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c-myc in the hematopoietic lineage is crucial for its angiogenic function in the mouse embryo

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The *c-myc* proto-oncogene, which is crucial for the progression of many human cancers, has been implicated in key cellular processes in diverse cell types, including endothelial cells that line the blood vessels and are critical for angiogenesis. The de novo differentiation of endothelial cells is known as vasculogenesis, whereas the growth of new blood vessels from pre-existing vessels is known as angiogenesis. To ascertain the function of *c-myc* in vascular development, we deleted *c-myc* in selected cell lineages. Embryos lacking *c-myc* in endothelial and hematopoietic lineages phenocopied those lacking *c-myc* in the entire embryo proper. At embryonic day (E) 10.5, both mutant embryos were grossly normal, had initiated primitive hematopoiesis, and both survived until E11.5-12.5, longer than the complete null. However, they progressively developed defective hematopoiesis and angiogenesis. The majority of embryos lacking *c-myc* specifically in hematopoietic cells phenocopied those lacking *c-myc* in endothelial and hematopoietic lineages, with impaired definitive hematopoiesis as well as angiogenic remodeling. *c-myc* is required for embryonic hematopoietic stem cell differentiation, through a cell-autonomous mechanism. Surprisingly, *c-myc* is not required for vasculogenesis in the embryo. *c-myc* deletion in endothelial cells does not abrogate endothelial proliferation, survival, migration or capillary formation. Embryos lacking *c-myc* in a majority of endothelial cells can survive beyond E12.5. Our findings reveal that hematopoiesis is a major function of *c-myc* in embryos and support the notion that *c-myc* functions in selected cell lineages rather than in a ubiquitous manner in mammalian development.

KEY WORDS: *c-myc*, Angiogenesis, Vasculogenesis, Mouse, Hematopoiesis, Myc, Vascular development

INTRODUCTION

The *c-myc* (*Myc* – Mouse Genome Informatics) proto-oncogene encodes a basic helix-loop-helix/leucine zipper transcription factor that is short-lived but rapidly induced upon serum stimulation. Its deregulation is associated with a wide range of human cancers (Adhikary and Eilers, 2005; Evan et al., 2005; Grandori et al., 2000; Pelengaris et al., 2002), and its overexpression is highly tumorigenic in many types of tissues in animals. A large body of work performed in cultured cells shows that c-Myc is expressed broadly and functions as a central regulator of normal cellular programs, including cell proliferation, differentiation, growth, survival and migration in many cell types (Grandori et al., 2000). Although these studies have made tremendous contributions to our understanding of the cellular mechanisms underlying the oncogenic effects of c-Myc, the physiological function of this protein remains largely unknown.

c-myc-null embryos exhibit severe developmental abnormalities in a wide range of organs and die early in gestation before 10.5 days post coitum (dpc) (Davis et al., 1993; Trumpp et al., 2001), supporting the notion that *c-myc* is essential for a broad range of organ development. Recent studies suggest that c-Myc is required for the proliferation of progenitor cells and the self-renewal of stem cells (Murphy et al., 2005). In the intestine, c-Myc is expressed in the proliferative zone of intestinal crypts, where putative intestinal stem cells reside, and is essential for the formation of these crypts (Bettess et al., 2005; Muncan et al., 2006). Similarly, in the skin epidermis, c-Myc is expressed in the proliferative basal layer and bulge region, where stem and progenitor cells are located (Bull et al., 2001). These new in vivo findings suggest that *c-myc* is uniquely required in the stem and progenitor cell compartments.

The role of *c-myc* in the development of the vascular system is of particular interest because it is crucial not only for all aspects of normal tissue function but also for pathological tumor growth and survival. Endothelial cells (ECs) line blood vessels and are the primary cell type responsible for blood vessel function and regeneration. Hematopoietic cells (HCs) give rise to the blood cells of the circulatory system. Differentiation of these two lineages first occurs in yolk sac blood islands, where ECs and HCs may arise from a common mesoderm-derived precursor: the hemangioblast (Cumano and Godin, 2007; Eichmann et al., 2002; Ema and Rossant, 2003). During vascular morphogenesis, ECs coalesce to assemble a primitive vascular network composed of a capillary plexus with uniform caliber and honeycomb appearance. This formation of blood vessels by de novo EC differentiation is known as vasculogenesis (Adams and Alitalo, 2007; Carmeliet, 2005). The primitive capillary plexus subsequently undergoes growth and remodeling to shape the mature vascular tree. Angiogenesis is the process of new blood vessel growth from existing vessels (Folkman, 2006; Hanahan and Folkman, 1996; Thurston et al., 2007). c-Myc has been shown to regulate angiogenesis by promoting the expression of pro-angiogenic factors such as VEGF in stromal cells,

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while inhibiting the expression of the anti-angiogenic factor thrombospondin 1 (Baudino et al., 2002; Dews et al., 2006; Knies-Bamforth et al., 2004; Mezquita et al., 2005; Shchors et al., 2006; Watnick et al., 2003). c-Myc is reportedly required for vasculogenesis during development, as *c-myc*-null embryos have no detectable blood vessels (Baudino et al., 2002). However, whether c-Myc in ECs plays an essential role in vasculogenesis or angiogenesis is currently unknown.

To ascertain the cell type-specific role of c-Myc during vascular development, we generated conditional knockouts (CKs) of the *c-myc* gene in *c-myc$^{flox/flox}$* mice using cell lineage-specific Cre lines. We were surprised to find that *c-myc* was not required for vasculogenesis, and that deleting *c-myc* in a majority of ECs was compatible with early embryo survival. By contrast, *c-myc* deletion was detrimental to hematopoietic lineages during development, and *c-myc* deletion in these lineages was sufficient to cause vascular developmental defects.

MATERIALS AND METHODS

Mice

Tie2-Cre, *Tie1-Cre*, *Sox2-Cre*, *Vav-iCre*, *Tie1-GFP*, *c-myc$^{flox/flox}$* and *c-myc$^{+/–}$* mice were previously described (Braren et al., 2006; de Boer et al., 2003; Gustafsson et al., 2001; Hayashi et al., 2002; Iljin et al., 2002; Trumpp et al., 2001). All animals were treated in accordance with the guidelines of the University of California San Francisco (UCSF) Institutional Animal Care and Use Committee.

Materials

Details of antibodies and real-time PCR primers used can be provided on request.

Imaging of embryos, whole-mount immunofluorescence staining, and EC isolation and culture

Previously established procedures (Braren et al., 2006) were followed.

Whole-mount *lacZ* staining

lacZ staining of embryos and yolk sacs was as previously described (Carpenter et al., 2005). Samples were fixed in 4% paraformaldehyde (PFA) overnight after *lacZ* staining. Specimens were then embedded in paraffin and sectioned at 5 μm. Sections were stained with Eosin and visualized using a Zeiss Axioskop 2 Plus microscope (Zeiss, Thornwood, NY). Images were captured using a DC 300 camera and IM50 software (Leica, San Jose, CA).

Cell proliferation and TUNEL assays

Cell proliferation and apoptosis were evaluated as previously described (Braren et al., 2006). TUNEL^{+}CD31^{+} ECs and TUNEL^{+}CD31$^{–}$ cells were counted, and the ratio of TUNEL^{+}CD31^{+} cells to total CD31^{+} ECs was obtained. Four pairs of embryos from two different litters were examined. Statistical analysis was performed using the *t*-test.

EC motility assay

ECs isolated from 10.5 dpc embryos were plated on a six-well plastic plate coated with 10 μg/ml fibronectin and cultured overnight, then labeled for 3 hours with Dil (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate)-labeled acetylated low-density lipoprotein (DiI-Ac-LDL, Biomedical Technologies, Stoughton, MA) at 2.5 μg/ml in medium. Time-lapse microscopy was performed using a Marianas time-lapse imaging system (Intelligent Imaging Innovations, Santa Monica, CA). EC migration was recorded for 4 hours at 6-minute intervals. *xy* coordinates of individual ECs were tracked with Slidebook software (Intelligent Imaging Innovations, Santa Monica, CA).

