being induced in medial regions

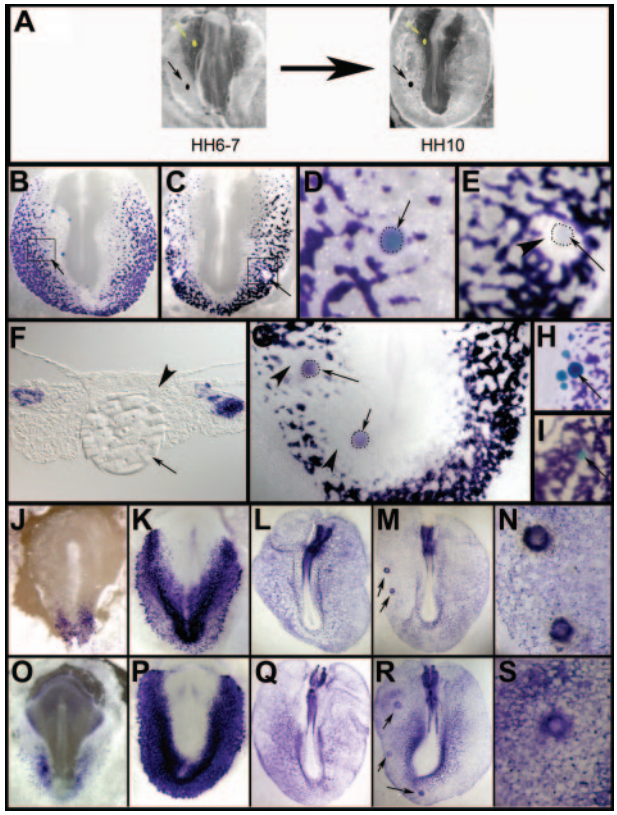

Figure 3. FGF inhibits ρ and enhances endothelial marker expression. (A) HH6-7 embryos are grafted with factor-soaked beads either within (black) or outside (yellow) the future ρ-expressing domain and cultured to HH10. (B-I) FGF4 beads inhibit ρ expression (C,E) and control beads have no effect (B,D). Boxed areas in panels B and C are magnified in panels D and E, respectively. Arrows point to grafted beads and arrowheads show the repression. F (section of E) shows presence of ρ^- cells around the bead. A similar effect is seen with FGF8 (G) but not BMP7 (H) or Noggin (I). (J-N) Expression of *Vegfr2* at HH4 (J), 6 (K), and 10 (L). FGF4 beads enhance its expression (M-N). Arrows point to grafted beads. (O-Q) Normal expression of *Ets1* at HH4 (O), 6 (P), and 10 (Q). FGF4 beads enhance its expression (R-S).

(Figure 4F), which was not observed in normal embryos or in graft experiments with secreted factors (Table 1). This prompted us to investigate the effect of SU5402 in more detail.

To confirm this effect, we cultured whole embryos with SU5402 dissolved in the albumen in the modified New culture setting.[68] As mentioned earlier, we chose to test the effect with embryos from HH6-7 onward. Earlier treatment resulted in malformations in the embryo proper as well as a greatly reduced area vasculosa, likely as a consequence of mesoderm induction and migration defects (data not shown). No obvious defect or delay in embryonic development was seen with the concentration used (DMSO: Figure 4G,I; SU5402: Figure 4H,J; *Paraxis* staining: I-J insets). Control-treated embryos showed normal ρ expression (Figure 4G,K). SU5402 caused dramatic medial expansion of the ρ-expressing domain (Figure 4H,L). Sections of treated embryos revealed ρ^+ cell clusters below the splanchnic mesoderm in the lateral plate (Figure 4Ni-v), far more medial than in control embryos (Figure 4M). In the endogenous hemogenic domain, the intensity of ρ expression and the pattern of BI distribution were not greatly affected (Figure 4G-H). In most cases, we saw ρ^+ clusters still surrounded by ρ^- cells with endothelial cell morphology (Figure 4Ni-v).

