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The transcription factor ZBP-89 controls generation of the hematopoietic lineage in zebrafish and mouse embryonic stem cells

Xiangen Li, Jing-Wei Xiong, C. Simon Shelley, Heiyoung Park and M. Amin Arnaout*

Hematopoietic development is closely linked to that of blood vessels and the two processes are regulated in large part by transcription factors that control cell fate decisions and cellular differentiation. Both blood and blood vessels derive from a common progenitor, termed the hemangioblast, but the factor(s) specifying the development and differentiation of this stem cell population into the hematopoietic and vascular lineages remain ill defined. Here, we report that knockdown of the Krüppel-like transcription factor ZBP-89 in zebrafish embryos results in a bloodless phenotype, caused by disruption of both primitive and definitive hematopoiesis, while leaving primary blood vessel formation intact. Injection of *ZBP-89* mRNA into *cloche* zebrafish embryos, which lack both the hematopoietic and endothelial lineages, rescues hematopoiesis but not vasculogenesis. Injection of mRNA for Stem Cell Leukemia (SCL), a transcription factor that directs hemangioblast development into blood cell precursors, rescues the bloodless phenotype in ZBP-89 zebrafish morphants. Forced expression of ZBP-89 induces the expansion of hematopoietic progenitors in wild-type zebrafish and in mouse embryonic stem cell cultures but inhibits angiogenesis in vivo and in vitro. These findings establish a unique regulatory role for ZBP-89, positioned at the interface between early blood and blood vessel development.

KEY WORDS: Hematopoiesis, Angiogenesis, *ZBP-89* gene, *SCL/tal-1* gene, Stem cells

INTRODUCTION

Vertebrate hematopoiesis occurs in two developmental waves: a short primitive wave, predominantly generating erythrocytes and primitive myeloid cells, and a definitive wave, producing long-term hematopoietic stem cells (Orkin and Zon, 2002). Hematopoietic stem cell (HSC) progenitors are believed to arise from bipotential fetal liver kinase-1$^+$ (FLK1$^+$; KDR – Zebrafish Information Network) mesoderm stem cells (Choi et al., 1998; Huber et al., 2004; Palis et al., 1999; Thompson et al., 1998), which also give rise to vascular progenitors. The genetic regulatory networks that control blood and blood vessel development have been extensively studied but the factors that regulate the generation of blood and blood vessels from FLK1$^+$ hemangioblasts are incompletely understood. One such factor is the product of the *cloche* (*clo*) gene, which is essential for generating both the hematopoietic and vascular progenitors (Kalev-Zylinska et al., 2002; Liao et al., 1998; Liao et al., 1997; Stainier et al., 1995; Thompson et al., 1998), but its nature remains to be defined. A second factor, SCL, is a basic helix-loop-helix transcription factor encoded by the *scl/tal1* gene that has been shown to be essential in directing hematopoietic fate commitment from hemangioblasts (Mikkola et al., 2003; Porcher et al., 1996; Robb et al., 1996), as well as in embryonic angiogenesis (Patterson et al., 2005; Visvader et al., 1998).

Hematopoietic and endothelial lineages can be produced in vitro from murine embryonic stem cell (ESC)-derived embryoid bodies (EBs), in a temporal pattern that recapitulates the development of these cell populations in vivo (Palis et al., 1999). Analysis of early EBs, between days 2.5 and 4 of ESC differentiation, prior to hematopoietic and endothelial lineage commitment, reveals the presence of a transient mesoderm-derived FLK1$^+$ SCL$^+$ progenitor, or blast colony-forming cell (BL-CFC), which represents the in vitro equivalent of the yolk sac hemangioblast (Chung et al., 2002; D'Souza et al., 2005; Fehling et al., 2003; Park et al., 2005). The expression of *c-kit* (*kita* – Zebrafish Information Network) in this population indicates a hematopoietic potential (Willey et al., 2006).

ZBP-89 (ZFP148) is the prototype of a novel class of transcription factors, phylogenetically conserved in mammals, that contains a characteristic array of three N-terminal C2H2 Krüppel-like zinc fingers and a fourth C2HC variant zinc finger. It shares homology with members of the Krüppel-like finger (KLF) protein family with three Krüppel-like zinc fingers (Bray et al., 1991); however, ZBP-89 has a fourth zinc finger and all four are located in the N-terminal region, in contrast to the conserved C-terminal location of the zinc fingers in the KLF protein family (Kaczynski et al., 2003). The *ZBP-89* gene is localized on chromosome 3q21, the site of breakpoints (Pekarsky et al., 1995) and translocations (Yamagata et al., 1997) in some cases of acute myeloid leukemia (Antona et al., 1998; Bernstein et al., 1986), but it is not clear whether any of these involve the *ZBP-89* gene itself. In the only in vivo study to date, haploinsufficiency of ZBP-89 caused infertility in normally developed male mice that was due to the growth arrest and apoptosis of fetal germ cells (Takeuchi et al., 2003). We have previously shown that ZBP-89 represses expression of the myeloid differentiation marker CD11b in vitro (Park et al., 2003). To explore its role in hematopoiesis in vivo, we cloned the zebrafish ortholog, analyzed its expression, and characterized the phenotype resulting from modulating its expression in zebrafish embryos and murine EB cultures. Our findings identify a crucial function of ZBP-89 in embryonic blood and endothelial cell development, and place it downstream of *clo* and upstream of *scl* in the genetic hierarchy of early hematopoiesis.

Nephrology Division, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129, USA.

*Author for correspondence (e-mail: arnaout@receptor.mgh.harvard.edu)

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MATERIALS AND METHODS

Fish strains and maintenance

Breeding zebrafish were raised, maintained and staged as described (Westerfield, 1993). The *clothe*^{m39} zebrafish mutant lines, *gata1:EGFP* and *flk1:EGFP* transgenic fishes have been described elsewhere (Cross et al., 2003; Long et al., 1997; Stainier et al., 1995).

Cloning of the zebrafish *zbp-89* full-length coding sequence

Zebrafish *zbp-89* was cloned by RT-PCR based on the sequence predicted from Sanger Center's zebrafish genomic DNA sequence (Sanger Institute), using the human ZBP-89 protein sequence as bait. The zebrafish *zbp-89* full-length coding sequence was all contained in Contig #25012. The predicted exon/intron boundary was obtained using the website: genes.mit.edu/GENSCAN.html. A series of PCR primers was designed accordingly and nested RT-PCR reactions were performed using total RNA from 24 hpf zebrafish embryos. A 1.3 kb cDNA encoding the N-terminal fragment of ZBP-89 was generated in first-round RT-PCR with the primers F1 (5′-TGCTGGAGGACATGAATCCACCAG-3′) and R1 (5′-TGGAGAGAGACTCTGGGACTGCTC-3′). The gel-purified (Qiagen) fragment was used as template DNA in a nested PCR reaction using the enzyme-restricted (underlined) primers EcoRI-F (5′-AAGAGAATTCATGAACATTGATGACAAGCTGG-3′) and XbaI-R (5′-CTGCTCTAGAGCCTGCTG-3′). The nested PCR products were cloned into the *Eco*RI-*Xba*I-restricted PSK$^+$ vector (PSK-1.3-ZBP-89) and sequenced. The 1.1 kb encoding the C-terminal fragment of ZBP-89 was cloned by following a similar strategy. The first round primers were F2 (5′-TCCCCACCTGGCAGCAGGCATCTTG-3′) and R2 (5′-AGCTTTTGTTCAGCCAAAGGTTTG-3′), and the nested PCR primers were XbaI-F (5′-CAGCAGGCTCTAGAGCAG-3′) and NotI-R (5′-AAGAGCGGCCGCTCAGCCAAAGGTTTGGCT-3′). The 1.1 kb C-terminal fragment was inserted into the PSK-1.3-ZBP-89 vector to reconstitute the cDNA encoding the full-length protein.