Fetal liver cell-isolation and flow cytometry analysis

Fetal livers at 11.5 dpc were dissociated mechanically and passed through a 40 μm nylon mesh. Cells were collected in 10% FBS/0.5% BSA/1× PBS-calcium and magnesium free (CMF). Cell viability was determined using a Trypan Blue dye exclusion assay. For flow cytometry analysis, cells (3-5×10^{5}) were diluted into 100 μl 5% FBS/0.5% BSA/1× PBS-CMF, and the antibody-cell suspension was incubated on ice for 30 minutes. A BD LSRII FACS Machine (BD Biosciences, San Diego, CA) was used to perform flow-cytometry, and FlowJo software was used for data analysis. Propidium iodide (1 μg/ml) staining was used to exclude dead cells.

Collection of embryonic peripheral blood cells

Embryonic peripheral blood (PB) was isolated by opening the embryonic vitelline vessels, dorsal aortae and the heart to release blood cells completely. The cells were passed through a 40 μm nylon mesh before use.

Immunofluorescence staining of sections and isolated cells

Rehydrated paraffin sections were blocked with 5% donkey serum in PBS for 2 hours at room temperature. They were then incubated with primary antibody at 4°C overnight, washed three times with PBS, followed by 1 hour secondary antibody incubation at 4°C in blocking solution (2% BSA, 3% normal donkey serum, 0.01% Triton X100, 1×PBS). Samples were washed with PBS and mounted with Vectashield containing DAPI (4′, 6 diamidino-2-phenylindole; Vector Laboratories. Burlingame, CA). Cells grown on six-well plates were fixed in 4% PFA/PBS for 20 minutes and permeabilized in 0.1% Triton X-100/1×PBS/2%BSA for 10 minutes at room temperature before blocking (2% BSA/0.01% of Triton X100/1×PBS). Images were captured using either air lenses or a 63× Anchroplan water immersion lens, and a Zeiss Axiovert2 Plus microscope equipped with a Sensicam CCD camera and Slidebook software (Intelligent Imaging Innovations, Santa Monica, CA).

Allantoic explants

Allantoic explant was performed as described (Braren et al., 2006). Allantoises were isolated at E8.0 at the 6- to 8-somite stage and cultured for 24-48 hours on FN-coated dishes. Cultures were stained with anti-CD31 as described above.

RESULTS

c-myc is essential for angiogenesis but not vasculogenesis

Gross abnormalities and developmental retardation by 9.5 dpc and death by 10.5 dpc have been previously observed in *c-myc$^{–/–}$* embryos (Davis et al., 1993; Trumpp et al., 2001). Multi-organ failure, including circulatory defects, is believed to underlie this early developmental arrest, although the specific cell lineages affected are largely unknown. We therefore analyzed *c-myc$^{–/–}$* embryos specifically for vascular defects, using whole-mount immunostaining against CD31, an EC-specific marker. As previously reported, these mutant embryos were developmentally retarded and displayed major developmental defects. However, we were surprised to find that major vessels such as the dorsal aorta had developed by 8.75 dpc. Smaller inter-somitic and cranial vessels had also formed in the embryo proper, although they were underdeveloped compared with wild-type vessels (Fig. 1A,B). In the yolk sac, primitive vascular networks had formed, although the mutant yolk sac contained more vascular plexuses and less organized hierarchal branches than the control (Fig. 1C,D). At 10.25 dpc, although the control vasculature had matured to an elaborate and well-organized system (Fig. 1E,G), the *c-myc$^{–/–}$* embryos retained a primitive vasculature in the head (Fig. 1F) and the yolk sac (Fig. 1H). Nonetheless, embryo cross-sections revealed that, like controls, *c-myc$^{–/–}$* embryos had ECs lining their dorsal aortae, common cardinal veins and other blood vessels (Fig. 1I,J). These results indicate that, in contrast to a previous report (Baudino et al., 2002), *c-myc$^{–/–}$* embryos contained differentiated ECs that were capable of assembling a primitive vasculature. Subsequent vascular remodeling, however, was defective in the mutants.

Because the detection of ECs in the *c-myc$^{–/–}$* mutant was surprising, we examined the vasculature in another mutant embryo, in which *c-myc* is deleted in the entire embryo proper but retained in the placenta. We created a CK using the *Sox2-Cre* mouse line, in

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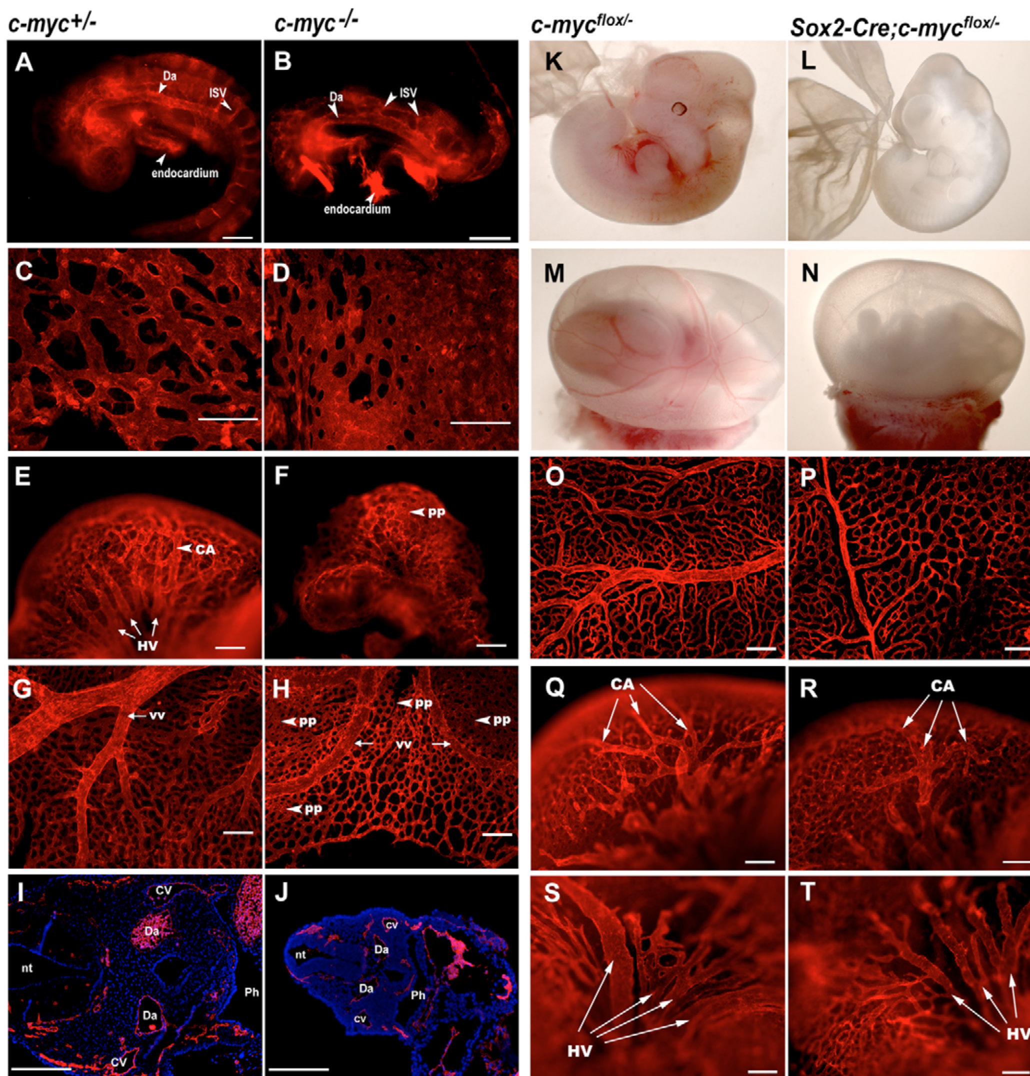