Since endothelial-like cells were still observed in treated embryos, we next investigated whether FGF activity is required for endothelial marker expression. We compared the effect of SU5402 with DMSO control using endothelial markers *Ets1*, *Vegfr2*, and *Vecad*. Expression of all 3 was potently inhibited by SU5402 (compare Figure 4Oi, Pi, and Qi with Figure 4Oii, Pii, and Qii), indicating that endothelial development was greatly compromised with the inhibition of FGFR activity. This was further confirmed by analyzing the effect on *Lmo2*. Analysis of *Lmo2* RNA expression (Figure 4Ri-iii) showed similar patterns as previously described.[8] At HH10, *Lmo2* was expressed in both blood and endothelial cells (Figure 4S,Ui-ii,Wi-ii) in control embryos. With SU5402 treatment, *Lmo2* expression in blood cells was not affected, whereas expression in endothelial cells was completely abolished (Figure 4T,Vi-ii,Xi-ii). In agreement with these observations, no vascular network or blood flow was observed (data not shown) when treated embryos were cultured until controls reached circulation stage (> HH13). These data indicated that inhibition of the FGF pathway severely blocked endothelial differentiation.

FGFR2 is a key negative regulator of primitive blood differentiation

SU5402 can inhibit tyrosine kinase activity of all FGF receptors. We performed further experiments to investigate which FGF receptor(s) may be mediating our observed effects. As in mammals, chicks have 4 FGFRs. The expression patterns of *FGFR1*, *2*, and *3* have been described in chicks[69] but not in the context of EEM differentiation. We first carried out detailed in situ analyses for *FGFR1-4* from HH4 to HH12, with special attention to their possible involvement in blood and endothelial differentiation. Among them, we found *FGFR2* (Figure 5Bi-vi) to be the likely candidate in mediating these observed effects. *FGFR2* was expressed in the endothelial cells in the extraembryonic region (Figure 5Bvi,F), whereas primitive blood cells were devoid of its transcripts (Figure 5F inset). The splanchnic mesoderm in the lateral plate and medial EEM was also positive for *FGFR2* (Figure 5F). *FGFR1* transcripts were not detected in EEM in either blood or endothelial cells (Figure 5Avi,E), although they were present in both somitic and lateral plate mesoderm (Figure 5E). *FGFR3* (Figure 5Ci) was expressed at HH4 in all ectoderm cells except those adjacent to the streak. At HH10, nascent somites and neighboring intermediate mesoderm were strongly positive (Figure 5Ciii,G). In lateral plate and medial EEM, signals can be detected in somatic and splanchnic mesoderm but not in endothelial cells or blood cells at any axial level (Figure 5G). Expression patterns of *FGFR1* and *3* indicated they may also play a role in restricting primitive blood differentiation to more lateral regions. For *FGFR4* (Figure 5Di-iii), we did not observe obvious expression in the extraembryonic region at any stage. *FGFR4* signals were seen in the neural plate from HH4 (Figure 5Di-iii).

Table 1. Effect of signal molecules on ρ expression

Factor	Induction (%)	Inhibition, no. beads with effect/ no. beads tested (%)
BMP2	0/13 (0)	0/9 (0)
BMP7	0/16 (0)	0/12 (0)
Chordin	0/37 (0)	1/12 (8)
Noggin	0/28 (0)	0/20 (0)
FGF4	0/62 (0)	40/53 (75)
FGF8	0/40 (0)	15/29 (52)
Nodal	0/24 (0)	0/18 (0)
Dkk1	0/29 (0)	0/22 (0)
VEGF165	0/27 (0)	0/15 (0)
Wnt3A	1/52 (2)	0/28 (0)
Epo	0/6 (0)	0/4 (0)

Beads are grafted as indicated (Figure 3A). The effect of each factor is assayed by its ability to either induce (in ectopic or endogenous domain) or inhibit (endogenous) ρ expression in adjacent cells.

