**Cbfa2 is required for the formation of intra-aortic hematopoietic clusters**

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**SUMMARY**

**Cbfa2** (*AML1*) encodes the DNA-binding subunit of a transcription factor in the small family of core-binding factors (CBFs). **Cbfa2** is required for the differentiation of all definitive hematopoietic cells, but not for primitive erythropoiesis. Here we show that **Cbfa2** is expressed in definitive hematopoietic progenitor cells, and in endothelial cells in sites from which these hematopoietic progenitors are thought to emerge. Endothelial cells expressing **Cbfa2** are in the yolk sac, the vitelline and umbilical arteries, and in the ventral aspect of the dorsal aorta in the aorta/genital ridge/mesonephros (AGM) region. Endothelial cells lining the dorsal aspect of the aorta, and elsewhere in the embryo, do not express **Cbfa2**. **Cbfa2** appears to be required for maintenance of **Cbfa2** expression in the endothelium, and for the formation of intra-aortic hematopoietic clusters from the endothelium.

Key words: CBF, AML1, hematopoietic stem cells

**INTRODUCTION**

Hematopoietic cells differentiate from mesoderm during embryogenesis, in close association with endothelial cells. In the mouse, the first blood cells (primitive erythrocytes) and endothelial cells differentiate from mesodermal masses in the yolk sac at about 7.5 days post coitus (dpc) (Haar and Ackerman, 1971; Moore and Metcalf, 1970). The concurrent development of both lineages led to the hypothesis, many years ago, that endothelial cells and hematopoietic cells share a common precursor called the hemangioblast (Murray, 1932; Sabin, 1920). A common origin of both lineages is further supported by recent molecular and genetic analyses. For example, several cell surface markers are common to endothelial cells and hematopoietic progenitors, including CD34, Flk-1 and TIE2 (Augustin et al., 1994; Takakura et al., 1998). In addition, cells of both lineages can be cultured from mouse embryonic stem cells from a common, transient progenitor (Choi et al., 1998; Nishikawa et al., 1998a). Mutations in several genes affect both hematopoietic and endothelial cell development. **Flk1** is required for the development of primitive and definitive hematopoietic cells and endothelial cells in the embryo, and is thought to be required at the level of the hemangioblast (Shalaby et al., 1997, 1995). Disruption of **Scl** disrupts primitive and definitive hematopoiesis, and perturbs angiogenesis in the yolk sac, but does not impair the initial formation of endothelial cells (Robb et al., 1995; Shivdasani et al., 1995; Visvader et al., 1998). The **cloche** gene is also required for normal blood and endothelial cell development (Stainier et al., 1995). The **Tie2** gene is required for normal vascular network formation, and for definitive hematopoiesis (Dumont et al., 1994; Sato et al., 1995; Takakura et al., 1998).

Definitive hematopoietic progenitors and stem cells have been found in several distinct sites in the embryo, including the yolk sac (Moore and Metcalf, 1970; Toles et al., 1989; Weissman et al., 1977), the umbilical and vitelline arteries, the para-aortic splanchnopleure and the aorta/genital ridge/mesonephros (AGM) region (Dieterlen-Liévre and Martin, 1981; Eren et al., 1987; Godin et al., 1995, 1993; Medvinsky and Dzierzak, 1996; Medvinsky et al., 1993; Müller et al., 1994; Ogawa et al., 1988; Tavian et al., 1996; Yoder et al., 1997). Some of these hematopoietic cells have been found in close association with endothelium. In humans, chicks and mice, clusters of hematopoietic cells were found associated with the endothelium on the ventral surface (floor) of the dorsal aorta (Dieterlen-Liévre and Martin, 1981; Garcia-Porrero et al., 1995; Tavian et al., 1996). In the mouse, the clusters are found at the developmental time when long-term repopulating hematopoietic stem cells (LTR-HSCs) appear in the AGM region (Medvinsky and Dzierzak, 1996). Histological pictures have also shown clusters of hematopoietic cells budding from the endothelium of the vitelline and umbilical arteries in 9.5 dpc–11.5 dpc mouse embryos (Garcia-Porrero et al., 1995; Wood et al., 1997). Grafting experiments in chicks suggest that endothelial cells and intra-aortic hematopoietic clusters that populate the floor of the dorsal aorta share a common hemangioblast precursor in the splanchnopleural mesoderm (Pardanaud et al., 1996). More recent experiments suggest that definitive hematopoietic cells differentiate directly from an
endothelial precursor. For example, Nishikawa et al. (1998b) showed that lymphoid cells could be cultured in vitro from VE-cadherin-positive endothelial cells isolated from the yolk sac and caudal region of mouse embryos. Lineage tracing experiments demonstrated that endothelial cells in the chick aorta marked with low-density lipoproteins (LDL) coupled to DiI gave rise one day later to LDL-marked hematopoietic clusters on the floor of the aorta (Jaffredo et al., 1998).

Cbfa2 and Cbfb encode two subunits of a core-binding factor (CBF) that is required at an early stage in definitive hematopoiesis. Cbfa2 encodes a DNA-binding CBFα subunit, and Cbfb encodes the non-DNA binding CBFβ subunit. Homozygous disruption of either Cbfa2 or Cbfb severely impairs definitive hematopoiesis, but does not affect primitive erythropoiesis or development of the yolk sac vasculature (Niki et al., 1997; Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996a,b). Differentiation of all definitive hematopoietic lineages is affected, suggesting that Cbfa2 and Cbfb are required at the level of definitive hematopoietic progenitors and/or stem cells.