Fig. 1. *c-myc* null and *Sox2-Cre*-mediated *c-myc* deletion result in anemia, vascular defects and lethality. (**A-H**) Anti-CD31-stained whole-mount embryos (A,B,E,F) and yolk sacs (C,D,G,H) dissected at 8.75 dpc (A-D) or 10.25 dpc (E-H). (**I**,**J**) Cross-sections (5 μm) of anti-CD31-stained whole-mount embryos. (**K-N**) Live embryos dissected at 11.5 dpc. (**O-T**) Anti-CD31-stained whole-mount yolk sacs (O,P) and heads (Q-T) at 11.5 dpc. Note the less developed vessels with reduced caliber (yolk sac) and number of branches (head) in the mutant animal. Da, dorsal aorta; ISV, intersomitic vessel; pp, primitive plexus; vv, vitelline vessels; CA, carotid artery; HV, primary head veins; CV, cardinal vein; nt, neural tube; Ph, pharynx. Scale bars: 200 μm.

which Cre is active in all cells of epiblast origin, including the entire embryo proper, the yolk sac mesoderm, the amnion membrane and the embryonic vessels in the placenta, but not the visceral endoderm or the extra-embryonic ectoderm (Vincent and Robertson, 2003). We verified Cre expression throughout the entire embryo by Cre reporter assay (data not shown). The *Sox2-Cre;c-myc*$^{flox/-}$ mutant progeny displayed growth retardation by 11.5 dpc and died between 11.5 and 12.5 dpc. These embryos, unlike the null mutants, appeared grossly normal without major organ defects before E10.5 (data not shown). However, at E11.5 they were anemic (Fig. 1L,N) and displayed abnormal vasculature (Fig. 1P,R,T). Anti-CD31 staining revealed abundant ECs in mutant yolk sacs and embryos proper (Fig. 1O-T), demonstrating that the mutant embryos were not defective in EC differentiation, although capillary remodeling was defective. Mutant yolk sac microvessels were more primitive, comprising disorganized capillary plexuses with larger intercapillary spaces than in the controls. Major vitelline vessels were narrower and underdeveloped in the mutant (Fig. 1O,P). Similarly, head capillaries were more primitive. The carotid arteries were less elaborate and the primitive head veins were narrower compared with controls (Fig. 1Q-T). These findings confirmed that *c-myc* is not required for the onset of vasculogenesis in embryos. However, it is essential for subsequent vascular morphogenesis.

The *Sox2-Cre;c-myc*$^{flox/-}$ mutant embryos that maintained *c-myc* in their visceral endoderm and placenta died 2 days later than *c-myc*$^{-/-}$ embryos, suggesting that expression of *c-myc* in these extra-embryonic tissues is essential to the survival of the embryos at this stage. Consistent with this notion, *c-myc* is expressed at high levels in the ectoplacental cone starting at 6.5 dpc (Downs et al., 1989), and c-Myc promotes trophoblast proliferation (Erlebacher et al., 2004). We found that by 8.25 dpc, *c-myc*$^{-/-}$ placentas contained fewer trophoblasts, exhibiting defective placental morphology and cellular composition lacking trophoblast integrity. In addition, the mutant chorionic plate was thinner, and the ectoplacental cone was not integrated (see Fig. S1 in the supplementary material). These findings suggest that *c-myc* is required for placental development, and that the cellular function of *c-myc* in this organ requires future investigation. In this report, we have focused on the function of *c-myc* in the embryo proper.

Loss of *c-myc* in endothelial and hematopoietic cells induces similar vascular defects as loss of *c-myc* in the entire embryo proper

To determine whether eliminating *c-myc* specifically from the circulatory system would result in developmental and vascular defects, we crossed *c-myc*$^{flox/flox}$ mice with *Tie2-Cre;c-myc*$^{+/-}$ mice,

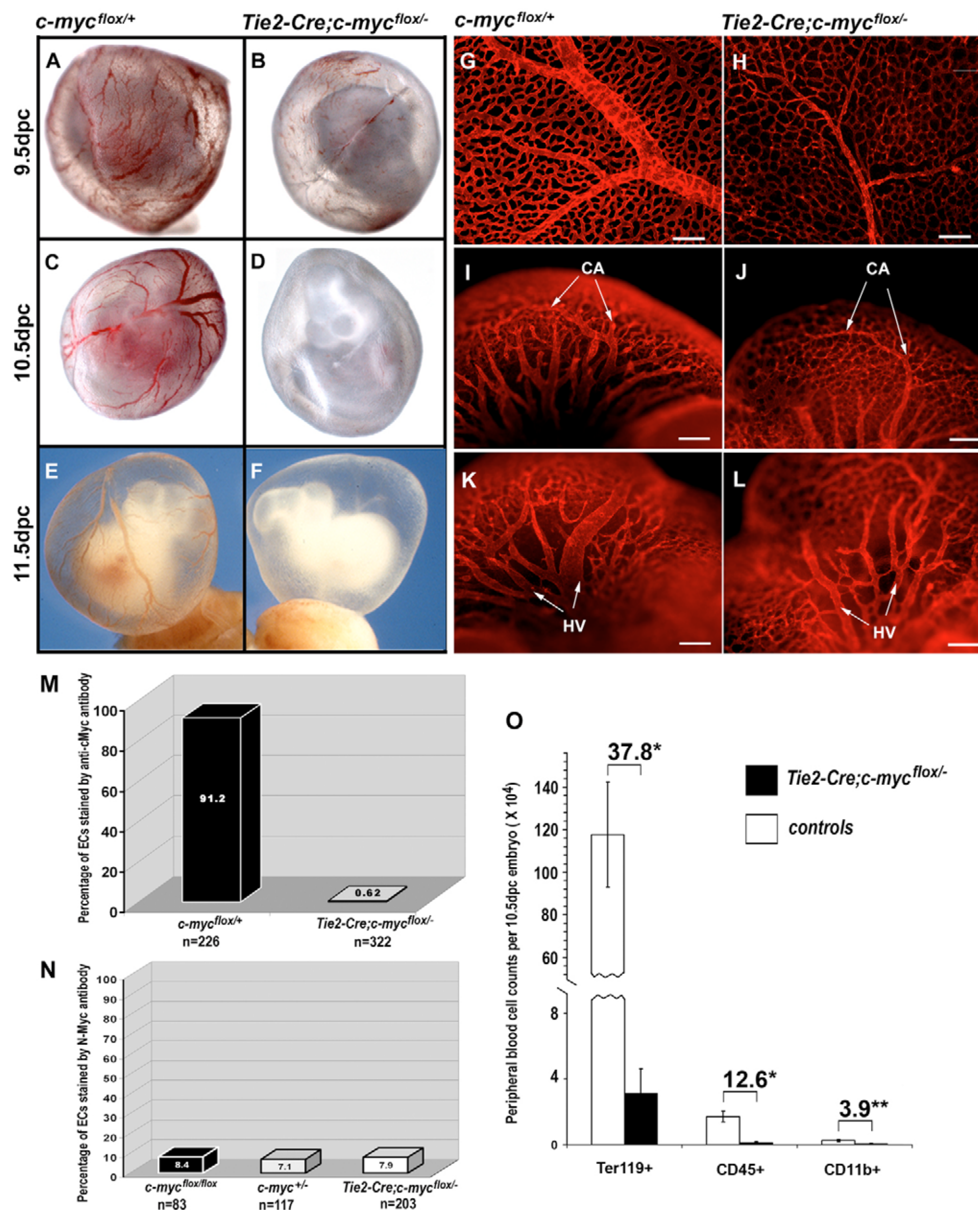

Fig. 2. *Tie2-Cre*-mediated *c-myc* deletion results in anemia, vascular defects and lethality. (**A-F**) Live embryos in yolk sac dissected at 9.5 dpc (A,B), 10.5 dpc (C,D) or 11.5 dpc (E,F). (**G-L**) Anti-CD31-stained whole-mount yolk sacs (G,H) and heads (I-L) at 11.5 dpc. Vasculature in the mutant is less developed with reduced caliber and number of branches. Scale bar: 200 μm. (**M**,**N**) Efficient c-Myc deletion by *Tie2-Cre* did not increase N-Myc expression in 10.5 dpc primary ECs. The percentage of c-Myc-positive ECs is indicated in M. The percentage of N-Myc positive ECs is shown in N. 'n' represents the number of cells counted in each assay. (**O**) Peripheral blood cell counts at 10.5 dpc from control (*n*=19) and *Tie2-Cre;c-myc*$^{flox/–}$ mutant (*n*=7) embryos. The numbers above the brackets show the fold decrease in the mutants compared with controls. **P*<0.01; ***P*<0.05 by Student's *t*-test.