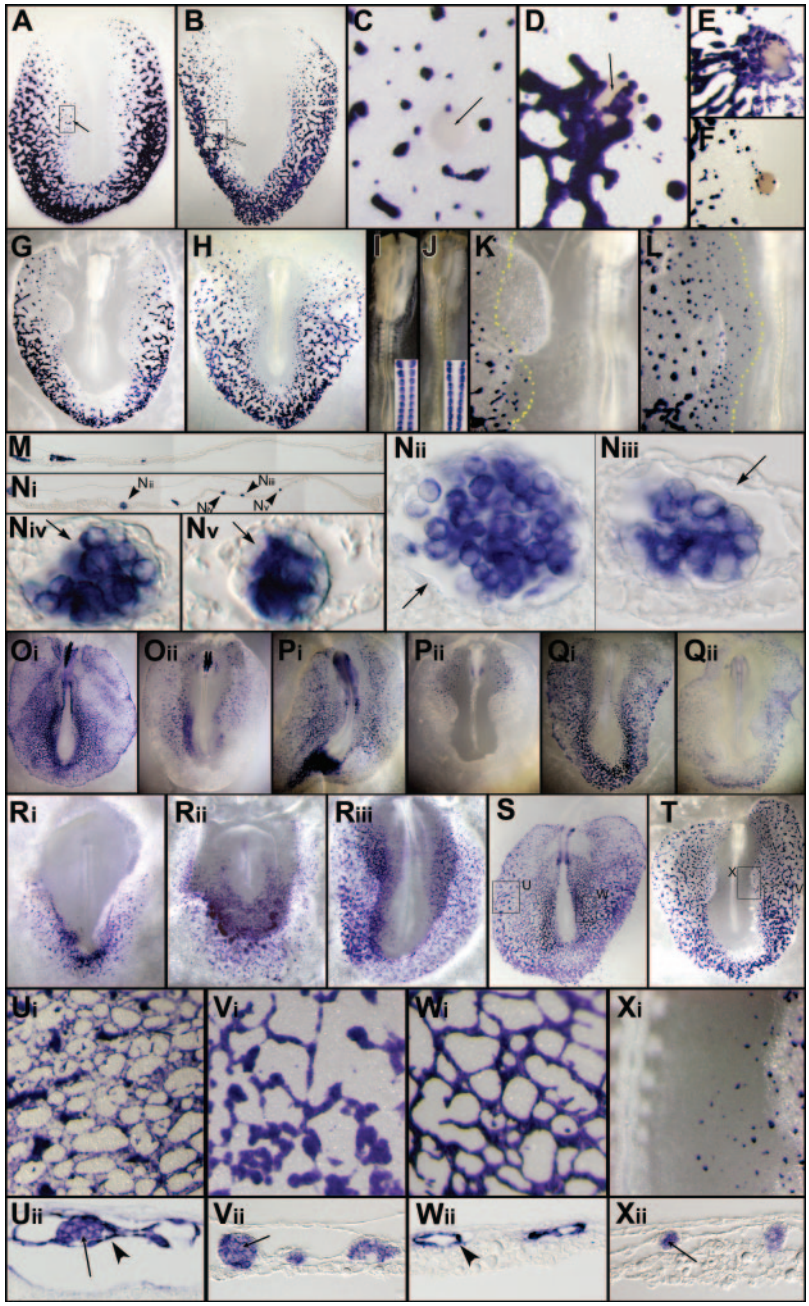

Figure 4. Inhibition of FGFR causes ectopic blood formation and down-regulation of endothelial markers. (A-F) Control DMSO bead has no effect (A,C) and SU5402 bead has excessive surrounding ρ^+ clusters (B,D). Panels E and F show additional examples of blood induction by SU5402 beads. Arrows point to grafted beads. (G-N) Whole embryo culture in the presence of DMSO (G,I) or SU5402 (H,J) does not affect general growth (I-J, insets: *Paraxis* in situ). SU5402 drastically expands medial limit of blood formation (H,L) compared with DMSO (G,K), as outlined by yellow dotted line. Sections of panels L (Ni-v) and K (M) show clear induction of ectopic ρ^+ clusters (arrowhead). Panels Nii-v show presence of endothelial-like cells (arrows) surrounding induced (Niii-v) and endogenous (Nii) clusters. (O-X) Down-regulation of *Ets1* (Oii), *Vegfr2* (Pii), and *Vecad* (Qii) by SU5402 compared with respective controls (Oi, Pi, and Qi). *Lmo2* expression is shown for HH4 (Ri), 5 (Rii), and 8 (Riii). At HH10 (S), it is expressed in both endothelial and blood cells laterally (Ui and section in Uii) and only in endothelial cells medially (Wi-ii). SU5402 abolishes endothelial expression of *Lmo2* while maintaining blood expression laterally (Vi-ii). Ectopic blood clusters in the medial region also express *Lmo2* (Xi-ii) but only in inner cells like ρ globin (Niii-v). Arrows indicate blood expression; arrowheads, endothelial expression.