To determine precisely when during definitive hematopoiesis Cbfa2 and Cbfb are required, we identified cells expressing Cbfa2 in developing mouse embryos, and examined the fate of these cells in Cbfa2-deficient embryos. We find that Cbfa2 expression marks a transient population of endothelial cells populating the floor of the dorsal aorta in the AGM region, as well as endothelial cells in other sites where definitive hematopoietic cells are thought to emerge. Intra-aortic hematopoietic clusters in the vitelline and umbilical arteries, and on the floor of the dorsal aorta are absent in Cbfa2-deficient mice. We hypothesize that Cbfa2 is required for the ‘budding’ of hematopoietic cells from hemogenic endothelium at these sites. Cbfa2 also appears to be required for maintenance of Cbfa2 expression in endothelial cells.

MATERIALS AND METHODS

Recombination constructs and targeting of ES cells

The targeting vector replaced a 10.0 kb fragment, including exons 7 and 8 of Cbfa2, with lacZ coding sequences, followed by the PGKNeo

3' end of lacZ coding sequences plus the SV40 small T antigen and poly(A) signals, into the targeting vector. The targeting vector replaced a 10.0 kb fragment, including exons 7 and 8 of Cbfa2, with lacZ coding sequences, followed by the PGKNeo gene. Nucleotides 702-970 from the pSV-β-galactosidase vector (Promega) were amplified by polymerase chain reaction (PCR), using the primers (sense) 5'-AATCTGAGTGCATCCCGTCTTACAA-CA-3' and (antisense) 5'-GTAACCGTGCA TCTGCCAGT-3'. The PCR product was cleaved with BsmI and BsaI, and subcloned along with a Bsa36I-BamHI fragment from the pSV-β-galactosidase vector, containing the 3' of lacZ coding sequences. A thymidine kinase gene (tk) under the regulation of the PGK promoter was incorporated at the 3' end of the homology region.

The targeting vector was linearized with NotI, electroporated into J1 ES cells, and Cbfa2+/−/F1 mice generated by standard protocols, and as described previously (Li et al., 1992; Wang et al., 1996a,b). Genotypes were determined by β-galactosidase activity, Southern blot analysis of tail, embryo, or yolk sac DNA (Fig. 1B), or by PCR analysis using primers specific for the lacZ gene (5'-AGTAGATAGGTAAGTGCCTC-3').

Cbfa2+/−/female mice were crossed with Cbfa2+/− males (Wang et al., 1996a) to generate Cbfa2+/−/embryos. The Cbfa2−/− allele was identified by β-galactosidase activity, and the Cbfa2−/− allele by PCR, using a primer from within neo (5'-TCGCCAGCGCATCGCCTTC-3'), in conjunction with a primer from intron 4 of Cbfa2 (5'-AGTAGATAGGTAAGTGCCTC-3'). The cross was designed so that PCR detection of the Cbfa2−/− allele was not complicated by maternal contamination of fetal tissues.

Light microscopy

Embryos were dissected at various stages of gestation and processed for β-galactosidase activity, as described by Miles et al. (Miles et al., 1997). 8 μm sections of entire paraffin-embedded embryos were prepared, and alternate slides were counterstained with hematoxylin and eosin to facilitate tissue identification. Forty embryos from 6.5 to 10.5 dpc were analyzed.

Semi-thin sections from AGM regions were prepared by fixing 10.5 dpc Cbfa2+/−/ and Cbfa2+/−/− embryos in 3% glutaraldehyde (GTA), 2% paraformaldehyde (PF), 2.5% dimethylsulfoxide (DMSO), and 0.1% CaCl2 buffered by 0.1 M sodium cacodylate (pH 7.4) for at least 3 hours at room temperature (RT). Samples were rinsed three times in 0.1 M sodium cacodylate containing 0.05% CaCl2 and 0.3 M sucrose at pH 7.4, a transverse cut was made 2-3 somites caudal to the developing forelimbs, and the posterior portion of the embryo (including the AGM region) was post-fixed in 2% OsO4 in 0.1 M sodium cacodylate/0.05% CaCl2/0.3 M sucrose at pH 7.4 for 2 hours at RT. Samples were dehydrated through a graded series of ethanol and propylene oxide, and embedded in epon (LX112). Semi-thin sections (0.5-0.7 μm) were prepared from similar areas in each block and stained with ‘Epoxy Tissue Stain’ (toluidine blue and basic fuschin; Electron Microscopy Sciences, Inc.).

Ultrastructure analysis

Embryos (10.5 dpc) were isolated in ice cold PBS and fixed for 2 hours at RT in 2% PF/0.25% GTA in 0.1 M piperoxine-N, N'-bis-[ethanesulfonic acid] (PIPES) buffer. Embryos were washed in PBS, then stained with 1 mg/ml Blue-gal (Sigma) for 6 hours at 37°C. Samples were rinsed in PBS, a transverse cut made 2-3 somites caudal to the developing forelimbs, and the posterior portion of the embryo post-fixed in 3% GTA, 2% PF, 2.5% dimethylsulfoxide (DMSO), 0.1% CaCl2 buffered by 0.1 M sodium cacodylate (pH 7.4) for at least 3 hours at RT. Samples were rinsed three times in 0.1 M sodium cacodylate at pH 7.4, post-fixed in 2% OsO4, 0.1 M sodium cacodylate (pH 7.4), embedded in epon (LX112), and 0.5-0.7 μm sections prepared as described above. Semi-thin sections were lightly stained (15-30 seconds) with ‘Epoxy Tissue stain’. Thin sections (60-90 nm) were prepared from each block, stained with 2% aqueous uranyl acetate (50 minutes), followed by Reynolds’s lead citrate (8 minutes). All electron micrographs were taken at 80 or 100 kV on a JEOL 100CX.