Morpholino oligonucleotide, mRNA and plasmid microinjection

Three morpholino antisense oligonucleotides (MO) targeting the *zbp-89* transcript were obtained from Gene-Tools, LLC. One oligomer (*atg*MO, 5′-CCTCCAGCTTGTCATCAATGTTCAT-3′) was designed to block translation of the mRNA, leading to knockdown of the protein. A second (*splice*MO, 5′-GTCAAAATATTACCTGATGGCAATA-3′) targeted an exon splice donor site in exon 8. A third MO contained a five-nucleotide mismatch in *atg*MO (Mismatched *atg*MO, 5′-CCaCCtGCTTGTgATCAATcTTgAT-3′; mismatched bases are in small letters). Each morpholino oligomer was diluted in 100 mmol/l KCl, 10 mmol/l HEPES, 0.1% Phenol Red (Sigma). Embryos were microinjected at the one- to two-cell stage with 4 ng of the morpholino oligomer (in a volume of 2 nl). RT-PCR analysis revealed the formation of an alternative splice product in *splice*MO-injected embryos that was predominant at 24 hpf and encoded a 237 amino acid ZBP-89 protein terminating after the second zinc finger domain.

The full-length human *ZBP-89* cDNA was directionally cloned into the *Eco*RI and *Xho*I sites of pCS2$^+$ for overexpression. Human *ZBP-89* sense RNA and zebrafish *scl* sense RNA (Gering et al., 1998) were transcribed from linearized pCS2$^+$-ZBP-89 using the mMessenge mMachine Kit according to the manufacturer's protocol (Ambion). *clo*$^{-/-}$ mutant embryos for microinjection were obtained from heterozygous in-crosses, and 100 pg of *ZBP-89* mRNA was injected into one- to two-cell embryos. The full-length human *ZBP-89* coding region was subcloned downstream of the zebrafish *flk1* promoter (Cross et al., 2003), using *Eco*RI and *Not*I sites, to generate the flk1-ZBP-89 plasmid. For morpholino rescue experiments, 300 pg of RNA or 4 ng of the linearized flk1-ZBP89 plasmid DNA were injected into one- to two-cell embryos immediately following the morpholino injection.

Whole-mount in situ hybridization

A 2.4 kb *Eco*RI-*Not*I-restricted zebrafish *zbp-89* fragment was cloned into the pBS (K$^+$) vector. Antisense mRNA was transcribed from the *Eco*RI-linearized plasmid using T3 polymerase, and sense mRNA was transcribed from the *Not*I-linearized plasmid using T7 polymerase as a control. RNA antisense probes were generated with UTP-digoxigenin (DIG), according to the manufacturer's instructions (Roche). Antisense riboprobes to *gata1*, *scl*, *lmo2*, *c-myb*, *flk1*, *fli1a*, *Tie1*, *cdx4*, *tif1g* (*moonshine*), *gata2*, *runx1*, *pu.1*, *l-plastin* and *mpo* have been described previously (Ransom et al., 2004; Davidson et al., 2003; Kalev-Zylinska et al., 2002; Liao et al., 1998; Thompson et al., 1998; Lyons, 1998). DIG-labeled riboprobes were detected using alkaline phosphatase-conjugated anti-DIG antibodies (Roche), followed by detection of alkaline phosphatase activity using NBT/BCIP substrate (Roche).

DAF staining

Embryos (36 hpf) were stained with DAF (2,7-diaminofluorene), which sensitively stains hemoglobin, as described (Weinstein et al., 1996). In brief, embryos were fixed in 4% paraformaldehyde (PFA) for 2 hours, washed three times in PBS, then pre-incubated in the DAF staining solution [0.01% diaminofluorene, 200 mM Tris (pH 7.0), 0.05% Tween 20] for 1 hour at room temperature in the dark. Hydrogen peroxide was added to a final concentration of 0.3%, and the embryos were incubated for 5-20 minutes. Embryos were washed three times in PBS then photographed.

RT-PCR analysis of *zbp-89* expression

A single wild-type (WT) and a clo$^{-/-}$ mutant zebrafish embryo were harvested at the 18 hpf stage, rinsed twice with PBS and transferred into an RNase-Free tube containing 100 μl Trizol RNA solution. Total RNA was extracted according to the manufacturer's instructions (Ambion). Total RNA (4 μl) was used as a template for cDNA synthesis, following the protocol from the Applied Biosystems manual. Two microliters from a total of 50 μl cDNA were used for regular RT-PCR with the zebrafish *ZBP-89* forward (5′-GAAAAGCCTTTCCAGTGCAATCA-3′) and reverse (5′-ATCTTTGACAGCTGTTTCTGCAC-3′) primers.

Mouse ZBP-89 expression in sorted cells from the FLK$^+$SCL$^+$, FLK$^-$SCL$^+$ and FLK$^+$SCL$^-$ population was carried out as follows: 1×10^5 cells from each subset were collected, total RNA extracted and cDNA synthesized as described above. RT-PCR was performed using the mouse ZBP-89 forward (5′-GAGATTTCCTTCAGCGTTTAC-3′) and reverse (5′-TTTGGAAGGGTCTGGTTGTC-3′) primers.

TUNEL assay

Zebrafish embryos were staged and fixed as for in situ hybridization and stored in methanol. After rehydration, embryos were permeabilized by proteinase K digestion, re-fixed in buffered 4% PFA and washed in PBT. Apoptosis was detected in embryos by terminal transferase dUTP nick-end labeling (TUNEL), according to the manufacturer's protocols (In Situ Cell Death Detection Kit: POD, Roche). The staining pattern was observed using light microscopy.

Real-time PCR

One million FLK1$^+$ mesoderm stem cells were dissociated from EBs at different time points, rinsed twice with PBS and transferred into an RNase-free tube containing 100 μl Trizol RNA solution. Total RNA isolation and cDNA synthesis were carried out as described earlier. Two microliters from a total of 50 μl cDNA were used for real-time PCR, which was performed according to the manufacturer's instructions (Stratagene). The ZBP-89 real-time PCR primers were: forward primer (ZBP-89RF1), 5′-CGGCATAGACGAAATGCAGTC-3′; and reverse primer (ZBP-89RR1), 5′-CCTGGTGAGGCAAACTTCGAT-3′. The internal control primers were: GAPDHF, 5′-TGACCACAGTCCATGCCATC-3′; and GAPDHR, 5′-GACGGACACATTGGGGGTTAG-3′.

ESC culture, differentiation and colony assays

J1 ES cells were maintained as described (Wang et al., 2004). Scl:hCD4 mouse ES cells (kindly provided by Dr K. Choi at the University of Washington, St Louis) (Chung et al., 2002) were generated by knock-in of the non-functional human CD4 receptor into the SCL locus, thus allowing quantitative counting of SCL$^+$ cells by FACS analysis using the anti-hCD4 monoclonal antibody. Mouse ESC clones D5 and E1 that stably overexpress ZBP-89, and a control stable ES cell line, were generated by transfecting the linearized plasmid encoding mouse ZBP-89 under control of the β-actin promoter (for the D5 and E1 ESCs) or the vector alone (control ESC), followed by selection in neomycin. The resulting ES cell lines were

maintained on the mouse feeder cell line SNL in ES medium containing Dulbecco's modified Eagle medium (DMEM), 10 ng/ml mouse leukemia inhibitory factor (mLIF; Chemicon International, Temecula, CA), 15% fetal calf serum (FCS; HyClone, Logan, UT), 1 mM sodium pyruvate, 2 mM glutamine, 0.1 mM nonessential acid, 100 μM monothioglycerol (MTG, Sigma, St Louis, MO), 50 U/ml penicillin and 50 μg/ml streptomycin. ESCs were cultured to about 50% confluence on gelatin-coated plates prior to EB induction.