Since *FGFR2* was expressed in endothelial cells and absent in primitive blood cells, we next tested whether activation of the FGF pathway mediated through FGFR2 can influence cell fate. For this purpose, we constructed an expression vector for the constitutive active form of FGFR2 (CA-FGFR2; see "Molecular biology"). A DNA construct expressing CA-FGFR2 together with a reporter GFP gene was cointroduced into posterior streak mesoderm at HH4 by electroporation. Control embryos were electroporated with the GFP construct alone. We scored the distribution of CA-FGFR2–positive cells at HH10 in comparison with control embryos. To exclude a possible secondary effect due to migratory defect, only cells that had reached the endogenous ρ^+ domain were scored. In control embryos (Figure 5H-I,L; electroporated cells, brown; blood cells, blue), GFP-positive cells were distributed among different lineages. For our analysis, we grouped the location of positive cells into 3 categories: primitive blood (ρ^+), endothelial (cells with flattened morphology surrounding ρ^+ cluster), and others (all other positive cells). Among 1571 cells scored (Table 2), 36.0% control GFP-positive cells (565) became blood cells, whereas 29.9% (470) went to endothelial lineage. The remaining 34.1% (536) contributed to other cell types. Among the CA-FGFR2–positive cells, distribution ratios were greatly altered (Figure 5J-K,M). In 1703 total cells scored (Table 2), we found fewer than 50 (2.8%) CA-FGFR2–positive cells differentiated into primitive blood, whereas the vast majority (1366, 80.2%) became endothelial cells.

To confirm these effects, we performed a knockdown experiment using *FGFR2*-specific splice-block morpholino (see "Molecular biology"). Antimorpholino staining did not yield sharp cell boundaries, making statistical analysis difficult. Nevertheless, the general distribution patterns of morpholino-positive cells were easily recognizable. Cells with control morpholino were distributed among different lineages (Figure 5N,P; morpholino cells, blue; blood cells, brown), similar to control GFP cells. *FGFR2* morpholino cells showed a distribution pattern opposite