Methylcellulose colony forming assays

Fetal livers, vitelline and umbilical arteries/cords were dissected from 11.5 dpc embryos and digested in 0.1% collagenase (Sigma, C2674), 20% fetal calf serum (FCS) in PBS at 37°C for 1-2 hours. Cells were washed with alpha minimum essential medium (GIBCO/BRL) and plated in 35 mm suspension dishes in alpha medium containing 0.8% methylcellulose, 30% FCS, 1% bovine serum albumin, 2 mM glutamine, 0.1% 2-mercaptopethanol (Methocult; Stemcell Technologies, Vancouver). Methylcellulose medium was supplemented with 1.3% pokeweed mitogen-stimulated spleen cell conditioned medium (Stemcell Technologies), 2 units/ml erythropoietin (R&D Systems), 20 ng/ml IL-3 (R&D Systems), 50 ng/ml stem cell factor (SCF; R&D Systems), and 5 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF; R&D Systems).

FACS analysis

Livers and AGM regions from 11.5 dpc embryos, and bone marrow from adult mice were prepared and loaded with di-(β-D-galactopyranoside) (FDG) as described by Sanchez et al. (Sanchez et al., 1996). Phycoerythrin (PE)-conjugated antibodies to c-kit (2B8),...
Mac-1 (M1/70), PECAM-1 (MEC13.3), and CD44 (IM7) were obtained from Pharmingen. PE-conjugated streptavidin (Pharmingen) was used to detect both biotinylated CD34 antibody (RAM34, Pharmingen) and a biotinylated anti-goat antibody (Vector Laboratories), after primary incubation with goat-anti-mouse SCF (G19, Santa Cruz Biotechnology, Inc.). FDG-loaded cells (1×10^5) from individual embryos, or pooled aliquots of cells from embryos with identical genotypes were stained with primary antibody, incubated on ice for 20 minutes, and washed with PBS/FCS. Secondary and tertiary incubations were performed when applicable, as described above. Appropriate isotope controls were used in each experiment. After the final wash, cells were resuspended in 500 µl PBS/FCS. The fluorescence created by the β-galactosidase reaction was detected on the FITC channel of a FACStar (Becton-Dickinson). PE was detected on the FL2 channel. Triple labeling of cells was performed using a Cy-Chrome (FL3) conjugated streptavidin (Pharmingen) to detect biotinylated antibodies, while PE-labeled c-kit and β-galactosidase expression were detected as described above.

**RESULTS**

*Cbfa2* and *Cbfb* are required for definitive hematopoiesis, indicating that both subunits of the heterodimer are essential for function in vivo. Since *Cbfa2* expression appears to be more temporally and spatially restricted than that of *Cbfb* (Ogawa et al., 1993; Satake et al., 1995; Simeone et al., 1995; Wang et al., 1993), it should be the more accurate indicator of where the active heterodimer is located. We introduced a marker, the bacterial lacZ gene, into the *Cbfa2* locus by homologous recombination. The targeting vector replaced exons 7 and 8 of *Cbfa2* with lacZ coding sequences (Fig. 1A,B). The splice acceptor site from exon 7 was preserved, so that a CBFα2-β-galactosidase fusion protein would be synthesized containing the N-terminal 242 amino acids from CBFα2 (Bae et al., 1993) (including its DNA-binding domain) fused to β-galactosidase sequences. Nuclear localization sequences from CBFα2 should be contained within the fusion protein (Kanno et al., 1998), and we found β-galactosidase activity concentrated in the nucleus (Fig. 1C).

*Cbfa2* expression in *Cbfa2^{lz/+}* embryos is first seen at 7.5 dpc, at the neural plate stage of development (Fig. 2A), in the endoderm and some extraembryonic mesodermal cells (not shown). At 8.0-8.5 dpc, *Cbfa2* expression coalesces into a tight band encircling the yolk sac (Fig. 2B). All newly emerging primitive erythrocytes in the yolk sac express *Cbfa2* at 8.0 dpc (not shown), but by 8.5 dpc, *Cbfa2* expression declines significantly (Fig. 2C), and disappears entirely by 10.5 dpc (Fig. 3C) in all primitive erythrocytes. The ring of *Cbfa2* expression remaining at 8.5 dpc consists of punctate expression in yolk sac endoderm, and intense expression in small numbers of endothelial cells in yolk sac capillaries, in hematopoietic cells closely associated with yolk sac endothelium, and in endothelial cells that appear to be budding into the lumina of yolk sac capillaries (Fig. 2C). *Cbfa2* is also expressed in mesenchymal cells in the distal portion of the allantois at 8.5 dpc, at the point where it joins the chorion (not shown). The allantois was recently shown to be a source of HSCs in birds (Caprioli et al., 1998). The allantois will form the body stalk, which includes the umbilical artery, a site where hematopoietic cells have also been seen histologically in mice (Garcia-Porrero et al., 1995; Wood et al., 1997).

In the 8.5 dpc embryo proper, *Cbfa2* is expressed in

![Fig. 1.](attachment:image_url) Generation of the *Cbfa2^{lz}* allele. (A) Targeting strategy used to replace exons 7 and 8 of the *Cbfa2* gene with lacZ coding sequences. Probe A was used to screen for homologous recombination events, and probe B to confirm the deletion of exon 7 sequences in *Cbfa2^{lz/lz}* embryos. The expected size of the EcoRI restriction fragments that hybridize with probe A, and the SpeI fragment that hybridizes with probe B are indicated. Black regions indicate *Cbfa2* coding sequences in exon 7 and within exon 8. S, SalI; E, EcoRI; P, PstI; Sp, SpeI; N, NaeI. (B) Southern analysis of genomic DNA from 11.5 dpc embryos. (C) Predominantly nuclear localization of β-galactosidase activity in mouse embryo fibroblasts isolated from 12.5 dpc *Cbfa2^{lz/+}* embryos.
endothelial cells of the vitelline artery, and in the ventral portion (floor) of the paired dorsal aortae (Fig. 2D). The vitelline artery is the caudal extension of the dorsal aorta at 8.5 dpc, connecting it to the yolk sac circulation (for a brief description of the development of the arterial system in the caudal region of the mouse embryo, see Garcia-Porrero et al., 1995). Cbfa2 expression in the vitelline artery is restricted to its intra-embryonic portion, and is not seen in the segment of the artery that extends into the yolk sac.