in which Cre is active in EC and HC lineages starting as early as 7.5 dpc in the common progenitor of these two lineages (Braren et al., 2006). To assess the efficiency of *Tie2-Cre*-mediated *c-myc* deletion, we measured nuclear-specific c-Myc expression in isolated ECs by immunostaining (see Fig. S3 in the supplementary material). Quantitative analysis showed that virtually no (0.62%) mutant ECs and 91.2% of control ECs had c-Myc staining by 10.5 dpc (Fig. 2M). These experiments demonstrate the success of *c-myc* deletion.

These embryos, like *Sox2-Cre* CKs, appeared grossly normal without major organ defects before E10.5 (data not shown). At 9.5 dpc, the *Tie2-Cre*; *c-myc*$^{flox/–}$ embryos were similar to controls, with all embryos showing blood-filled vasculatures, although some were slightly paler (Fig. 2A,B). However, at 10.5 dpc, the mutants appeared anemic (Fig. 2D). At 11.5 dpc, the mutant yolk sacs and embryos were completely white, whereas control yolk sacs displayed vessels filled with red blood cells (Fig. 2E,F). Mutant embryos were smaller than controls (Fig. 2F). No mutant embryos survived beyond 12.5 dpc (see Table S1 in the supplementary material). The gross abnormalities and the stage at which the phenotype occurred in *Tie2-Cre* CK closely resembled that of the *Sox2-Cre* CK embryos.

Anti-CD31 staining on 11.5 dpc *Tie2-Cre* CK embryos also revealed a very similar vascular phenotype to the *Sox2-Cre* CK, with abundant ECs but more primitive capillaries and smaller major vessels in mutant yolk sacs and heads compared with the controls (Fig. 2G-L). Quantitative analysis of the yolk sac vascular defects is summarized in Fig. S2 (see supplementary material). These findings from *Tie2-Cre* CK embryos confirm that vasculogenesis occurred without *c-myc*, but further capillary remodeling was defective. Additionally, the allantois is vascularized by vasculogenesis, and we did not detect any apparent vascular defects in the *Tie2-Cre* CK allantoic explants (Fig. 3A,B), further suggesting that vasculogenesis occurs in the absence of *c-myc*.

Expression of N-Myc (Mycn – Mouse Genome Informatics), another Myc family member, driven by the *c-myc* promoter can functionally replace c-Myc activity *in vivo* (Malynn et al., 2000). To

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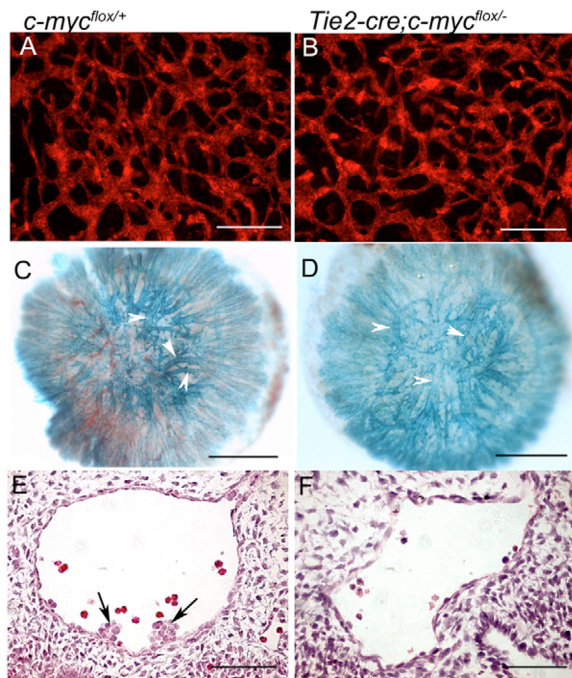

Fig. 3. *Tie2-Cre*-mediated *c-myc* deletion did not affect allantoic or placental vasculature but diminished hemogenic endothelial cells in the aorta. (**A**,**B**) Allantoic explants stained for CD31 show no obvious difference between the control and mutant. (**C**,**D**) Placental vasculature at 11.5 dpc revealed by *Tie2-lacZ* reporter assay shows no obvious differences between the control and mutant. Arrowheads indicate the blood vessels. (**E**,**F**) H&E stained cross-section of dorsal aorta shows hemogenic endothelial cells (arrows) in the control but not in the mutant. Scale bars: 50 μm.

rule out the possibility that *n-myc* expression might compensate for the loss of *c-myc* in ECs, we stained purified ECs from 10.5 dpc *Tie2-Cre* CKs with anti-N-Myc antibodies (Fig. S3 in the supplementary material). We found that N-Myc was expressed in a small fraction (7.1-8.4%) of ECs, but that this fraction was independent of the EC genotype (Fig. 2N). To rule out a possible compensatory effect from *L-myc*, we analyzed *L-myc* expression by real time RT-PCR in *Sox2-Cre* CK yolk sacs. Whereas c-myc expression was significantly reduced, L-myc expression was unchanged in the mutant (data not shown). These results demonstrate that neither N-Myc nor L-Myc is likely to compensate functionally for c-Myc in ECs.

To examine whether placental vasculature is defective in this mutant, we analyzed the blood vessel structure in the placenta using a *Tie2-lacZ* reporter. By 11.5 dpc, although the mutant embryo was already pale and blood vessels in the yolk sac and embryo proper were already defective, the placental blood vessels appeared indistinguishable from the control (Fig. 3C,D). These data indicate that *c-myc* deletion in the ECs and HCs did not significantly affect the placental vasculature, suggesting that the death of this mutant embryo is unlikely to be due to a placental failure.

To characterize defects in the HC lineage, we counted the peripheral red blood cells (Ter119$^+$), hematopoietic cells (CD45$^+$) and myeloid cells (CD11b$^+$) from yolk sacs and embryos and found that by 10.5 dpc *Tie2-Cre*; *c-myc*$^{flox/–}$ mutants exhibited a 38-fold reduction in total blood cell number, a 13-fold reduction in CD45$^+$ cells and a fourfold reduction in CD11b$^+$ cells (Fig. 2O). Hematoxylin and Eosin staining on paraffin sections also confirmed a nearly complete absence of blood cells by 11.5 dpc (data not shown). Hemogenic ECs that reside in the ventral side of the dorsal aorta at around 10.5 dpc are thought to give rise to HSCs (de Bruijn et al., 2002; Taoudi and Medvinsky, 2007). We examined serial cross-sections of dorsal aortae from four pairs of embryos at 10.5 dpc. Cells located at the ventral wall of the aorta and morphologically resembling hemogenic ECs were seen in all controls but in none of the mutants (Fig. 3E,F). These findings suggest that removing *c-myc* from ECs and HCs is sufficient to induce the hematopoietic, angiogenic and survival defects observed in embryos harboring a global *c-myc* deletion.

c-myc deletion in HCs is sufficient to induce vascular defects and embryonic lethality

To delineate the effect of *c-myc* deletion in hematopoietic lineages on vascular development, we examined embryos in which *c-myc* had been deleted specifically in HC lineages using *Vav-iCre. Vav-iCre* has been shown to mediate gene excision in adult HCs (de Boer et al., 2003). We thus analyzed *Vav-iCre* activity in embryos using a *Rosa26R-lacZ* reporter according to our established method (Braren et al., 2006). At 11.5 dpc, we found that *Vav-iCre* was active almost exclusively in fetal liver HCs (Fig. S4A,C in the supplementary material) and in some circulating blood cells in the yolk sac (Fig. S4B in the supplementary material), but not in the endothelium or any other tissues (see Fig. S4D in the supplementary material). This result indicates that *Vav-iCre* mediated *c-myc* deletion in HCs without affecting ECs or any other cell types. We assessed *Vav-iCre* activity in individual embryos by FACS analysis in 11.5 dpc fetal liver HCs using the *Rosa26YFP* reporter (Srinivas et al., 2001). The fraction of HCs expressing Cre varied among individual embryos in all three HC lineages tested, with averages around 50% (see Fig. S4K in the supplementary material).