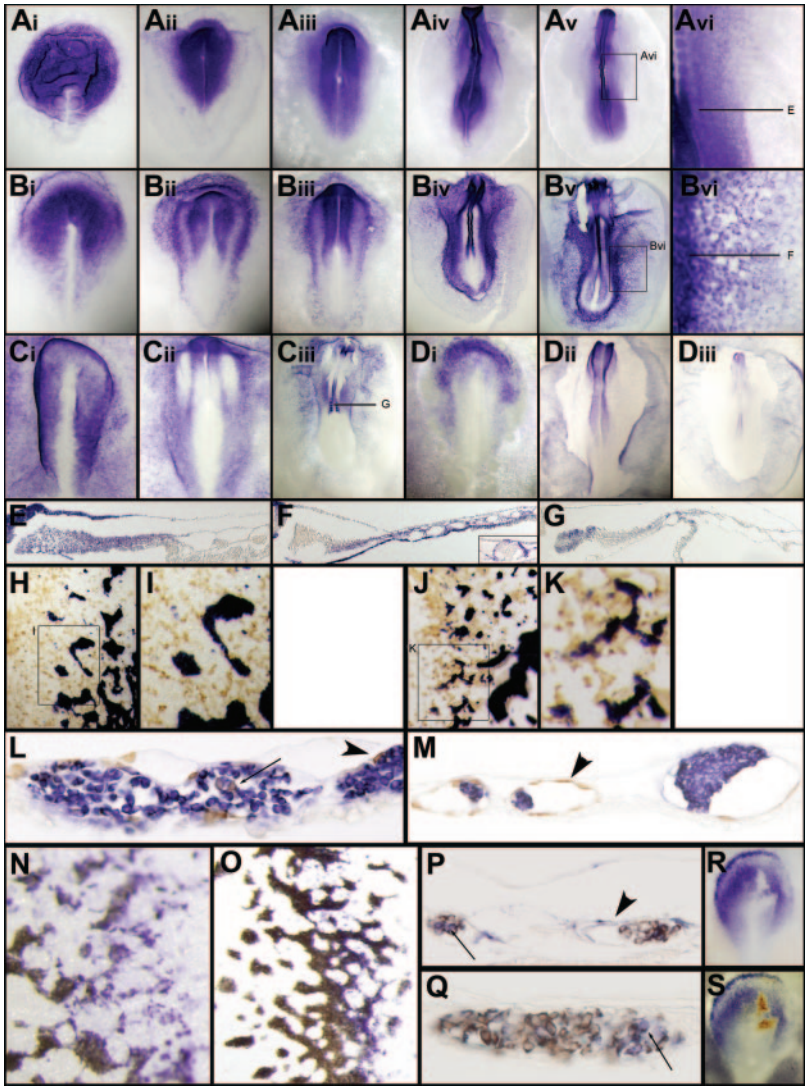

Figure 5. FGFR2 is the key mediator. (A) *FGFR1* at HH4 (Ai), 5 (Aii), 6 (Aiii), 8 (Aiv), and 10 (Av). (B) *FGFR2* at HH4 (Bi), 6 (Bii), 7 (Biii), 9 (Biv), and 10 (Bv). (C) *FGFR3* at HH4 (Ci), 6 (Cii), and 10 (Ciii). (D) *FGFR4* at HH4 (Di), 7 (Dii), and 10 (Diii). (E-G) Sections of *FGFR1* (E), *FGFR2* (F), and *FGFR3* (G). Inset in panel F shows *FGFR2* in endothelial but not blood cells. (H-M) Brown indicates electroporated cells; blue, blood cells stained with ρ; arrowheads, endothelial distribution; arrows, blood distribution. CA-FGFR2 cells contribute mainly to endothelium (J,K,M), whereas control cells are seen among different lineages (H-I,L). See Table 2. (N-S) Blue indicates morpholino electroporated cells; brown, blood cells stained with ρ. Knockdown of FGFR2 with a splice block morpholino leads to blood localization (O,Q), and control morpholino cells are distributed among different lineages (N,P). Arrowheads indicate endothelial distribution; arrows, blood distribution. *FGFR2* splice morpholino blocks *FGFR2* mature transcript accumulation in neural ectoderm at HH4 (compare R with Bi). (S) Brown indicates morpholino-positive cells and blue *FGFR2* expression (R shows embryo before morpholino staining).

to that of CA-FGFR2 and contributed primarily to blood cells (Figure 5O,Q). The efficacy of *FGFR2* morpholino was confirmed by down-regulation of the mature *FGFR2* transcript in neural ectoderm at HH4 (Figure 5R-S). These observations demonstrated that FGFR2 plays a critical role in preventing primitive blood differentiation and promoting endothelial formation, consistent with phenotypical analysis of *fgfr2* null mice.[70]

Relationship with the VEGFR signaling pathway

Some of our observed effects may be explained by proposed roles of VEGFR signaling during primitive hematopoiesis. *VEGFR2* is initially expressed in blood and endothelial precursors and *vegfr2* null embryos die lacking both lineages.[34,35] In our analyses, FGF can induce the *VEGFR2* expression and SU5402 resulted in its suppression, suggesting some effects were mediated via VEGFR. In bead graft with VEGF165 protein, however, we did not see inhibition of ρ (Table 1). Since SU5402 can block kinase activity of both FGFRs and VEGFRs, we used a VEGFR-specific inhibitor SU5416[71] to assess its contribution. As a control, we incubated 15–hours after fertilization (hpf) zebrafish embryos with SU5416 until 30 hpf and observed enlarged pericardial cavity and failure of the establishment of circulation (data not shown), similar to what has been previously described.[72] We then cultured chick embryos from HH6-7 in the presence of SU5416. Unlike in treatment with SU5402, no obvious effect on ρ expression was observed with SU5416 (Figure 6A,E), nor did we observe any remarkable difference with *Lmo2* (Figure 6D,H). Interestingly, we observed a slight up-regulation of endothelial marker *Ets1* and *Vegfr2* with SU5416 treatment (Figure 6B-C, F-G), contrary to strong inhibition seen with SU5402. This evidence suggested that FGFR plays important roles during hematopoietic and endothelial differentiation, distinct from those mediated via VEGFR.