Cbfa2 expression in 10.5 dpc embryos can be seen in the vitelline and umbilical arteries, the fetal liver, the AGM region, and in sites unrelated to hematopoiesis (Fig. 2E). In the vitelline (Fig. 2F) and umbilical arteries (not shown), grape-like clusters of hematopoietic cells expressing Cbfa2 can be seen closely associated with endothelial cells in the lumen. The extraembryonic portion of these arteries from 10.5-11.5 dpc embryos gave rise to large, predominantly myeloid or mixed lineage colonies in colony forming unit culture (CFU-C) assays (Fig. 2G,H; Table 1).

Fetal livers from 10.5 dpc embryos express Cbfa2 in the liver capsule mesoderm (Fig. 5A,B), and in hematopoietic cells (Fig. 3A). Fluorescence activated cell sorting (FACS) analysis demonstrates that CBFα2+ fetal liver and adult bone marrow cells express cell surface markers associated with definitive hematopoietic stem cells and early progenitors (Fig. 4A-C,G-I). In fetal livers from 11.5 dpc embryos, 36% of the cells co-express Cbfa2 and CD34. The latter is expressed on fetal liver and bone marrow hematopoietic stem cells and early progenitors (Andrews et al., 1992; Katz et al., 1985; Krause et al., 1994) (Fig. 4A). 25% of fetal liver cells express both c-kit and Cbfa2 (Fig. 4A). Sanchez et al. (1996) showed that fetal liver LTR-HSCs reside mainly in the c-kit+CD34+ population. 14% of fetal liver

Fig. 2. Cbfa2 expression during mouse development in Cbfa2+/+ embryos. (A) Cbfa2 expression in the extraembryonic tissue of a 7.5 dpc embryo, at the neural plate stage. (B) Cbfa2 expression in developing blood islands at 8.5 dpc. The negative embryo on the right is a Cbfa2+/- littermate. (C) Detail of the yolk sac (8.5 dpc) showing Cbfa2 expression in endothelial cells, and in hematopoietic cells closely associated with the endothelium (black arrows). Note that primitive erythrocytes express only low levels of Cbfa2 (outlined arrows). Punctate staining is also seen in the yolk sac endoderm (e). (D) Section of an 8.5 dpc embryo showing Cbfa2 expression in vitelline artery endothelial cells (v), ventral walls of the paired dorsal aortae (a), and yolk sac (y) vessels. Note that Cbfa2 expression is in the intraembryonic segment (open arrow), but not the extraembryonic segment (black arrow) of the vitelline artery. (E) View of a 10.5 dpc embryo showing Cbfa2 expression in the vitelline (v) and umbilical (u) arteries, AGM region (agm), and fetal liver (fl). Expression is also seen in the olfactory epithelium (o), spinal ganglia (g), and maxillary processes (m). Expression in external genitalia is not visible from this angle. (F) View of the vitelline artery intra-abdominal segment (10.5 dpc) with a hematopoietic cell cluster expressing Cbfa2. (G,H) Cytocentrifuge preparations of myeloid (G) and mixed lineage (H) colonies from in vitro cultures of umbilical arteries from 11.5 dpc Cbfa2+/+ embryos, showing neutrophils (n), macrophages (m), eosinophils (eo), and enucleated erythrocytes (er) (Wright Giemsa stain).
Cbfa2 and intra-aortic hematopoietic cells

Cbfa2 is expressed at 10.5 dpc in the ventral portion of the dorsal aorta within the AGM region (Fig. 3A,B). The anterior and posterior boundaries of Cbfa2 expression in the AGM region coincide with the location of the mesonephros, but do not extend in the rostral direction into the region flanked by pronephros. Four discrete cell types in the AGM region express Cbfa2: mesenchymal cells, endothelial cells, small numbers of hematopoietic cells in the lumen (Fig. 3C), and a few cells in the mesonephros (not shown). Almost all endothelial cells in the floor of the dorsal aorta express Cbfa2, while very few endothelial cells in the roof of the aorta express Cbfa2.

Cbfa2 expression in endothelial cells was confirmed by ultrastructural analysis, using a β-galactosidase substrate (Bluo-gal), the product of which forms an electron dense precipitate that adheres to cell membranes (Weis et al., 1991) (Fig. 3E). CBFα2+ cells in the floor of the dorsal aorta participate in the formation of zonula occludens type (tight) junctions to adjacent endothelial cells. Many CBFα2+ endothelial cells in the floor of the aorta have a very rounded appearance, and appear to be budding into the lumen of the aorta. In multiple sections from six embryos we failed to identify any endothelial cells in mitosis, suggesting this region of the dorsal aorta may expand via recruitment of cells from the surrounding mesenchyme.

FACS analysis of cells isolated from the AGM region of 11.5 dpc embryos shows that 10% of CBFα2+ cells express CD34 (Fig. 4D), and approximately 27% of CBFα2+ cells express c-kit. LTR-HSCs isolated from the AGM region of 11 dpc embryos reside in the c-kit+ CD34+ population (Sanchez et al., 1996). Within the c-kit+CD34+ population, 35% express Cbfa2 (Fig. 4E).