Differentiation of ESCs into EBs and colony assays were carried out as described (Wang et al., 2004). Briefly, EBs were generated in either liquid or 1% methylcellulose cultures (1×10^4 ESCs per 35-mm Petri dish) in ESC differentiation medium containing Iscove Modified Dulbecco's medium (IMDM), 15% FCS, 2 mM glutamine, 450 μM MTG, 50 μg/ml ascorbic acid and 20% BIT [1% bovine serum albumin (BSA), 10 μg/ml insulin and 200 μg/ml transferrin (StemCell Technologies)]. BL-CFCs were counted from EBs at 4 days of culture. To generate blast colonies from hemangioblasts, 1×10^4 EBs/ml were replated on 35-mm petri dishes in 1% methylcellulose in the presence of IMDM, 2 mM glutamine, 450 μM MTG, 25 μg/ml ascorbic acid, 20% BIT, 5 ng/ml human vascular endothelial growth factor (hVEGF), 50 ng/ml SCF, 10 ng/ml human fibroblast growth factor 2 (hFGF2), and 2 U/ml human erythropoietin (hEPO). BL-CFCs can be recognized as loose clusters of cells after 4 days of culture. Primitive erythroid progenitors were obtained from day 6 EBs. Definitive myeloid progenitors were obtained from day 10-12 EBs in 1% methylcellulose matrix. Hematopoietic colonies were counted 7-10 days after replating. For vascular-like EB culture, EBs were initially generated in 1% methylcellulose culture for 11 days, then transferred into collagen matrix for 3 days before being examined.

Immunostaining, flow cytometric analysis and sorting

Single-cell suspensions were prepared from EBs cultured for different time periods by trypsinization for 2 minutes at 37°C and then passing through a 21-gauge needle. Cells were immunostained (15 minutes, 4°C in PBS/0.1% BSA buffer) with phycoerythrin (PE)- or allophycocyanin (APC)-rat anti-mouse monoclonal antibodies against c-KIT (APC), FLK1 (PE), SCA1 (PE), CD45 (FITC), VE-Cadherin, CD31 (PE) (PharMingen, Becton, San Diego, CA), or, in the case of Scl-hCD4 ESCs, with an additional biotinylated mouse monoclonal antibody to human CD4 (CALTAG), followed by streptavidin-APC (Sav-APC; Pharmingen). Cells stained with anti-VE-cadherin were visualized using a secondary PE-labeled goat anti-rat IgG. Cells were then analyzed using FACS Caliber, or sorted using FACS MoFlo (Becton Dickinson).

Western blotting

EBs were lysed with RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl (TBS), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, freshly protease inhibitor cocktail (Roche)] for 30 minutes on ice then spun for 10 minutes at 4472 ***g***. Samples were loaded onto 7.5% SDS-polyacrylamide gels together with molecular weight markers (Invitrogen), and transferred to nitrocellulose membrane. The blots were incubated with goat polyclonal IgG antibody to the conserved C terminus of human ZBP-89 (Santa Cruz; diluted 1:200 in TBS containing 0.05% Tween-20) overnight and washed extensively. After incubating with a donkey anti-goat IgG-horseradish peroxidase (HRP)-conjugated secondary antibody (Dako; diluted 1:2000) at room temperature for 1 hour, the blots were visualized using the ECL kit (BioRad), according to the manufacturer's instructions. A mouse monoclonal anti-β-actin antibody (Sigma) at a dilution of 1:500 was used to document equal loading per lane.

RESULTS

Knockdown of *ZBP-89* produces a bloodless phenotype in zebrafish embryos

To explore its role in hematopoiesis in vivo, we cloned the zebrafish ortholog of *ZBP-89* (see Fig. S1 in the supplementary material). Zebrafish ZBP-89 shares a 74.1% similarity and 61.8% amino acid identity with human ZBP-89. Whole-mount in situ hybridization showed that *zbp-89* is expressed in the posterior lateral mesoderm, the head mesenchyme and the intermediate cell mass (ICM, equivalent to the yolk sac in mammals) of 24-hour postfertilization (hpf) zebrafish embryos (Fig. 1A-C), an expression pattern resembling that of *scl* (Liao et al., 1998). We used antisense morpholinos (MOs) targeting the translation start site (*atg*MO) or the splice donor site (*splice*MO) in exon 8 in *zbp-89*, the latter of which causes a splicing defect, to knockdown expression of this gene in developing zebrafish embryos. No blood cells were observed either within or outside of the vasculature in either *atg*MO- or *splice*MO-injected live 48 hpf embryos (Fig. 1D-G; see also Movies 1 and 2 in the supplementary material), although all embryos displayed a beating heart. Over time, pericardial edema developed in MO-injected embryos, presumably because of the absence of blood circulation, and the morphants began to exhibit axis deformities, with the vast majority dying before 4 days post fertilization (dpf). Zebrafish one- to two-cell embryos injected with an MO containing five mismatches in *atg*MO (mismatched *atg*MO) developed normally (Fig. 1D,E). Subsequent analyses were carried out in the *atg*MO-generated morphants. The bloodless phenotype caused by the depletion of ZBP-89 was rescued by co-injecting *atg*MO with wild-type human *ZBP-89* mRNA into wild-type embryos (data not shown), or with a plasmid in which ZBP-89 is expressed under the control of the *flk1* promoter into transgenic *Tg* (*gata1:GFP*) embryos (Fig. 1H-J).

zbp-89-depleted zebrafish embryos fail to develop primitive or definitive blood

In zebrafish, primitive hematopoiesis arises from two regions of the lateral mesoderm: the anterior lateral mesoderm located rostrally in the head region that gives rise to the myeloid lineage, and the posterior lateral mesoderm, which forms the ICM (the equivalent of the extraembryonic mammalian yolk sac blood island) just ventral to the notochord, where erythroid development takes place (Al-Adhami and Kunz, 1977; Fouquet et al., 1997; Gering et al., 1998; Herbomel et al., 1999). Expression of the early hematopoiesis markers *scl*, *lmo2* and *gata2* (Liao et al., 1998) was reduced in 12-24 hpf embryos depleted of *zbp-89* (Fig. 1L,N,P; data not shown) when compared with controls (Fig. 1K,M,O). This loss was not caused by defects in mesoderm conversion into blood and blood vessel precursors, as reflected by the normal expression of the caudal *hox*-related gene *cdx4* (Davidson et al., 2003) (Fig. 1Q,R). Whole-mount TUNEL staining of 22 hpf *zbp-89* morphants did not reveal a significant increase in the apoptosis of cells in the embryonic blood island and tail bud region when compared with wild-type embryos (data not shown), suggesting that ZBP-89 may be required for fate specification rather than survival of early hematopoietic precursors. Expression of the primitive erythroid markers *gata1* and *tif1g* (*moonshine*) in the ICM (Fig. 2A,C) (Ransom et al., 2004) was lost almost entirely in *zbp-89* morphants (Fig. 2B,D, respectively). However, expression of *tif1g* in the central nervous system was unaffected (Fig. 2C,D), reflecting the specificity of the *atg*MO-induced defects for regions of active hematopoiesis. Expression of the primitive myeloid lineage markers *pu.1*, *mpo* and *l-plastin* (Fig. 2E,G,I) was also markedly downregulated in 24 hpf *zbp-89* morphants (Fig. 2F,H,J).