Table 2. Statistical distributions

	Control, no. cells (%)	CA-FGFR2, no. cells (%)
Total	1571 (100)	1703 (100)
Blood	565 (36.0)	47 (2.8)
Endothelial	470 (29.9)	1366 (80.2)
Others	536 (34.1)	290 (17.0)

FGFR activity modulates expression level of hematopoietic marker Gata1

Primitive blood progenitors express earlier lineage markers Gata2, Scl, and Gata1 before hemoglobin expression, indicating that events to separate the endothelial and hematopoietic lineages take place prior to terminal differentiation. Gata1 has been suggested as a definitive marker for the hematopoietic lineage, before the

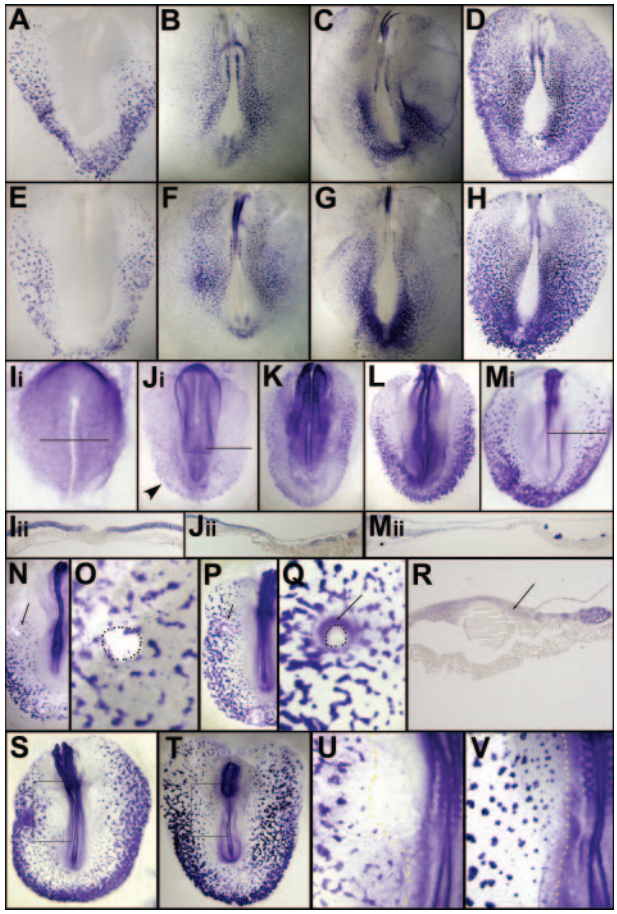

Figure 6. Contributions of VEGFR and hematopoietic marker Gata1 in FGFR-mediated effects. (A-H) Specific inhibition of VEGFR pathway by SU5416 does not affect ρ (E) or *Lmo2* (H) expression and slightly up-regulates *Vegfr2* (F) and *Ets1* (G) compared with their respective controls (A,D,B,C). (I-M) Gata1 expression at HH4 (Ii-ii), 6 (Ji-ii), 7 (K), 8 (L), and 10 (Mi-ii). Arrowhead points to weak but broad expression in EEM. Sections of panels Ii, Ji, and Mi at indicated levels are shown in Iii, Jii, and Mii, respectively. (N-R) Control bead does not affect *Gata1* expression (N-O), which at HH10 is up-regulated in blood and absent in nonblood cells (Mi-ii). FGF4 bead maintains low-level expression of *Gata1* in adjacent cells (P-Q and section in R). These cells are completely negative for ρ (Figure 3F). Dotted line marks bead and arrow points to weak expression. (S-V) SU5402 treatment of whole embryo results in up-regulated and expanded *Gata1* expression (T,V) compared with control (S,U), similar to the effect on ρ globin (Figure 4L).