Emergence of LTR-HSCs from the AGM region peaks at approximately 11 dpc in the mouse (Sanchez et al., 1996). We examined AGM regions from 14.5 dpc Cbfa2lz/+ embryos to determine whether Cbfa2 expression in endothelium persists at later stages of development. Cbfa2 expression in the dorsal aorta of 14.5 dpc embryos was found immediately rostral to the bifurcation of the dorsal aorta into the iliac arteries. The dorsal aorta elongates in the caudal direction as the embryo grows, and Cbfa2 expression at all stages examined (8.5-14.5 dpc) appears to coincide with the actively growing caudal portion of the aorta. Histological sections show that Cbfa2 expression in the vicinity of the dorsal aorta at 14.5 dpc is restricted to mesenchymal cells (Fig. 3D). The endothelium, which at this time is separated from the mesenchyme by a layer of smooth muscle, does not express Cbfa2. No budding of hematopoietic cells from the endothelium is apparent at 14.5 dpc. Cbfa2 expression in the umbilical cord of 17.5 dpc...
embryos was also confined to the mesenchyme (not shown). Therefore, endothelial cells that express Cbfa2 appear only transiently during development, coincident with the emergence of definitive hematopoietic progenitor and stem cells.

**Cbfa2 is required for the formation of intra-aortic hematopoietic clusters**

To pinpoint when during hematopoiesis Cbfa2 is required, we crossed Cbfa2lz/+ mice with mice heterozygous for a nonfunctional Cbfa2 allele that lacks exon 5 (Cbfa2rd+)/ (Wang et al., 1996a). Neither fetal livers nor the vitelline and umbilical arteries from 11.5 dpc Cbfa2lz/rd embryos gave rise to definitive hematopoietic colonies in CFU-C assays (Table 1). Therefore, the Cbfa2lz allele, which lacks coding sequences for the CBFα2 C terminus (including the transactivation domain (Kanno et al., 1998), is a non-functional allele. We compared the histology of normal (Cbfa2lz+/) and Cbfa2-deficient (Cbfa22/d/) embryos. All embryos contain only one copy of the Cbfa2lz allele, thus β-galactosidase expression levels can be directly compared.

**Bone Marrow**

![Image of surface antigens on Cbfa2-positive cells from the fetal liver and AGM region of 11.5 dpc mouse embryos.](image)

**Fig. 4.** Surface antigens on Cbfa2-positive cells from the fetal liver and AGM region of 11.5 dpc mouse embryos. (A) Expression on fetal liver cells. Data are presented as FACS dot plots, and percentages of cells in each quadrant are indicated. Isotype control antibodies (PE and Cy) and FDG (FITC) substrate double staining are shown for fetal livers from Cbfa2++/ littermates. Triple staining was performed with FDG (FITC) to detect CBFα2-β-gal expression. Cy-streptavidin to detect biotinylated antibodies to CD34 and SCF, and PE directly coupled to an anti-c-kit antibody. Analysis was performed on 10^5 cells from pooled fetal livers isolated from Cbfa2lz/+ embryos, and data from one representative sample is shown. The mean values (range in parentheses) of 5-12 independent samples are: c-kit^{+}CBFα2-β-gal: 24.1% (18.6%–27.5%); CD34^{+}CBFα2-β-gal: 34.4% (33.1%–36.5%); SCF^{+}CBFα2-β-gal: 35.0% (33.2%–36.4%). (B) Triple staining was performed on fetal liver cells as described in A, and the c-kit^{+}CD34^{+} and c-kit^{+}SCF^{+} gated populations in the upper right quadrant analyzed for CBFα2-β-gal expression. The average number of c-kit^{+}CD34^{+} cells was 12.8% (8.8%–16.5%), and c-kit^{+}SCF^{+} cells was 16.7% (12.2%–20.6%). Percentages of CBFα2-β-gal-positive cells within the c-kit^{+}CD34^{+} and c-kit^{+}SCF^{+} populations are indicated. (C) Isotype control antibodies (PE) and FDG (FITC) substrate double staining are shown for pooled fetal livers from Cbfa2++/+ embryos. Mean values (range in parentheses) of 5-12 independently pooled fetal liver samples from Cbfa2lz/+ embryos are: CD44^{+}CBFα2-β-gal: 58.4% (55.3%–61.2%); Mac-1^{+}CBFα2-β-gal: 9.5% (8.3%–10.6%); PECAM-1^{+}CBFα2-β-gal: 30.8% (18.0%–42.1%). (D) AGM cells. Experiments were performed as described in A. Mean percentages: c-kit^{+}CBFα2-β-gal: 2.5% (1.6%–3.7%); CD34^{+}CBFα2-β-gal: 6.1% (4.6%–8.2%); SCF^{+}CBFα2-β-gal: 8.8% (6.3%–11.3%). (E) Triple staining of AGM cells, as described in B. The average number of c-kit^{+}CD34^{+} cells was 5.9% (4.2%–8.0%), and of c-kit^{+}SCF^{+} cells was 7.8% (6.5%–10.0%). (F) AGM cells, as in C. Mean values (range in parentheses): CD44^{+}CBFα2-β-gal: 2.4% (1.9%–2.9%); Mac-1^{+}CBFα2-β-gal: 2.5% (2.1%–3.1%); PECAM-1^{+}CBFα2-β-gal: 3.4% (3.0%–4.0%). (G) Adult bone marrow cells. Experiments were performed as described in A. Mean percentages: c-kit^{+}CBFα2-β-gal: 7.6% (6.6%–9.6%); CD34^{+}CBFα2-β-gal: 5.4% (4.2%–7.1%); SCF^{+}CBFα2-β-gal: 22.9% (13.6%–35.8%). (H) Triple staining of bone marrow cells, as described in B. The average number of c-kit^{+}CD34^{+} cells was 2.3% (1.9%–2.7%), and of c-kit^{+}SCF^{+} cells was 3.7% (2.9%–5.4%). (I) Bone marrow cells, as in C. Mean values (range in parentheses): CD44^{+}CBFα2-β-gal: 83.2% (79.6%–86.5%); Mac-1^{+}CBFα2-β-gal: 64.9% (53.9%–72.6%); PECAM-1^{+}CBFα2-β-gal: 12.4% (11.6%–13.7%).
Fetal livers of 10.5 dpc Cbfa2 lz/rd embryos contain CBFa2 + mesenchymal cells, but no CBFa2 + hematopoietic cells (Fig. 5B). FACS analysis of 11.5 dpc Cbfa2 lz/rd embryos indicates severe depletion of CD44 + hematopoietic progenitors (Fig. 6A). Significant decreases are also seen in the proportions of CBFa2 + c-kit +, CBFa2 + CD34 +, CBFa2 + SCF +, and CBFa2 + CD44 + fetal liver cells.