Definitive hematopoiesis in zebrafish embryos occurs by 32 hpf in the ventral wall of the dorsal aorta, a region equivalent to the mammalian aorta-gonad-mesonephros (AGM) region (Burns et al., 2002; Kalev-Zylinska et al., 2002). We examined expression of the definitive hematopoiesis markers *runx1* and *c-myb*, ablation of either one of which results in a complete absence of definitive hematopoiesis (Burns et al., 2002; Mucenski et al., 1991; Okuda

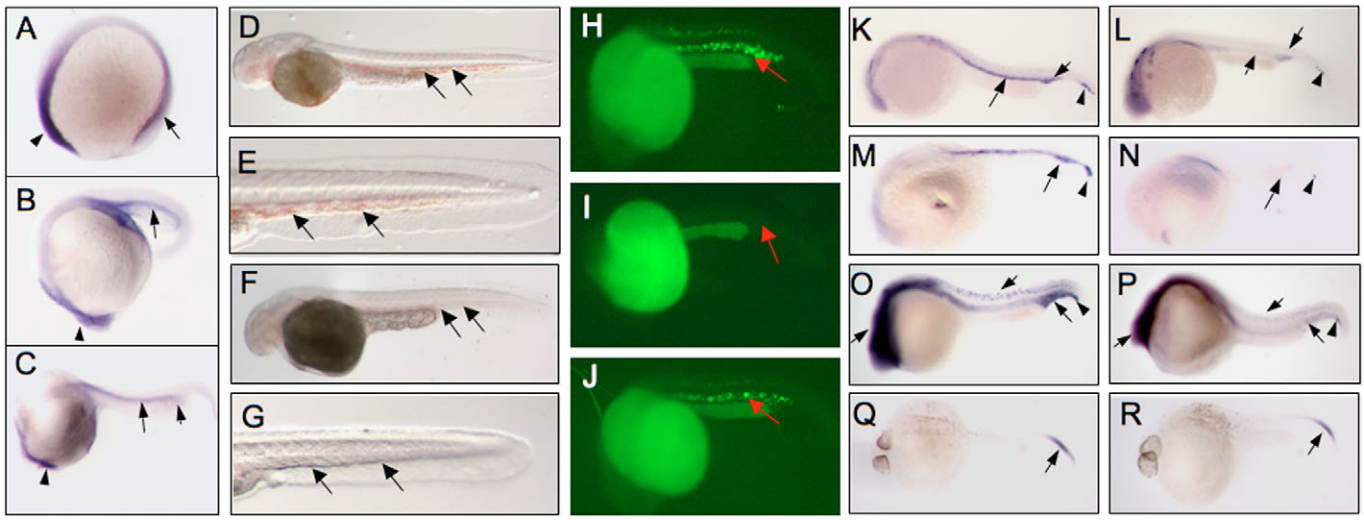

Fig. 1. Expression profile of *zbp-89* in wild-type zebrafish embryos and the phenotype of *zbp-89* morphants. (**A-C**) Tissue expression of *zbp-89* by whole-mount in situ hybridization of 12 hpf (A), 18 hpf (B) and 24 hpf (C) embryos. Lateral views, dorsal upward, anterior to the left are shown. By 12 hpf (A), *zbp-89* is expressed in the anterior (arrowhead) and posterior (arrow) lateral plate mesoderm. At 18 hpf (B), it is strongly expressed in the anterior ICM (arrow) and the brain region (arrowhead). At 24 hpf (C), expression in the anterior ICM (arrow) and the wedge region of the anterior ICM (short arrow) is seen together with expression in the brain (arrowhead). No signal was detected with the sense probe (not shown). (**D-G**) Loss of ZBP-89 results in a bloodless phenotype. DAF staining of 48 hpf whole-mount zebrafish embryos. Blood (arrows) is present in the representative control (mismatch *atg*MO; D,E) but not in *atg*MO-injected (F,G) embryos (different embryos are shown in F and G). The bloodless phenotype was present in 78% and 62% of the 100-115 embryos injected at the one- to two-cell stage with either *ZBP-89 atg*MO or *splice*MO, respectively. All views are lateral with anterior left and dorsal top. (**H-J**). Rescue of *zbp-89* morphants by tissue-specific expression of human ZBP-89 under control of the *flk1* promoter. *Gata1*-driven GFP in 22 hpf transgenic (*gata1:EGFP*) zebrafish embryos that were untreated (H), injected with *atgMO* only (I) or injected with *atgMO* plus flk1-ZBP-89 plasmid (J) at the one- to two-cell stage. Single 22 hpf embryos were examined under a fluorescent microscope revealing *gata1* expression (red arrows) in the ICM of the untreated (H) and ZBP-89-rescued (J), but not in *atgMO* only-injected (I), embryos. (**K-R**) Effect of ZBP-89 knockdown on expression of early hematopoiesis markers. Whole-mount in situ hybridization in wild-type (WT; K,M,O,Q) and ZBP-89-depleted (L,N,P,R) 24 hpf embryos. Embryos were hybridized with digoxigenin-labeled RNA probes for *scl* (K,L), *lmo2* (M,N) and *gata2* (O,P). In wild type, *scl* is expressed in the anterior ICM (K, arrow), posterior ICM (arrowhead in K) and the wedge region of anterior the ICM (K, short arrow). Only minimal expression remains in the wedge region and the posterior ICM in the *zbp-89* morphants (L). *Lmo2* (M) and *gata2* (O) display a similar expression pattern to *scl* in wild-type embryos, and expression of both is markedly reduced in the ICM of *zbp-89* morphants (N and P, respectively). *Gata2* is also expressed in the brain and spinal ganglia (short arrows in O,P); its expression at these sites is somewhat reduced by the loss of ZBP-89. (Q,R) Expression of *cdx4* in the posterior ICM of 24 hpf wild type (Q) and *zbp-89* morphants (R). *cdx4* expression is not affected by the loss of ZBP-89.

et al., 1996). Expression of *runx1* and *c-myb* was reduced by depletion of ZBP-89 in 24 hpf and 48 hpf zebrafish embryos (Fig. 2K-R). This was particularly evident in the stem cell population associated with the dorsal aorta; the few cells that continued to express these markers were mostly confined to the posterior-most portion of the ICM. Thus, ZBP-89 depletion phenocopies the defects in primitive and definitive hematopoiesis seen in *scl* null mice (Porcher et al., 1996; Robb et al., 1996) and in *scl* zebrafish morphants (Patterson et al., 2005). Expression of *fli1a* and *flk1*, which are indicative of primary blood vessel formation, was minimally affected by the loss of ZBP-89 in 18-20 hpf embryos (Fig. 2S,T; data not shown).

ZBP-89 acts downstream of *clo* but upstream of *scl*

To position *ZBP-89* in the regulatory gene cascade leading to blood formation, we injected human *ZBP-89* mRNA into one- to two-cell stage embryos collected from the clo^{fv087b} mapping cross. The injected embryos were fixed at 3 dpf and stained with DAF, and those with positive DAF-staining were genotyped with the SSR marker z1496, which is very tightly linked to clo^{fv087b} (Fig. 3). Rescue of the hematopoietic program, detected by DAF staining, was observed in homozygous *clo* mutant embryos (Fig. 3E,F), indicating that *ZBP-89* overexpression can rescue hematopoiesis in the complete absence of *clo* function. This finding was also confirmed by overexpressing ZBP-89 in embryos from $fv087b^{+/-}$ *clo* Tg (*gata1:GFP*) crosses. As shown in Fig. 3H, this treatment rescued the $GATA1^{+}$ cell population in the anterior ICM.

To assess whether forced expression of ZBP-89 also reconstitutes blood vessel formation, we used a transgenic zebrafish line, Tg (*flk1:EGFP*), in which the *flk1* promoter directs the expression of EGFP (Cross et al., 2003). *Flk1:EGFP* is expressed in the blood vasculature of wild-type embryos (Fig. 3I), but is not expressed in *clo* embryos from $fv087b^{+/-}$:Tg (*flk1:EGFP*) crosses (Fig. 3J). Injection of *ZBP-89* mRNA into one-cell stage embryos from such crosses did not rescue *flk1* expression (Fig. 3K). Thus, *ZBP-89* also acts functionally downstream of *clo*, but, in contrast to SCL, is able to rescue the hematopoietic but not the vascular lineage. We next evaluated the *ZBP-89* transcript levels in *clo* mutants by RT-PCR. As shown in Fig. 3L, *ZBP-89* mRNA was significantly reduced in *clo* null mutant embryos when compared with wild-type embryos, which is consistent with the above functional data.