About 60% of *Vav-iCre*;*c-myc* $^{flox/–}$ mutants (27/43) appeared anemic at 11.5 dpc (Fig. 4B), with paler and smaller fetal livers compared with controls (see Fig. S4I,J in the supplementary material). A similar % of mutants died around 12.5dpc, a stage similar to the *Tie2-Cre* and *Sox2-Cre* CKs. About 16% of mutants (7/43) showed various degrees of hemorrhaging in the embryo proper (see Fig. S4E,G in the supplementary material). About 21% of mutants (9/43) survived through 12.5 dpc but developed anemia and died by 15.5 dpc. None of the *Vav-iCre* mutants survived to birth. It is likely that non-uniform Cre activity among individuals resulted in these variable phenotypes (see Fig. S4K in the supplementary material). Nonetheless, deletion of *c-myc* solely in HCs resulted in complete embryonic lethality of all embryos examined.

To visualize the vasculature of the *Vav-iCre*;*c-myc* $^{flox/–}$ mutant embryos, we performed whole-mount anti-CD31 staining on 11.5 dpc embryos. We found that the vascular defects in these anemic embryos resembled those in *Tie2-Cre-* and *Sox2-Cre* CKs (see Fig. 1P,R,T, Fig. 2H,J,L). Although the overall vascular patterning in *Vav-iCre*;*c-myc*$^{flox/–}$ embryos proper and yolk sacs was in place, the vessels were narrower and the vascular network appeared underdeveloped and primitive compared with controls (Fig. 4D,F,H). Taken together, these results suggest that *c-myc* deletion in HC lineages alone likely accounts for the anemia, embryonic lethality and vascular developmental defects induced by *c-myc* deletion in ECs and HCs combined.

To quantify the hematopoietic defects in 11.5 dpc *Vav-iCre* CKs, we performed HC counts in peripheral blood (Fig. 4I) and fetal liver cells (Table 1). We found a several fold decrease of cells (7.6 fold in Ter119$^+$ cells, 4.3 fold in CD45$^+$ and 4.6 fold in CD11b$^+$ cells) in the mutant peripheral blood. The mutants' fetal livers had cytopenia and

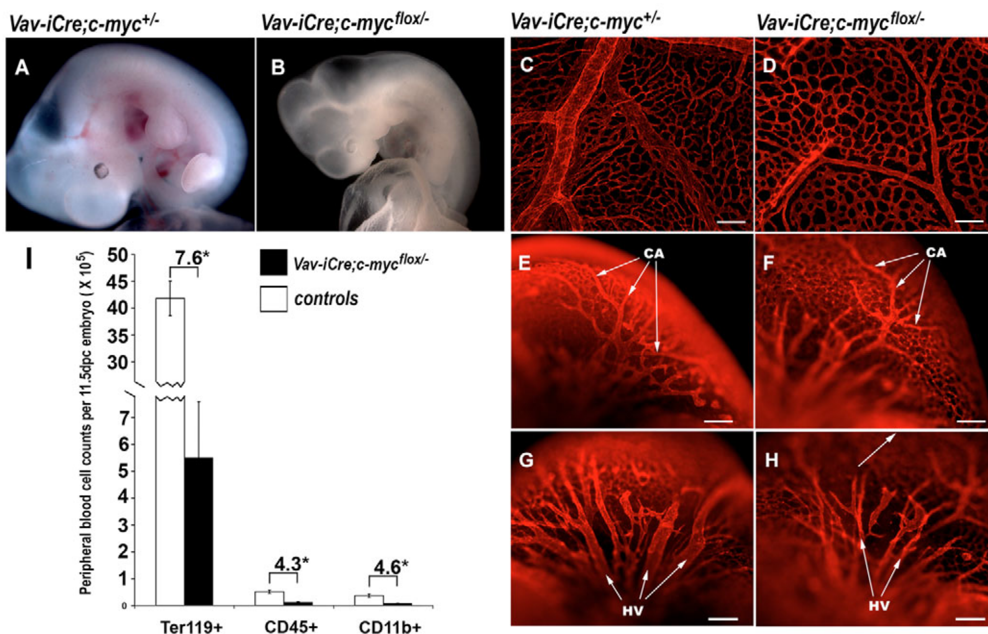

Fig. 4. *Vav-iCre*- mediated *c-myc* deletion results in cytopenia, vascular defects and lethality. (**A**,**B**) Live embryos at 11.5 dpc. (**C**-**H**) Anti-CD31-stained whole-mount yolk sacs (C,D) and heads (E-H) at 11.5 dpc. CA, internal carotid arteries; HA, primary head veins. Scale bars: 200 μm. (**I**) Peripheral blood cell counts of control (n=16) and *Vav-iCre;c-myc*$^{flox/–}$ mutant (n=8) embryos. The numbers above brackets show the fold decrease in the mutants compared with controls. *P<0.01 and **P<0.05 by Student's t-test.

contained 12.5-fold fewer cells than their littermate controls. In addition, we used Lin markers, containing CD3e, CD11b, CD45R/B220, Ter119, Ly-6G and Ly-6C to label the committed hematopoietic lineages, which include T lymphocytes, B lymphocytes, monocytes/macrophages, NK cells, erythrocytes and granulocytes. We found the proportion of committed (Lin$^+$) cells was reduced while that of uncommitted cells (Lin$^–$) was increased in *Vav-iCre*;*c-myc*$^{flox/–}$ embryos. Among the Lin$^–$ cells, the proportion of KLS-HSCs (Kit$^+$, Lin$^{–/lo}$, Sca1$^+$) (Ivanova et al., 2002) in the *Vav-iCre*;*c-myc*$^{flox/–}$ fetal liver was increased, while the proportion of Kit^{hi}, Lin$^–$ cells was significantly decreased. In summary, these results demonstrate that elimination of *c-myc* in HCs by *Vav-iCre* compromised definitive hematopoiesis.

c-myc deletion in the majority of ECs and a subset of HCs results in partial survival

As *c-myc* deletion in HCs alone using *Vav-iCre* resulted in defects similar to *c-myc* deletion from both HCs and ECs (with *Sox2-Cre* or *Tie2-Cre*), we investigated the effect of endothelial *c-myc* expression on vascular development. For this experiment, we needed a Cre line that would remove *c-myc* in the ECs but not the HCs; however, such a reagent is not currently available. We therefore chose the *Tie1-Cre* line, with Cre active in the majority of ECs and a subset of HCs by 10.5 dpc (Gustafsson et al., 2001) (see Fig. S5 in the supplementary material). We verified *Tie1-Cre* activity using the *Rosa26R* reporter and double immunostaining for CD31 and β-galactosidase. Approximately 80% of CD31-positive cells were Cre active at 10.5 dpc (see Fig. S5 in the supplementary material). We also characterized *Tie1-Cre* expression in HCs by FACS using the *RosaYFP* reporter. Cre was active in ~54% of myeloid cells, 26% of erythroid cells and 15% of lymphocyte precursor cells at 11.5 dpc (Fig. S6 in the supplementary material). These results suggest that *Tie1-Cre* mediated *c-myc* deletion in the majority of ECs and a subset of HCs.