The absence of fetal liver hematopoiesis does not appear to result from failure of definitive hematopoietic cells to home properly to the fetal liver, since hematopoietic cells expressing Cbfa2 are also undetectable in circulating blood (not shown). Rather, there appears to be a defect in the formation of intra-aortic hematopoietic clusters. No clusters of hematopoietic cells are present in the vitelline arteries of 10.5 dpc Cbfa2 lz/rd embryos (Fig. 5F). Furthermore, both the numbers of endothelial cells expressing Cbfa2, and the intensity of Cbfa2 expression in endothelial cells is dramatically reduced in the vitelline (Fig. 5F) and umbilical (not shown) arteries. This defect is not caused by failure of CBFa2 + endothelial cells to migrate to these arteries, since CBFa2 + endothelial cells are present one day earlier, at 9.5 dpc (Fig. 5D). Cbfa2 expression in yolk sac endothelial cells is, however, absent in 9.5 dpc Cbfa2 lz/rd embryos, and no CBFa2 + hematopoietic cells are visible (Fig. 5H).

Significant defects are also observed in the AGM region of Cbfa2 deficient embryos. At 9.5 dpc, endothelial cells and para-aortic mesenchymal cells expressing Cbfa2 are present in the floor of the dorsal aorta in both Cbfa2 lz/+ and Cbfa2 lz/rd embryos (Fig. 7A,B). However, whereas by 10.5 dpc Cbfa2 expression has intensified in endothelial cells of Cbfa2 lz/+ embryos and hematopoietic cells are apparent, Cbfa2

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**Table 1. Colonies arising from fetal liver, vitelline artery and umbilical artery hematopoietic progenitors isolated from 11.5 dpc Cbfa2+/+ and Cbfa22lz/rd embryos**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Colony number per embryo‡</th>
<th>Embryos analyzed</th>
<th>Artery§</th>
<th>Colony number per embryo‡</th>
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<td></td>
<td>4</td>
<td>Umbilical</td>
<td>105 (37) 373 (105) 248 (37)</td>
</tr>
<tr>
<td>Cbfa2 lz/rd</td>
<td></td>
<td>10</td>
<td>Umbilical</td>
<td>0 0 0</td>
</tr>
</tbody>
</table>

*The extraembryonic portions (between the yolk sac and embryo proper) of the vitelline and umbilical arteries, representing approximately 10⁴ cells, were dissected and analyzed.

‡Colonies (>30 cells in size) were scored on days 7-8 of incubation. E, erythroid colonies; M, myeloid colonies; Mix, erythroid-myeloid mixed colonies.

Average number of colonies per embryo is indicated, followed by standard deviation in parentheses.

§Umbilical artery and vitelline arteries were isolated from the same embryos.

¶Small numbers of erythroid colonies were seen in separate experiments from pooled 10.5 dpc and 11.5 dpc Cbfa2+/+ embryos (not shown).
expression fades significantly in endothelial cells of Cbfa2lz/+ embryos (Fig. 7C,D). Endothelial cells in the floor of the dorsal aorta in Cbfa2lz/rd embryos are relatively flat and elongated (Fig. 7H-I), compared with those in Cbfa2lz/+ embryos (Figs 7G, 3E). There is also significant crowding of para-aortic mesenchymal cells immediately adjacent to the ventral endothelium in Cbfa2lz/rd embryos that is not seen in more dorsal sections of the aorta, nor in Cbfa2lz/+ embryos (Fig. 7E,F and G-H). The mesenchymal cells are elongated and flattened and in close contact with the overlying endothelium (Fig. 7H-I), but do not form desmosomes or junctional complexes with the endothelium (not shown).

The numbers of CBFα2+c-kit+ CBFα2+CD34+, CBFα2+SCF+, and CBFα2+CD44+ cells in AGM regions from Cbfa2lz/+ and Cbfa2lz/rd embryos are not statistically different (Fig. 6B). The presence of CBFα2+ cells in the AGM region that express c-kit, CD34 and SCF suggests that Cbfa2 is not required for the expression of these three cell surface markers.