To evaluate the functional relationship of ZBP-89 with SCL, *scl* RNA was co-injected with *atg*MO into one- to two-stage transgenic Tg (*gata1:EGFP*) zebrafish embryos, where *EGFP* is

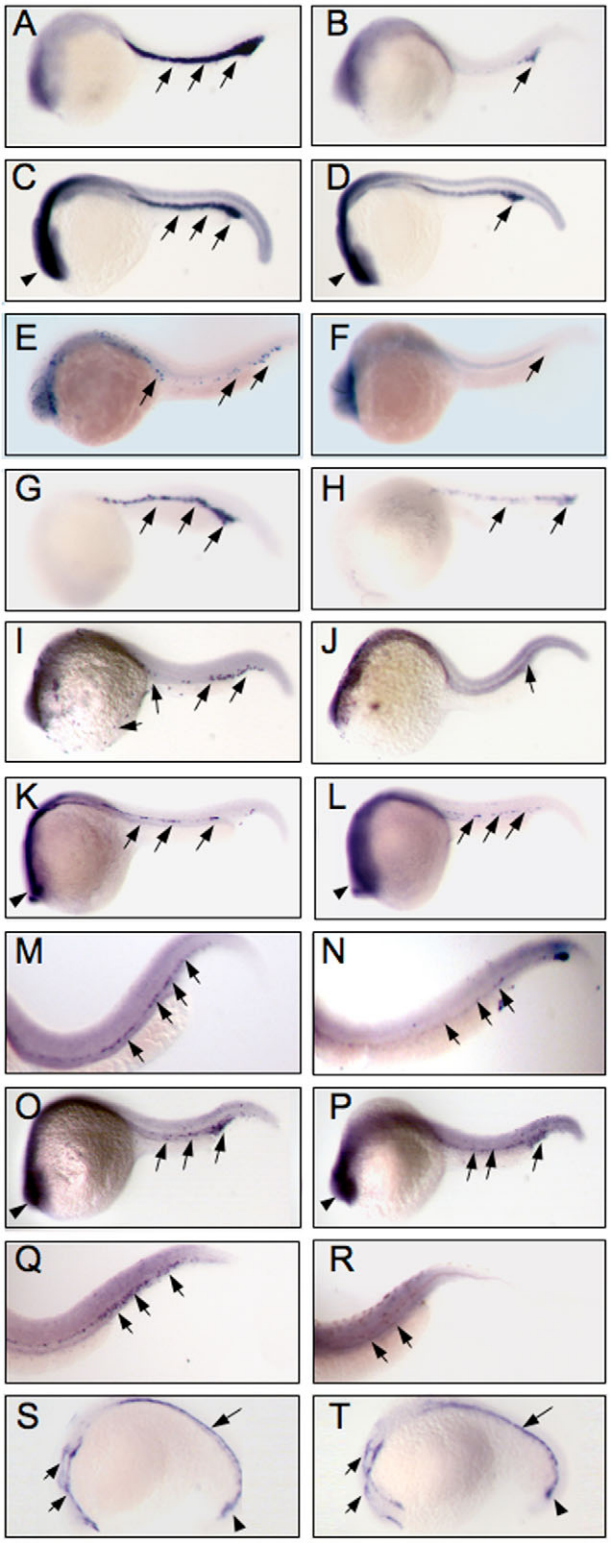

Fig. 2. Expression of hematopoietic and vascular markers in wild type and *zbp-89* morphants. (**A-R**) Expression of primitive erythroid (A-D), primitive myeloid (E-J) and definitive hematopoietic markers (K-R). Normal expression (arrows) of the primitive erythroid genes *gata1* (A) and *tif1g* (C) in the anterior ICM of wild-type embryos is almost completely lost in *zbp-89* morphants (B,D, respectively). Expression of *tif1g* in neural tissue (arrowheads) is not affected. Expression of the primitive myeloid markers *pu.1*, *mpo* and *l-plastin* in wild type (E,G,I) and in *zbp-89* morphants (F,H,J). The normal expression of *pu.1* in primitive macrophages in the anterior ICM (arrow) in 24 hpf embryos (E) is markedly reduced by depletion of ZBP-89 (F). Its expression is also reduced in 20 hpf embryos in the head, rostral blood islands and ICM (not shown). *mpo* (G) and *l-plastin* (I) are normally expressed in the ICM of 24 hpf embryos (arrows) and in the anterior yolk region (*l-plastin*). Both markers are severely reduced by the loss of ZBP-89 (H,J, respectively). (K-R) Expression of the definitive hematopoietic markers *runx1* and *c-myb*. Expression of *runx1* begins in the ICM (K, arrows) at 24 hpf and is well developed in the ventral dorsal aorta at 48 hpf (M, arrows). Loss of ZBP-89 markedly reduces expression of *runx1* in 24 hpf (L) and 48 hpf (N) embryos. *c-myb* is normally expressed in the ICM of wild-type embryos at 24 hpf (O, arrows). In 48 hpf embryos, cells expressing *c-myb* are found scattered along the ventral wall of the dorsal aorta (Q, arrows), within the first progenitors of definitive hematopoiesis. *c-myb* expression is significantly reduced in *zbp-89* morphants in both 24 and 48 hpf embryos (P,R). Non-hematopoietic expression of *runx1* (K,L) and *c-myb* (O,P) in neural tissue (arrowheads) was not affected by the loss of ZBP-89. (**S**,**T**) *flk1* expression in 20 hpf wild type and in *zbp-89* morphants. *flk1* is normally expressed in cells located in two strips of the anterior lateral mesoderm (short arrows), and in the forming anterior (arrows) and posterior (arrowheads) ICM (S). This expression was not affected by the loss of ZBP-89 (T). All views are lateral with anterior left and dorsal top.

under control of the *gata1* promoter, a line that strictly labels erythrocytes (Long et al., 1997). We found that *scl* RNA rescued GATA1 expression in 22 hpf embryos, as well as pericardial edema and circulating blood in 48 hpf embryos (Fig. 4), and axial deformities in 3 dpf embryos (not shown). Thus, ZBP-89 acts upstream of *scl* in the transcriptional hierarchy of early hematopoiesis.

Ectopic expression of ZBP-89 in wild-type zebrafish embryos expands the hematopoietic markers but impairs vascular remodeling

Forced expression of ZBP-89 in wild-type zebrafish embryos caused significant expansion of the early hematopoietic markers *scl*, *lmo2* and *gata1* (Fig. 5B,D,F). However, expression levels of the *wt1* and *pax2.1* markers of the pronephros and pronephric duct, respectively (Gering et al., 2003), were unchanged (data not shown), suggesting that ectopic *ZBP-89* expression did not change the fates of kidney mesoderm in the early lateral mesoderm. ZBP-89 overexpression induced a simultaneous reduction in *flk1* (Fig. 5H,L) and *tie1* (Fig. 5J) expression in the intersomitic and axial blood vessels in 24 hpf embryos when compared with control (Fig. 5G,I,K).

Expression profile of *ZBP-89* in ESCs, hemangioblasts, and hematopoietic and angioblast progenitors

To assess whether ZBP-89 is also crucial for hematopoietic development in mammals, we analyzed the expression of *ZBP-89* in mouse embryonic stem cells (ESCs) undergoing differentiation into hematopoietic stem cells, and determined the consequences of its stable overexpression on hematopoietic and vascular development in vitro. The *ZBP-89* transcript was not detected in undifferentiated ESCs but was rapidly induced in early (day 1) EBs, peaking in day 2 EBs then declining afterwards (Fig. 6A). Under similar conditions, expression of the early hematopoietic markers *runx1*, *scl* and *gata1* begins at or after day 3 of culture (Lacaud et al., 2002), suggesting that in the mouse, as in zebrafish, *ZBP-89* acts upstream of these factors. The ZBP-89 protein was prominently expressed in day 3