expression of terminal differentiation genes. To investigate whether Gata1 plays a role in FGF-mediated inhibition of terminal differentiation, we first analyzed *Gata1* expression carefully during early development (Figure 6Ii-ii,Ji-ii,K-L,Mi-ii). The earliest expression of *Gata1* in ventral mesoderm was detected at HH5-6 (Figure 6Ji-ii), which covered the entire EEM area. Compared with globin genes, *Gata1* was expressed both earlier and in a wider region. Up-regulation of *Gata1* expression was observed from HH6-7 in future globin-positive cells (Figure 6Ji-ii,K). This suggested that the expression or activity level of Gata1 is critical for its role as terminal activator. We then investigated the effect of FGF bead graft on *Gata1* expression. Control beads did not affect *Gata1* expression (0/40; Figure 6N-O), which at HH10 is up-regulated in blood cells and down-regulated in nonblood cells. In cells adjacent to FGF4 beads, *Gata1* can be detected at low levels (Figure 6P-Q), which were weaker than in blood cells (Figure 6R) but comparable to the level seen at HH5-6 in the EEM (48/60). These Gata1-expressing cells adjacent to the graft beads were completely devoid of globin expression (Figure 3E-F). This suggested that FGF activity can block the up-regulation of Gata1, which is critical in determining whether to initiate the terminal differentiation program. Supporting this hypothesis, we observed up-regulated *Gata1* expression and expanded *Gata1*-positive blood island clusters in medial EEM and lateral plate mesoderm in SU5402-treated embryos (compare Figure 6T,V with Figure 6S,U), similar to the effect on ρ expression (Figure 4L).

Discussion

The early phase of primitive hematopoiesis is dedicated to generating erythrocytes, providing the sole source for circulating erythrocytes until embryonic day 12 (E12) in mice[73] and day 10 in chicks.[74] Hemoglobin serves as a critical marker for its terminal differentiation. In this study, we report that 4 of the globin genes are expressed from early stages of primitive hematopoiesis. Each globin is expressed in all blood cells, suggesting no allelic exclusion of embryonic globins within single blood cells. The initiation of ρ expression always takes place in a couple of cells followed by expression in surrounding cells, suggesting a stepwise terminal differentiation from precursor cells.

At HH7, ρ^+ cells are limited to the posterior and outer edge of area vasculosa. The majority of EEM cells, having migrated extensively from their origin in the streak, are still ρ^- at this stage. Our data suggest that migration and differentiation events are distinctly regulated. Nevertheless, early steps of differentiation marked by Gata2, Scl, and Gata1 for hematopoiesis and Vegfr2, Est1, and Vecad for vasculogenesis take place during migration, indicating differentiation itself is a multistep process. Aside from Gata1, however, these markers are initially expressed in both lineages and only later resolve to a specific one. Other described terminal transcriptional regulators for hematopoiesis, such as EKLF, have their onset of expression prior to the up-regulation of Gata1. The delayed expression of hemoglobin indicates a tight regulation of the onset of terminal differentiation. Further investigation will be needed to understand the precise mechanism of this delay.

We found that none of the factors tested can ectopically induce hemoglobin expression and only FGFs cause inhibition. Since we have not exhausted the list or tested combinations, we do not rule out the involvement of pathways that did not elicit an effect. The effects we observe with FGF are achieved through crosstalk with the VEGFR pathway, as Vegfr2 is up-regulated by high FGFR activity and down-regulated by its inhibition. However, our data suggest the FGF pathway plays a distinct role and is the primary mediator in this process, as specific inhibition of VEGFR did not cause similar phenotypes. Crosstalk between these 2 pathways as well as their distinct roles have been well documented in other systems.[48,75-77]

Our evidence suggests FGF acts by modulating Gata1 expression level or activity. Gata1 expression is considered the last step before terminal differentiation. However, it is expressed both earlier and more widely than globins, suggesting the requirement of intermediate steps. A recent study on the global gene regulation profile of *gata1* null erythroblasts demonstrated a significant delay of globin gene transcription after reintroduction of Gata1, suggesting the involvement of additional signals.[78] Up-regulation of Gata1 expression, however, coincides with its purported role as a terminal activator, suggesting the importance of Gata1 level in terminal differentiation. A reduction of 4- to 5-fold in Gata1 expression was shown to markedly impair erythroid cell maturation in mice.[79] In our analyses, FGF strongly inhibits globin gene expression, although the cells surrounding FGF beads still express weak but detectable levels of Gata1. The up-regulation of Gata1 expression, however, is inhibited by high FGF activity, and inhibition of FGFR activity prominently up-regulates Gata1 expression in ectopically induced blood cells. Whether this is achieved by a direct or indirect effect of the FGF signaling pathway will require further investigation.