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**Fig. 6.** Expression of hematopoietic markers in 11.5 dpc Cbfa2lz/+ and Cbfa2lz/rd embryos. (A) Fetal livers, analyzed as described in Fig. 4. Data are from individual, representative embryos. The average values (range in parentheses) for 8-13 embryos of CBFα2-βgal+ cells expressing other cell surface markers (upper right quadrants) are as follows: Cbfa2lz/+; c-kit+ 22.6% (19.1%-28.0%), CD34+ 42.0% (37.6%-47.6%), SCF+ 40.3% (36.1%-40.9%), CD44+ 56.6% (55.1%-63.6%). Cbfa2lz/rd; c-kit+ 6.2% (4.8%-8.2), CD34+ 16.4% (14.5%-18.3), SCF+ 13.6% (11.1%-18.3), CD44+ 1.7% (0.8%-3.1%). The differences between Cbfa2lz/+ and Cbfa2lz/rd embryos in all double positive populations was significant at p<0.01, as determined by a two tailed t-test. (B) AGM regions from individual embryos. Average values for CBFα2-βgal double positive cells (upper right quadrants) are as follows: Cbfa2lz/+; c-kit+ 3.4% (1.2%-4.7%), CD34+ 8.9% (5.4%-15.1%), SCF+ 4.8% (2.2%-6.7%), CD44+ 3.5% (1.8%-4.7%). Cbfa2lz/rd; c-kit+ 2.0% (1.1%-2.8%), CD34+ 8.2% (7.5%-9.8%), SCF+ 4.2% (3.5%-4.8%), CD44+ 1.6% (1.5%-2.0%).
DISCUSSION

*Cbfa2* is required for the differentiation of all definitive hematopoietic cells, but not for primitive erythropoiesis. Here we show that *Cbfa2* is expressed in a small, transient population of endothelial cells in the yolk sac, the vitelline and umbilical arteries, and in the floor of the dorsal aorta. Soon after *Cbfa2* is expressed in these sites, hematopoietic clusters appear that are closely associated with *Cbfa2*-positive endothelium. The timing of *Cbfa2* expression and appearance of intra-aortic hematopoietic clusters parallels the emergence of definitive hematopoietic cells, as detected previously by histology and in vivo functional analyses (Garcia-Porrero et al., 1995; Godin et al., 1995; Medvinsky and Dzierzak, 1996; Müller et al., 1994). For example, definitive hematopoietic progenitor cells with B-lymphoid potential emerge autonomously at 8.5 dpc in mice, prior to the establishment of embryonic circulation, both in the yolk sac and the para-aortic splanchnopleure, the latter of which includes the paired dorsal aortae and intraembryonic portion of the vitelline artery (Godin et al., 1995). The first long-term repopulating HSCs with the potential to engraft irradiated adult recipients emerge autonomously within the AGM region at 10.0 dpc. (*Medvinsky and Dzierzak, 1996; Müller et al., 1994*).

*Cbfa2* expression is apparent as early as 8.5 dpc in endothelial cells lining the vitelline artery and in the floor of the dorsal aortae. *Cbfa2* expression in mesenchymal cells at the distal tip of the allantois at 8.5 dpc precedes formation of the umbilical cord and later endothelial cell expression at that site. Clearly demarcated regions of *Cbfa2* expression are thus present as early as 8.5 dpc in all sites where definitive hematopoietic cells later emerge, supporting the hypothesis that definitive hematopoietic progenitors and/or

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**Fig. 7.** Defects in the AGM region of *Cbfa2 lz/rd* embryos. (A,B) Views of the dorsal aorta ventral wall showing endothelial cell *Cbfa2* expression in both *Cbfa2 lz/+* (A) and *Cbfa2 lz/rd* (B) embryos (9.5 dpc). (C,D) High power views of the dorsal aorta ventral wall in *Cbfa2 lz/+* (C) and *Cbfa2 lz/rd* (D) embryos (10.0 dpc), showing *Cbfa2* expression in the para-aortic mesenchyme in both embryos, endothelial cell expression in the *Cbfa2 lz/+* embryo, and reduced endothelial cell expression in the *Cbfa2 lz/rd* embryo. (E,F) Comparative semi-thin (0.6 μm) sections through the AGM regions of *Cbfa2 lz/+* (E) and *Cbfa2 lz/rd* (F) embryos (10.5 dpc), showing the relative cellular accumulation throughout the para-aortic mesenchyme in *Cbfa2 lz/rd* embryos (arrows) and the absence of accumulation in *Cbfa2 lz/+* embryos (see also G,H). (G,H) Comparative high power views of the para-aortic AGM regions of *Cbfa2 lz/+* and *Cbfa2 lz/rd* embryos (10.5 dpc) showing the Bluo-gal reaction product (dark blue precipitate on cell membranes) in both endothelial and subendothelial cells. Note the relatively flattened appearance of endothelial cells in *Cbfa2 lz/rd* embryos (H), most of which do not express *Cbfa2*, as well as the subendothelial accumulation of *Cbfa2*-positive cells throughout the para-aortic mesenchyme. Cells closer to the coelomic epithelium (not shown) tend to orient their long axes perpendicular to the endothelium (arrows), suggestive of movement towards the aortic wall. In contrast, *Cbfa2*-positive cells closer to the aortic wall orient their long axes parallel to the endothelium, suggesting sub-endothelial accumulation. (I) Electron micrograph of the region outlined in H showing few positive cells in the aortic endothelium, the lack of underlying cystic separation, and the concentration of para-aortic cells with their long axes parallel to the endothelium (asterisks).
stem cells emerge autonomously from distinct sites in the embryo.

**Cbfa2 appears to be required for budding of hematopoietic cells from hemogenic endothelium**

Jaffredo et al. (1998) recently showed that CD45<sup>+</sup> endothelial cells in the chick embryo, labeled with Dil-coupled LDL, give rise to LDL-labeled, CD45<sup>+</sup> intra-aortic hematopoietic clusters, demonstrating a precursor/progeny relationship between endothelial cells and intra-aortic hematopoietic clusters. Here we show that Cbfa2 is expressed in endothelial cells prior to the emergence of intra-aortic hematopoietic cells and clusters, and is required for the formation of these hematopoietic cells. Taken together, the data suggest that Cbfa2 is required for the budding of hematopoietic cells from a definitive hemogenic endothelium. We envision that Cbfa2 acts as a molecular switch specifying the conversion from an endothelial to hematopoietic cell fate.