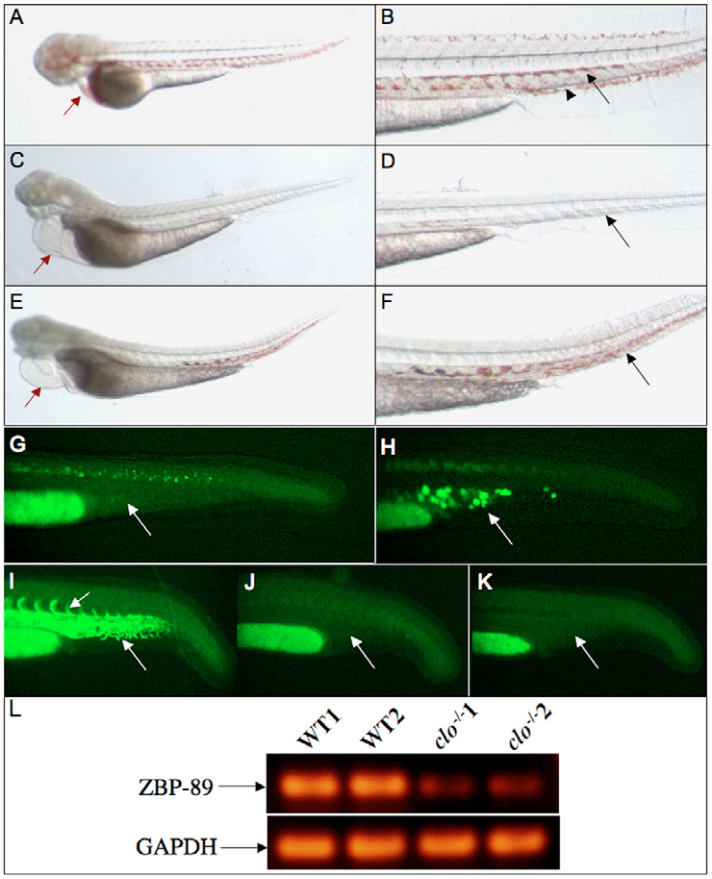

Fig. 3. ZBP-89 rescues the hematopoietic but not the vascular phenotype in *clo*$^{-/-}$ mutant zebrafish embryos. (**A**,**B**) Wild-type whole-mount 3 dpf embryos stained with DAF and examined at 4× (A) and 10× (B) magnification, showing blood cells in the heart (red arrow), the dorsal aorta (arrow, B) and the posterior cardinal vein (arrowhead, B). (**C**,**D**) *Clo*$^{-/-}$ embryo examined at at 4× (C) and 10× (D) magnification. No extravascular or circulating blood is seen. Red arrow in C indicates the dilated bloodless heart. (**E**,**F**) *ZBP-89* sense RNA injected into *clo*$^{-/-}$ embryos, shown at two different magnifications. Blood formation in the trunk is evident (arrow in F), but the heart chamber remains dilated and devoid of erythrocytes (E), consistent with the absence of blood vessels. (G,H) *Gata1*-driven GFP in *clo*$^{-/-}$ 48 hpf embryos transgenic (Tg) for *gata-1:EGFP*, before (**G**) and after (**H**) overexpression of *ZBP-89*. *Gata1* is found in the GFP-labeled erythroid lineage (arrow in H) in the anterior ICM of *clo*$^{-/-}$ embryos overexpressing *ZBP-89*. (**I-K**) Tg (*Flk1:EGFP*) 48 hpf embryos showing fluorescence in the axial (arrow in I) and intersomitic (short arrow in I) blood vessels; fluorescence in the yolk extension is nonspecific. (J,K) *flk1*-driven GFP in *clo*$^{-/-}$ 48 hpf embryos transgenic for *flk1:EGFP* before (J) and after (K) overexpression of *ZBP-89*. Arrows show a lack of blood vessel formation in both cases. (**L**) *ZBP-89* expression profile in two different 18 hpf wild-type (WT1 and WT2) and *clo*$^{-/-}$ (*clo*$^{-/-}$1 and *clo*$^{-/-}$2) embryos, assessed using RT-PCR. *GAPDH* expression was examined simultaneously as a reference.

EB-derived FLK1$^+$ mesoderm precursors (Fig. 6A, inset), and its transcript was also present in FLK1$^+$SCL$^+$ hemangioblasts (BL-CFC), FLK1$^-$SCL$^+$ hematopoietic progenitors and in FLK1$^+$SCL$^-$ angioblasts derived from day 4 EBs (see Fig. S2 in the supplementary material).

Overexpression of ZBP-89 in mouse ESCs leads to increased hematopoiesis but reduced sprouting angiogenesis

We evaluated the role of ZBP-89 in hematopoietic and endothelial lineage commitment using the mouse ES cell in vitro differentiation system. Stable ectopic expression of ZBP-89 in ESC/EBs led to a significant increase in the hemangioblast (blast colony forming cell, BL-CFC) population (Fig. 6B). Stable overexpression of ZBP-89 in ESC/EBs also induced a 5-fold increase in the number of SCA1$^+$/c-KIT$^+$ hematopoietic progenitors in 3 day and 4 day EB cultures (not shown), a 2-fold expansion of primitive erythroid colonies (BFU-Es) (Fig. 6B), definitive erythrocyte (CFU-E), macrophage (CFU-M), granulocyte-macrophage-megakaryocyte (CFU-GEMM) and granulocyte-macrophage (CFU-GM) colonies (Kennedy et al., 1997) (Fig. 6C), and a 3-fold increase in the number of the cell population expressing the hematopoietic marker CD45 (Fig. 6D). By contrast, formation of the vascular plexus (Feraud et al., 2001) by the cultured ZBP-89-overexpressing EBs

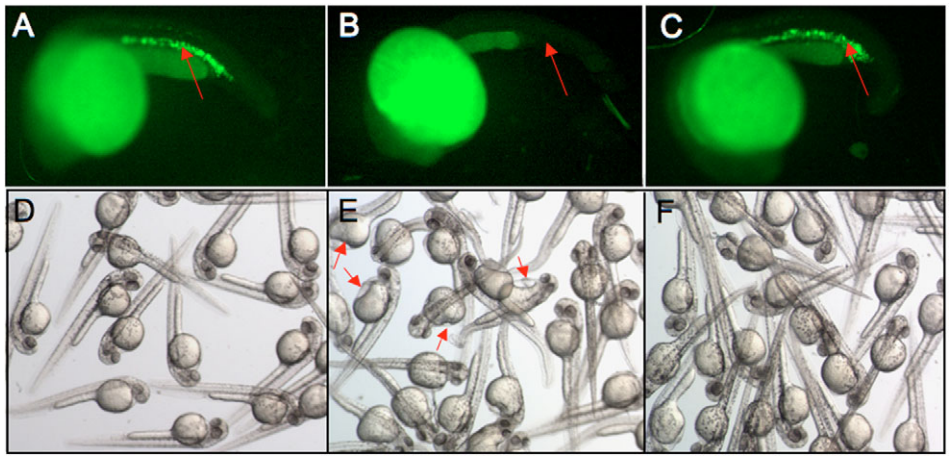

Fig. 4. Rescue of *zbp-89* morphants by zebrafish *scl* sense RNA. (**A-F**) *Gata1*-driven GFP in 22 hpf transgenic (*gata1:EGFP*) zebrafish embryos that were untreated (A,D), injected with *atgMO* only (B,E), or injected with *atgMO* plus *scl* RNA (C,F) at the one- to two-cell stage. (A-C) Single 22 hpf embryos examined under a fluorescent microscope revealing *gata1* expression (red arrows) in the ICM of the untreated (A) and *scl*-rescued (C) embryos, but not in the *atgMO* only-injected (B) embryos. (D-F) Bright-field low magnification images of the respective untreated and treated Tg *gata1:EGFP* embryos at 48 hpf. Short arrows in E point to pericardial edema in the *zbp-89* morphants.