By 12.5 dpc, we did not detect any defects in *Tie1-Cre;c-myc*$^{flox/–}$ mutants. By 17.5 dpc we observed anemic, dying mutant embryos. Remarkably, half of the mutants survived to birth and a third to post-weaning (Table 2). Surviving mutant adults appeared normal. These results demonstrate that *c-myc* deletion in a majority of ECs is compatible with embryo survival.

To examine the HC development in these mutants, we performed HC counts from the peripheral blood (Fig. 5) and fetal livers (Table 3) of 11.5 dpc *Tie1-Cre* CKs. We found a less than twofold decrease in peripheral blood cell lineages (Ter119$^+$, CD45$^+$, CD11b$^+$) in the mutants (Fig. 5). The mutant fetal liver cell number, composed mainly of HCs, was half of that in the control. The proportion of committed (Lin$^+$) and un-committed (Lin$^–$) cells in fetal livers was similar between the mutant and control (Table 3). Therefore, HC lineages were much less depleted in *Tie1-Cre*;*c-myc* $^{flox/–}$ than in *Vav-iCre*;*c-myc* $^{flox/–}$ embryos (Fig. 4K,L). The mild HC defects probably permitted the partial survival of the *Tie1-Cre* CKs.

c-Myc-depleted ECs show no significant cell biological defects

Most *Tie1-Cre* CKs examined at 11.5 dpc showed no detectable abnormalities, suggesting that *c-myc* expression in the majority of ECs is not essential for vascular development. To

Table 1. Impaired differentiation and proliferation of hematopoietic cells in *Vav-iCre*-mediated *c-myc* deletion in 11.5 dpc fetal livers

	CD45$^+$	Ter119$^+$	CD11b$^+$	Lin$^+$	Lin$^{lo/–}$	KLS within Lin$^–$	Kit^{hi} within Lin$^–$	Kit^{lo} within Lin$^–$	Live cells/ fetal liver
Controls	14.3 (1)*	22.5 (3.0)‡	3.8 (0.5)*	44.0 (2)*	56.0 (1.9)*	0.08 (0.02)†	19.7 (2.2)*	5.1 (0.4)†	258753 (12175)*
Mutants	3.5 (1.1)*	14.5 (2.4)‡	1.8 (0.5)*	25.0 (4.6)*	75.0 (4.7)*	0.2 (0.1)†	5.1 (1.6)*	11.8 (2.8)†	20693 (1062)*

Data represent the mean percentage of live cells (±s.e.m.) and the mean of live cell numbers per fetal liver (±s.e.m.) from 10 mutant fetuses and 20 control fetuses. Differences between mutant and control fetuses were significant at *P<0.01 and $^†P$<0.05, or not significant at $^‡P$=0.112. Controls were *Vav-iCre;c-myc*$^{+/–}$ and *c-myc*$^{flox/–}$; mutants were *Vav-iCre;c-myc*$^{flox/–}$.

Table 2. *Tie1-Cre* mediated *c-myc* deletion resulted in partial lethality

Ratio	Mutants*	Controls†
Survival after weaning (48 mice from seven litters)		
Expected‡	25%	75%
Experimental§	8%	92%
Expmt/Expt¶	32%	122%
Newborn survival (56 mice from eight litters)		
Expected‡	25%	75%
Experimental§	12.5%	87.5%
Expmt/Expt¶	50.0%	116.6%

*Mutants include *Tie1-Cre;c-myc*$^{flox/flox}$ and *Tie1-Cre;c-myc*$^{flox/\Delta}$.
†Controls include *c-myc*$^{flox/-}$, *c-myc*$^{flox/+}$, *c-myc*$^{+/+}$, *c-myc*$^{+/-}$, *c-myc*$^{flox/flox}$, *Tie1Cre;c-myc*$^{+/-}$, *Tie1Cre;c-myc*$^{+/+}$, *Tie1Cre;c-myc*$^{flox/+}$.
‡Expected indicates the expected Mendelian genetic ratio of genotypes.
§Experimental indicates genotypes from surviving animals.
¶Expmt/Expt indicates the ratio of experimental over expected.

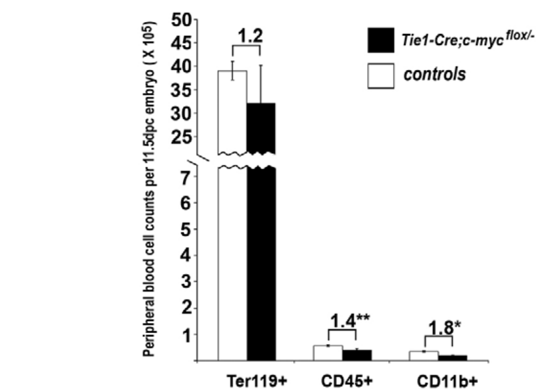

Fig. 5. *Tie1*-Cre-mediated *c-myc* deletion results in partial survival. Peripheral blood cell counts from control (*n*=20) and *Tie1-Cre;c-myc*$^{flox/-}$ mutant (*n*=6) embryos. The numbers above brackets show the fold decrease in the mutants over controls. *P<0.01 and **P<0.05 by Student's *t*-test.

verify whether *c-myc* is required in ECs for blood vessel formation, we examined the in vivo and in vitro behaviors of *Tie2-Cre* CK ECs.

We analyzed EC proliferation in vivo by BrdU incorporation and did not observe any significant difference in proliferation rates of aortic endothelium between control and mutant embryos (Fig. 6A). We also did not detect an obvious proliferative difference in isolated ECs from BrdU-labeled embryos following maternal BrdU injection (data not shown).

To determine whether loss of *c-myc* affected EC survival in vivo, we performed TUNEL assays on cryosections of yolk sacs. We found a significant increase in TUNEL staining in *Tie2-Cre* CKs compared with the controls at 10.5 dpc (Fig. 6B). However, increased TUNEL staining affected both ECs and non-ECs, even though non-ECs had a functional *c-myc* gene. These data suggest that the increase in EC death could be a secondary effect resulting from general poor embryo health rather than a direct result of *c-myc* deletion in these cells. In summary, we were unable to detect significant changes in cell proliferation and survival that could be ascribed to loss of *c-myc* specifically in ECs.

To examine the morphology and behavior of *c-myc*-deficient ECs, we isolated ECs from *Tie2-Cre* CKs at 10.5 dpc and cultured them for 5 hours on fibronectin-coated culture plates. Using anti-CD31 and phalloidin staining, we observed neither morphological differences nor changes in the organization of the actin cytoskeleton between control and *c-myc*-null ECs (Fig. 6C,D).

Normal angiogenesis relies on the ability of ECs to migrate (Beck and D'Amore, 1997). To assess the motility of *c-myc*-deficient ECs, we performed time-lapse video-microscopy of *c-myc*-null and control ECs isolated from 10.5 dpc embryos and cultured on fibronectin. The paths of both mutant and control ECs were random and indistinguishable from one another, as shown by our measurements of net path length and average speed (Fig. 6E,F,G). These results demonstrate that *c-myc*-null ECs are not defective in cell motility in vitro.