Disruption of the Cbfa2 gene also impairs the maintenance of Cbfa2 expression in endothelium. Endothelial cells expressing Cbfa2 appear in the arteries of Cbfa2 deficient embryos by 9.5 dpc, but a marked decline in Cbfa2 expression occurs soon thereafter. The reasons for this decline are unclear. One possible explanation is that further incorporation of CBFo<sub>2</sub><sup>+</sup> endothelial cells into these arteries is impaired, which is suggested by the significant accumulation of CBFo<sub>2</sub><sup>+</sup> para-aortic mesenchymal cells in Cbfa2-deficient embryos. The dorsal aorta, vitelline and umbilical arteries in Cbfa2-deficient embryos may continue to expand via selective incorporation of CBFo<sub>2</sub><sup>+</sup> cells, thereby diluting out the contribution from CBFo<sub>2</sub><sup>+</sup> cells. Another possibility is that the protein product of the Cbfa2 gene may positively regulate Cbfa2 expression. The human Cbfa2 gene (CBFA2, or AML1) contains two promoters, one of which has three potential CBF binding sites (Ghozi et al., 1996). Cbfa2 may contain a promoter active in hemogenic endothelial cells that initiates transcription of the gene. Subsequent maintenance of Cbfa2 expression in hemogenic endothelial cells, and stable commitment of these cells to the hematopoietic lineage may require the active CBFo<sub>2</sub>:CBFβ heterodimer.

**Cbfa2 expression may mark cells derived from definitive hemangioblasts in the splanchnopleural mesoderm**

Fate mapping studies in birds demonstrated that intra-aortic hematopoietic clusters and endothelial cells that populate the floor of the dorsal aorta differentiate from the splanchnopleural mesoderm, presumably from hemangioblasts in the para-aortic mesenchyme (Pardanaud and Dieterlen-Liévre, 1993; Pardanaud et al., 1996, 1989). Cells lining the roof of the aorta differentiate from angioblasts in the somatopleural mesoderm committed to the endothelial lineage. Cbfa2 is the first marker that differentiates between endothelial cells in the roof and floor of the dorsal aorta, and may specifically mark endothelial cells and hematopoietic cells derived from putative hemangioblasts in the splanchnopleural mesoderm. Many cells in the ventral para-aortic mesenchyme also express Cbfa2, some or all of which may be definitive hemangioblasts.

The hemangioblast is most commonly described as a mesenchymal precursor capable of differentiating directly into either or both endothelial and hematopoietic cells. For example, Choi et al. (1998) showed that nonadherent blast colony-forming cells generated from mouse embryonic stem cells could give rise to either or both endothelial cells and hematopoietic progenitors, and suggested that the blast colony-forming cells are hemangioblasts. Eichmann et al. (1997) demonstrated that Flk-1<sup>+</sup> mesodermal cells isolated from the posterior area of presomitic chick embryos could be differentiated into Flk-1<sup>+</sup> endothelial cells or Flk-1<sup>+</sup> hematopoietic cells when cultured in vitro in the presence or absence of vascular endothelial growth factor, respectively. However, we and others (Garcia-Porrello et al., 1995; Jaffredo et al., 1998) describe what appear to be newly emerging hematopoietic cells in the dorsal aorta and vitelline arteries that are connected via tight junctions to adjacent cells. These cells form an integral part of the endothelium lining the lumen of these arteries, and by this criterion are bona fide endothelial cells, forming a hemogenic endothelium. Thus, we and others (Jaffredo et al., 1998; Nishikawa et al., 1998b) propose that the differentiation of at least some definitive hematopoietic progenitors and/or stem cells from 'definitive hemangioblasts' appears to involve an endothelial intermediate. This process may differ from that by which the first wave of primitive erythrocytes and endothelial cells develop. Thus, the two views of differentiation from the hemangioblast are not necessarily incompatible.

The yolk sac gives rise to both primitive erythrocytes and progenitors for definitive hematopoietic cells. Cbfa2 expression in the primitive erythroid lineage is quite transient, and by 8.5 dpc has largely declined. Thereafter, Cbfa2 is expressed in a punctate pattern in the yolk sac, in a small population of endothelial cells, in hematopoietic cells in close contact with the endothelium, and in hematopoietic cells that appear to be budding from the endothelium. Young et al. (Young et al., 1995) described a small population of CD34<sup>+</sup> hematopoietic cells in the yolk sac in close contact with the endothelium, although details of hematopoietic budding would have been difficult to see since all yolk sac endothelial cells express CD34. In Cbfa2-deficient embryos, the first burst of Cbfa2 expression and development of primitive erythrocytes from the extraembryonic mesodermal mass occurs normally, but the subsequent budding of hematopoietic cells from endothelial cells lining yolk sac capillaries is impaired. We propose that at least some definitive hematopoietic progenitors in the yolk sac may differentiate via an endothelial cell intermediate in yolk sac capillaries, by a process similar to that observed in the vitelline and umbilical arteries and in the AGM region.

We thank Alice Given, Gary Ward and Steve Fiering for their excellent technical advice. We are especially grateful to Elaine Dzierzak for her many helpful comments, and for critically reading this manuscript. M. M.-P. is supported by a Jacob Javits Neuroscience Investigator Award, Public Health Service grant NS22897. T.N. is supported by T32 GM08704-01 from the NIH/GM. N. A. S. is supported by Public Health Service grant R01 CA58343. Flow cytometry was done at Dartmouth Medical School in The Herbert C. Engler Cell Analysis Laboratory, established by a grant from the Fannie E. Rippel Foundation and supported in part by the Core Grant of the Norris Cotton Cancer Center (CA 23108). N. A. S. is Leukemia Society of America Scholar.


The sialomucin CD34 is expressed on hematopoietic cells and blood vessels during murine hematopoiesis. Blood 85, 96-105.