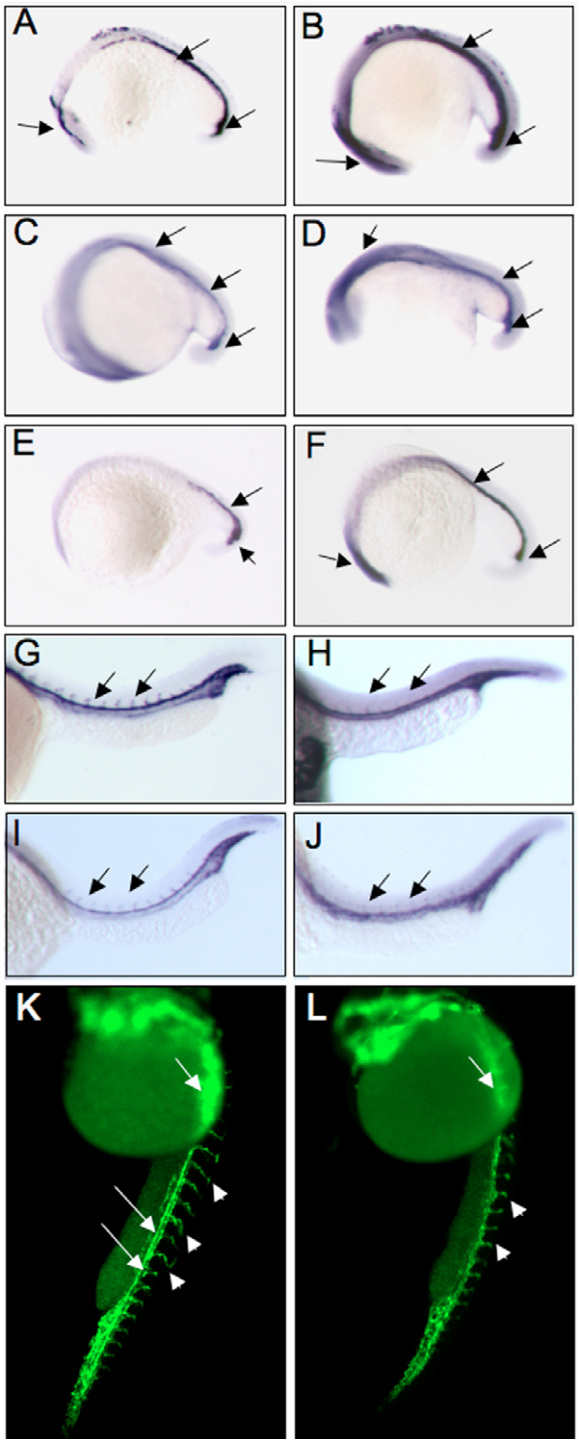

Fig. 5. Effect of forced expression of ZBP-89 on hematopoietic and vascular development in zebrafish embryos. (**A-J**) Wild type (A,C,E,G,I) and ZBP-89-overexpressing (B,D,F,H,J) 18 hpf (A-F) or 24 hpf (G-J) zebrafish embryos. In situ hybridization of wild-type embryos overexpressing ZBP-89 reveals a marked increase in the expression of *scl* (B), *lmo2* (D) and *gata1* (F), but a marked reduction in intersomitic expression of the endothelial markers *flk1* (H) and *tie1* (J), when compared with the respective untreated wild-type embryos (A,C,E,G,I), (**K**,**L**) Wild-type (K) and ZBP-89-overexpressing (L) Tg (*flk1:EGFP*) zebrafish embryos examined at 24 hpf. Overexpression of ZBP-89 caused a significant reduction in *flk1* expression in the axial (arrows), brain (short arrow) and intersomitic (arrowheads) blood vessels when compared with the control embryo.

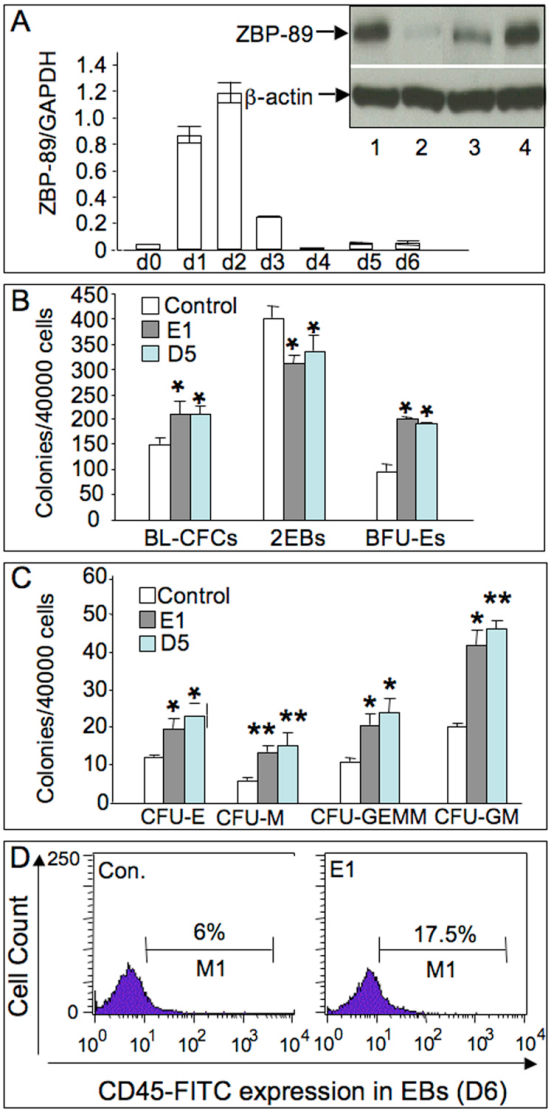

Fig. 6. Expression profile of ZBP-89 and effects of its overexpression on hematopoiesis in mouse EB cultures. (**A**) *ZBP-89* expression profile in undifferentiated ESCs and differentiating EBs quantified with real-time PCR. Numbers indicate the day of differentiation. Results represent mean±s.d. of three independent experiments. Inset, western blot analysis showing induction of the ZBP-89 protein mainly in FLK1$^+$ mesoderm precursors in day 3 EBs. Lane 1, positive control; lane 2, uninduced ESCs; lane 3, day 3 FLK1$^-$ mesodermal cells; lane 4, FLK1$^+$ mesoderm precursors. Equivalent amounts of cell lysate were loaded per lane as reflected by the β-actin signal. (**B**) Histograms showing the number of blasts (BL-CFCs), secondary EBs (2°EBs) and primitive erythroid (BFU-Es) colonies generated from control, D5 and E1 clones (bars represent the mean number of colonies±s.d. from two independent experiments). (**C**) Histograms (mean±s.d., n=3) showing the numbers of definitive erythroid (CFU-E), macrophage (CFU-M), granulocyte-macrophage-megakaryocyte (CFU-GEMM) and granulocyte-macrophage (CFU-GM) colonies. *P<0.01; **P<0.001 (paired t-test). (**D**) Flow cytometric analysis of a single-cell suspension from E1-derived EBs (see Materials and methods) stained with FITC-labeled rat anti-mouse CD45 monoclonal antibody. *ZBP-89* overexpression significantly increased the number of CD45$^+$ hematopoietic progenitors.