To further address the angiogenic potential of *c-myc*-null ECs, we tested their ability to form endothelial tubes in vitro (Fig. 6H,I). Because this assay requires a large number of ECs, we isolated ECs from the vena cava of adult *c-myc*$^{flox/flox}$ mice and removed their *c-myc* gene using an adenovirus-mediated Cre (AdCreGFP) deletion system. FACS analysis of GFP expression in the cultured ECs showed that the efficiency of adenovirus infection was 98% (Fig. 6J), and PCR analysis of genomic DNA confirmed that the *c-myc* gene was excised in the majority of the cells (Fig. 6K). However, we found no statistically significant difference in the number of branch points (data not shown) or the lengths of tubes formed by mutant and control ECs on the Matrigel surface (Fig. 6I,J). These findings suggest that *c-myc*-null ECs are not defective in cell migration or capillary morphogenesis.

Deletion of *c-myc* in HCs leads to reduction of pro-angiogenic factors crucial for vascular morphogenesis

HCs have been increasingly recognized as significantly contributing to angiogenesis by modulating the production of proangiogenic factors (Tordjman et al., 2001; Kopp et al., 2006; Shojaei et al., 2007). We therefore examined the mRNA levels of various proangiogenic factors, including *Pdgfa*, *Mmp2* and *Il1b*, in whole embryos, using quantitative-PCR analysis. We found that mRNA levels of these genes were significantly decreased in the anemic *Vav-iCre;c-myc*$^{flox/-}$ embryos compared with their control

Table 3. *Tie1-Cre* mediated *c-myc* deletion led to mild defects in hematopoiesis in 11.5 dpc fetal livers

	CD45$^+$	Ter119$^+$	CD11b$^+$	Lin$^+$	Lin$^{lo/-}$	KLS within Lin$^-$	Kit^{hi} within Lin$^-$	Kit^{lo} within Lin$^-$	Live cells/ fetal liver
Controls	15.4 (1.3)	24.0 (4.7)	4.2 (0.5)	51 (4.6)	49 (4.5)	0.09 (0.01)	21.0 (2.8)	3.5 (0.3)†	412,591 (47807)*
Mutants	17.8 (1.6)	20.9 (3.1)	4.7 (0.9)	49.5 (5.9)	50.5 (5.8)	0.05 (0.01)	16.9 (3.0)	5.2 (0.4)†	195,350 (43767)*

Data represent the mean percentage of live cells (±s.e.m.) and the mean of live cell numbers per fetal liver (±s.e.m.) from seven mutant fetuses and 24 control fetuses. Differences between mutant and control fetuses were significant at *P<0.01, †P<0.05. Controls were *Tie1-Cre;c-myc*$^{+/-}$, *Tie1-Cre;c-myc*$^{flox/+}$ and *c-myc*$^{flox/-}$. Mutants were *Tie1-Cre;c-myc*$^{flox/-}$.

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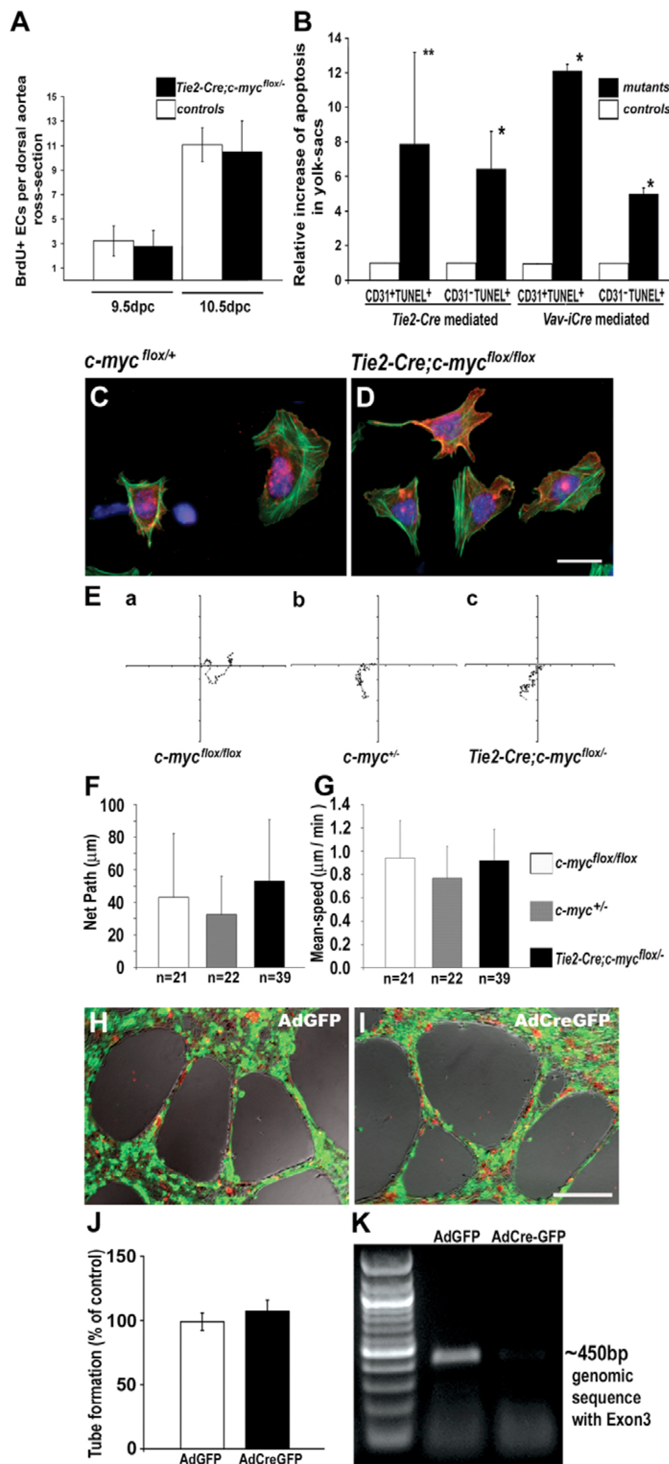

Fig. 6. *c-myc*-deficient ECs exhibit no detectable defects in proliferation, survival, morphology, motility or tube formation. (**A**) BrdU+ ECs in the dorsal aortae. Cross-sections were stained with anti-CD31 and anti-BrdU antibodies. (**B**) Fold increases in apoptosis in yolk sac ECs and non-ECs. Cross-sections of *Tie2-Cre*;*c-myc*$^{flox/–}$ (at 10.5 dpc) and *Vav-iCre*;*c-myc*$^{flox/–}$ (at 11.5 dpc) mutant (black bars) and control (white bars) yolk sacs were stained with anti-CD31 and for TUNEL. TUNEL$^+$ CD31$^+$ or TUNEL$^+$ CD31$^–$ cells were quantified. $^{**}P$<0.05; $^{*}P$<0.01 by Student's *t*-test. Data were collected from four pairs of embryos from two different litters. (**C**,**D**) Phalloidin (green), anti-CD31 (red) and DAPI (blue) stained ECs isolated from 10.5 dpc embryos. (**E-G**) Random movement of cultured ECs from 10.5 dpc embryos monitored by timelapse microscopy. Representative EC migration paths are shown (E) and quantitative analysis demonstrated no significant difference in either the net-path of cell migration (F, *P*=0.083) or in the average migration speed (G, *P*=0.090) in two independent experiments. The ANOVA two-tailed test was used in the statistical analysis. (**H-K**) No detectable defects in EC tube formation. *myc*$^{flox/flox}$ ECs were purified from adult vena cava. Cells 48 hours after infection with GFP fusion Cre-expressing adenovirus AdCreGFP or GFP expression control virus AdGFP were plated on Matrigel, and cultures were photographed 20 hours later (H,I). The results from four independent experiments were analyzed by two-tailed *t-test* (J, *P*=0.1916). Green, GFP; red, DiI-AcLDL labeled ECs. Genomic DNA PCR analysis demonstrates the deletion of *c-myc* floxed sequence by AdCreGFP but not AdGFP (K). Scale bar: 10 μm in C,D; 200 μm in H,I.

littermates (Fig. 7A). By contrast, levels of VEGF transcripts were dramatically increased, indicating a hypoxic response in the embryos suffering anemia (see Fig. 4A,B). Similarly, elevated VEGF protein was found in *Tie2-Cre*;*c-myc*$^{flox/–}$ embryos (Fig. 7B). These results demonstrate that c-Myc deficiency-induced hematopoietic defects may have reduced expression of certain proangiogenic factors, thereby hindering normal vascular morphogenesis.