In chicks, prestreak-stage marginal zone tissue can be induced to form blood by bFGF.[51] This is likely due to FGF's role in early mesoderm induction and reflects different FGF functions at different stages of early development. A similar report with dissociated quail prestreak-stage epiblast cells showed that FGF can induce both blood and endothelial cells in culture.[80] In *Xenopus,* midblastula animal cap cells can be induced by a combination of FGF and BMP to form Gata1-positive cells.[52] Another *Xenopus* study reported that FGF inhibits blood island formation and dominant-negative FGFR expands blood islands to the lateral plate mesoderm.[54] Although our analyses tend to agree with the latter observation, and beads soaked with both BMP and FGF caused similar inhibition of ρ expression as with FGF alone (F.N. and G.S., unpublished data, 2006), these effects in *Xenopus* cannot be easily compared with ours. The FGF pathway is well documented to play pleiotropic roles in multiple early developmental processes and for this reason we have chosen to study only the terminal differentiation step within a defined developmental window. Our analyses, however, support studies on cultured progenitor cells. bFGF was reported to maintain the immature state of primitive blood progenitors while blocking final differentiation.[55] Removal of FGF signaling effectively promoted final maturation.[55] Similarly, FGF activity was recently shown to block the final maturation of presomitic mesoderm (PSM) cells during somitogenesis.[81] In addition, our study provides strong evidence that FGFR2 is the main mediator in this process. It is premature, however, to hypothesize which ligand/receptor pair may be involved, as there are at least 22 FGF ligands and 2 FGFR2 isoforms.[82] FGF4 and 8 used in this study were described to be expressed medially in mesoderm precursor cells[83,84] and possibly create an FGFR2 activity gradient along the medio-lateral axis to control the timing of differentiation. Progressive decay of *FGF8* mRNA can serve as a key mechanism for creating a morphogenetic gradient of FGF8 protein.[83] Both FGFs were shown to bind and activate FGFR2 effectively.[70] In addition to these 2 FGFs, other FGF transcripts (3, 10, 12, 13, 14, and 19) were reported to be expressed in ventral mesoderm precursors.[84,85] None has been studied in detail in the context of EEM differentiation. Through gene array analysis, we have identified 5 additional FGFs expressed medially and at least 3 FGFs expressed more laterally in differentiating EEM cells (M.S., F.N., and G.S.; unpublished data, 2006). Additional complexity was highlighted by specific roles played by different isoforms.[86] Comprehensive analysis of dynamic expression patterns of different FGF molecules and their isoforms during EEM differentiation is currently being carried out.

Blood and endothelial differentiation are developmentally linked. The existence of bipotential precursor cells has been shown with in vitro differentiation assays. Whether bipotential precursor cells exist at the single-cell level remains to be shown in vivo. Within a population of undifferentiated blood island cells, the bias toward a particular lineage, the restriction of differentiation potentials, and the terminal differentiation happen progressively and may require a delicate balance of inputs from transcriptional regulators and signaling molecules. A surprising find in our studies is the tightness of this link. In our SU5402-treated embryos, we found strong inhibition of all endothelial markers tested, yet endothelial-like cells still surround blood cells. However, they are not functional endothelial cells and fail to establish a proper vascular network later in development. In medial regions where ectopic blood cells are induced, globin-negative cells with endothelial morphology are still observed within the same BI, no matter how few cells the BI contains. These observations suggest that primitive hematopoiesis is tightly regulated to occur in conjunction with endothelial differentiation, possibly controlled by yet unknown mechanisms to ensure that all blood cells generated are properly connected to the circulatory system.

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