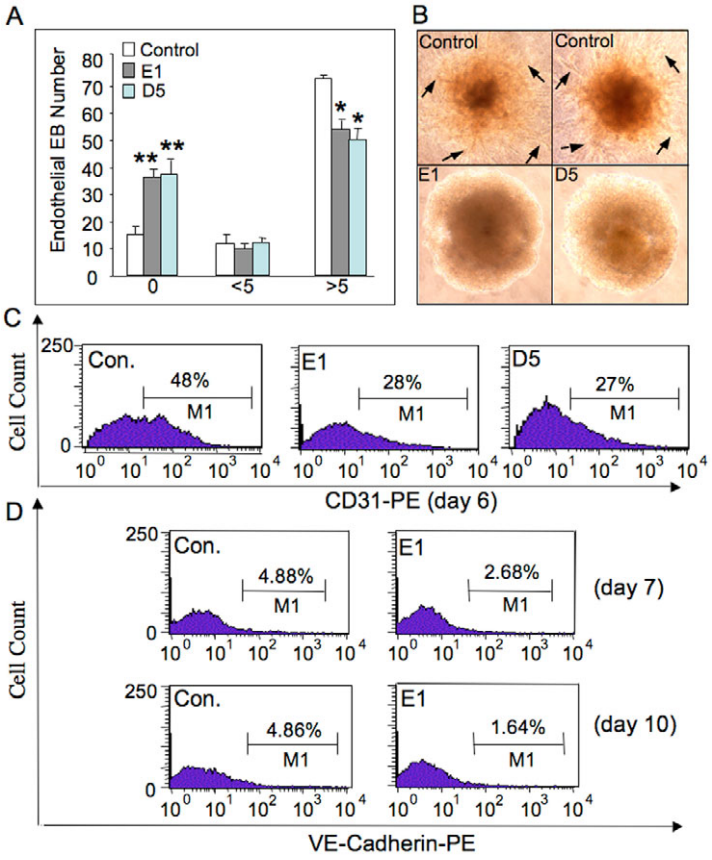

Fig. 7. Ectopic expression of ZBP-89 impairs angiogenesis in mouse EB cultures. (**A**) Primary day 11 EBs were dissociated and cultured in collagen matrix with growth factors for endothelial cell differentiation for four days. Histograms (mean±s.d., *n*=3) show the number of primary day 11 EBs with 0, <5 or >5 branching vessels/EB derived from the D5 and E1 clones, compared with the control. **P*<0.01; ***P*<0.001; NS, not significant. (**B**) Representative images of control-, E1- or D5-derived sprouting EB colonies. The reduction in the branching vessels (arrows in controls) in EBs derived from overexpressing ZBP-89 clones E1 and D5 is also clearly reflected by the compactness of the respective colonies; fewer branches are produced compared with the controls. (**C**,**D**) Flow cytometric analysis of a single-cell suspension of EBs overexpressing ZBP-89 and stained with PE-labeled anti-CD31 (day 6 culture) or anti-VE-Cadherin (day 7 and day 10 cultures) antibodies. ZBP-89 overexpression significantly reduced the number of CD31$^+$ and VE-Cadherin$^+$ angioblasts (similar results were obtained in two other independent experiments).

was significantly reduced (Fig. 7A,B), as was the number of the CD31$^+$ (Fig. 7C) and VE-Cadherin$^+$ (Fig. 7D) endothelial cell populations.

DISCUSSION

A major finding in this report is that the Krüpple-like zinc finger transcription factor ZBP-89 is essential for blood formation in zebrafish embryos and in vitro from differentiating mouse ESCs. *zbp-89* depletion in zebrafish impairs expression of the early hematopoietic genes *scl*, *gata2* and *lmo2*, the primitive erythropoietic genes *gata1* and *tif1g* (*moonshine*), the primitive myeloid genes *pu.1*, *mpo* and *l-plastin*, and the definitive hematopoietic genes *runx1* and *c-myb*. Forced expression of ZBP-89 in ESCs expanded the hematopoietic cell lineages. We place ZBP-89 upstream of *scl* and downstream of *clo* in controlling blood formation in zebrafish on the basis of the following findings. First, the bloodless phenotype in *zbp-89* morphants is rescued by *scl* RNA. Second, upregulation and depletion of *zbp-89* lead, respectively, to overexpression or loss of *scl* expression in zebrafish embryos. This finding, together with the similar expression profiles of both genes in zebrafish and their co-expression in hematopoietic progenitors, suggests that SCL may be a direct target of ZBP-89. Third, the *zbp-89* transcript is reduced in *clo*$^{-/-}$ mutants and *zbp-89* mRNA rescues the hematopoietic, but not the vascular, phenotype in *clo*$^{-/-}$ mutants.

The placement of *zbp-89* upstream of *scl* in the transcriptional hierarchy of hematopoiesis probably explains the hematopoietic defects seen in *zbp-89* morphants. Loss- and gain-of-function studies in vertebrate models have shown that SCL is essential for hematopoietic development from FLK1$^+$ hemangioblasts (Mikkola et al., 2003): *Scl*$^{-/-}$ mouse embryos die at embryonic day 9.5 because of a complete absence of primitive (yolk sac) and definitive (bone marrow) blood cells (Porcher et al., 1996; Robb and Begley, 1997; Robb et al., 1996; Shivdasani et al., 1995). SCL-depleted zebrafish embryos are also defective in primitive and definitive hematopoiesis, much like the *zbp-89* morphants, as a result of the loss of expression of *gata1*, *pu.1* and *runx1* (Patterson et al., 2005). SCL binds LMO2 directly, which bridges it to GATA1 and to E-proteins within a multicomponent complex that mediates the function of SCL in development of the definitive erythroid progenitors (Wadman et al., 1997). However, neither the DNA-binding nor the N-terminal transactivation domains of SCL are required for specification of hematopoietic cell fate from FLK1$^+$ mesoderm progenitors (Porcher et al., 1999), thus reflecting differences in the composition of the SCL complex. In support of this is the finding that, in the more primitive hematopoietic progenitors, GATA2 may replace GATA1 as a component of the SCL complex, which also contains SP1 (as has been observed in SCL-dependent activation of the c-*kit* promoter) (Lecuyer et al., 2002). These findings are consistent with the defects in primitive and definitive hematopoiesis observed in *gata2* null mice (Tsai et al., 1994), and with the known role of SP1 in hematopoiesis (Tenen et al., 1997). Interestingly, expression of *gata2* and *lmo2* is normal in SCL-depleted zebrafish embryos, but is significantly reduced in *zbp-89* morphants, suggesting that the expression of these factors, in addition to SCL, is also regulated by ZBP-89 in early hematopoietic progenitors. ZBP-89 contains a DNA-binding domain, as well as a transactivation domain (Hasegawa et al., 1997; Passantino et al., 1998). It may thus act as a transcriptional activator of *scl*, and/or as a component of the multi-factorial SCL complex, thereby endowing it with a DNA-binding function.

Gain-of-function experiments show that ZBP-89 acts as a negative regulator of angiogenesis both in vivo and in vitro. Although ZBP-89-depleted zebrafish embryos displayed no

detectable defects in vasculogenesis, as reflected by the normal expression of *flk1* and *fli1* markers, forced expression of ZBP-89 in zebrafish lead to angiogenic remodeling defects, as reflected by the impaired *tie1* and *flk1* expression in the intersomitic (Fig. 5H,J) and axial blood vessels (Fig. 5L). Furthermore, ectopic expression of ZBP-89 in ESCs lead to defective sprouting angiogenesis and a reduction in the number of $CD31^+$ and VE-Cadherin$^+$ endothelial cells. By contrast, the late angiogenic remodeling in mice and the loss of *flk1* expression in the dorsal aorta in zebrafish are both seen when SCL function is lost. One interpretation for these contrasting findings is that ZBP-89 may act as a transcriptional repressor of SCL in $FLK1^+SCL^-$ angioblasts, where ZBP-89 is expressed, leading to the observed angiogenic defects. This scenario may explain the inability of overexpressed ZBP-89 to rescue the vascular phenotype in $clo^{-/-}$ mutants, in contrast to SCL. Many transcription factors, including ZBP-89 (Merchant et al., 1996), SCL (Grutz et al., 1998; Lahlil et al., 2004) and GATA1 (Rodriguez et al., 2005) can function as activators or repressors, in part through the differential recruitment of co-activators and co-repressors depending on the cellular context. Alternatively, enforced expression of ZBP-89 in angioblasts might interfere with endogenous SCL complexes through sequestration, leading to the same phenotype that is produced by the loss of function of SCL. Because loss of ZBP-89 function is not associated with defects in vasculogenesis, its downregulation of SCL when overexpressed appears to be limited to the angiogenic phase of vascular development. Conditional loss of ZBP-89 function will help to distinguish between these possibilities.

In addition to its role in hemangioblast fate commitment towards hematopoietic progenitors shown here, ZBP-89 may have additional roles at other branching points in the hematopoietic transcriptional hierarchy. Conditional knockout studies in mice have demonstrated that sustained *scl* expression is essential for hematopoietic differentiation towards the erythroid-megakaryocytic pathway (Hall et al., 2003; Mikkola et al., 2003; Sanchez et al., 2001). Whether ZBP-89 is involved in the sustained expression of *scl*, and/or participates in the function of the SCL complex at this stage, remains to be determined.

In summary, our results suggest that ZBP-89 is a lineage-determining transcription factor that not only activates hematopoietic lineage-specific genetic programs, but may also suppress endothelial cell differentiation. Its position upstream of SCL in the transcriptional hierarchy of hematopoiesis suggests that it may also be involved in some of the effects that SCL mediates in adult hematopoiesis.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/18/3641/DC1

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