DISCUSSION

To ascertain the physiological functions of c-Myc in development, we examined embryos lacking *c-myc* completely, in the embryo proper but not in extra-embryonic tissues, in hematopoietic and endothelial lineages, and in hematopoietic cells specifically. Our findings demonstrate that *c-myc* is not required for vasculogenesis in the embryo but can indirectly control angiogenesis through its vital role in hematopoiesis. Deletion of *c-myc* in HCs alone is lethal and sufficient to elicit both hematopoietic and vascular defects.

c-Myc plays an essential role in embryonic hematopoiesis

Fetal hematopoiesis begins with primitive hematopoietic differentiation in blood islands of the yolk sac at 7.5 dpc and lasts until 10.5 dpc in mice. Definitive hematopoiesis, which generates enucleated erythrocytes among other hematopoietic lineages like HSCs, starts at 10.5 dpc in the aorta-gonad-mesonephros region. Concurrently, HSCs colonize the developing fetal liver. The murine placenta also harbors HSCs during midgestation. Around birth, hematopoiesis translocates to the bone marrow (BM). Adult and fetal hematopoiesis differ in the types of niches in which they occur, as well as in the capacity of adult versus fetal HSCs to renew, proliferate and differentiate (Cumano and Godin, 2007; Mikkola and Orkin, 2006; Wilson and Trumpp, 2006). The role of c-Myc in adult hematopoiesis in BM has been reported (Wilson et al., 2004). Our experiments demonstrate the essential function of c-Myc in fetal hematopoiesis.

Our data suggest that HCs undergo primitive differentiation in the absence of *c-myc*. At 9.5 dpc, prior to definitive hematopoiesis, the majority of *Tie2-Cre* CKs were indistinguishable from the controls, with blood-filled circulatory systems. This phenotype is in sharp contrast to that of embryos lacking *Scl*, a gene required for the

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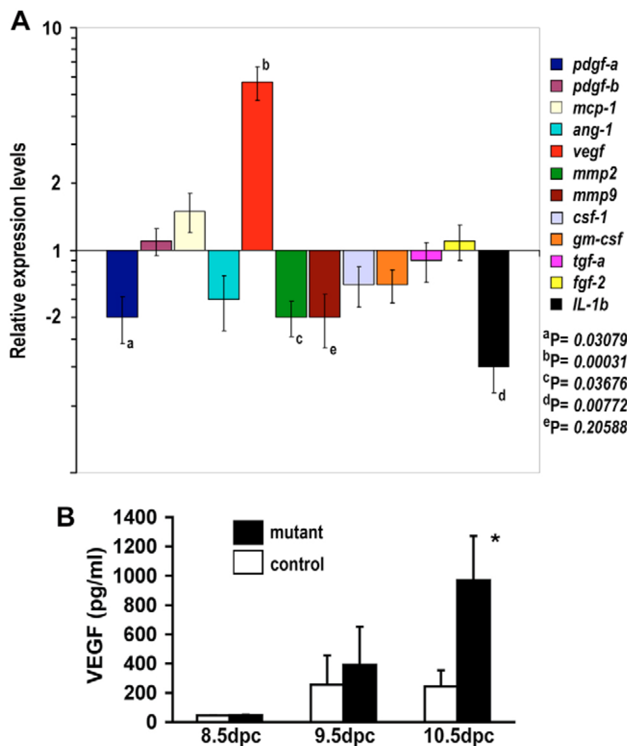

Fig. 7. Expression of proangiogenic factors in *Vav-iCre;c-myc*$^{flox/–}$ and *Tie2-Cre;c-myc*$^{flox/–}$ mutant embryos. (**A**) mRNA levels of proangiogenic factors in *Vav-iCre;c-myc*$^{flox/–}$ at 11.25 dpc. Real-time PCR was performed using total RNA from five mutants and 15 control embryos (*Vav-iCre;c-myc*$^{flox/+}$, *c-myc*$^{+/–}$ or *Vav-iCre;c-myc*$^{+/–}$). The statistical significance of the differences in means between controls (relative expression=1) and mutants are listed by their *P* values. (**B**) Increase in VEGF protein levels by ELISA in control and *Tie2-Cre;c-myc*$^{flox/–}$ embryos (*n*=5). VEGF levels in the *Tie2-Cre* mutant embryos were significantly elevated (*P<0.01).

differentiation of primitive hematopoietic cells. *Scl*-null embryos are devoid of blood cells with no sign of hematopoiesis at 9.5 dpc (Robb et al., 1995). One concern is whether *Tie2-Cre* mediated *c-myc* deletion occurs early enough to assess its requirement in primitive hematopoiesis. We have previously reported that this *Tie2-Cre* is active early in the blood island precursors and mediates efficient gene excision by 9.5 dpc (Braren et al., 2006). The presence of blood cells in both the *c-myc* null (Davis et al., 1993) and *Sox2-Cre* CKs (data not shown) further supports the idea that primitive hematopoiesis occurs in the absence of *c-myc*. From 9.5 dpc, the *Tie2-Cre* CKs developed progressive cytopenia. The likely cause of the primitive hematopoietic failure is the reduced survival of HCs (Dubois et al., 2008). Thus, *c-myc* is not required for the initiation of primitive hematopoiesis but is essential to sustain primitive hematopoiesis.

Our data from *Vav-iCre* CKs provide evidence that c-Myc is also required for definitive hematopoiesis, through a cell-autonomous mechanism. The *Vav1* promoter induces transgene expression in definitive but not primitive HCs (Ogilvy et al., 1999). Furthermore, we show that *Vav-iCre* mediates gene excision specifically in fetal liver HCs. The fact that the *Vav-iCre* CK developed severe cytopenia demonstrates that *c-myc* in HCs is essential for definitive hematopoiesis.

The absence of c-Myc in definitive hematopoiesis seems to affect the differentiation of HSCs, resembling *c-myc*-deficient adult hematopoiesis in BM (Wilson et al., 2004). The proportion of uncommitted HCs, KLS-HSCs and Kit^{low} progenitor cells increased in the *Vav-iCre* fetal liver. However, the proportion of Kit^{hi} progenitor cells decreased. This reduction could be a genuine decrease of Kit^{hi} progenitors in mutants or a loss of cell surface Kit expression on otherwise functional HSCs, as has been observed following myeloid ablation (Randall and Weissman, 1997). However, the proportion of the committed HCs, including Ter119^{+}, CD45^{+} and CD11b^{+} lineages, decreased significantly in *Vav-iCre* CKs. In addition, the total number of isolated fetal liver cells, which are composed primarily of HCs, was decreased. These data suggest that the mutant HSCs and progenitor cells can survive and divide but subsequent differentiation into HCs are defective. Our findings provide crucial evidence that c-Myc is required in a cell-autonomous fashion for HSC differentiation. This finding is complementary to the finding in *Sox2-Cre* CKs (Dubois et al., 2008).

The fact that mutant embryos in which *c-myc* is deleted in the entire embryo proper but not the placenta survived 2 additional days beyond the survival of the complete null embryo shows that *c-myc* is essential for placental development. It is currently unknown which specific cell lineage(s) c-Myc may function in and what precise role c-Myc may play in the placenta. However, this finding is intriguing in light of recent discoveries that the placenta is an active site for HSC development (Gekas et al., 2005; Mikkola et al., 2005). Given the crucial function of c-Myc in both fetal and adult hematopoiesis, it is plausible that c-Myc may function in placental HSCs. The c-Myc CKs described here may serve as an excellent model to elucidate the molecular control of placental hematopoiesis.

c-Myc is not required for embryonic vasculogenesis

Previous reports suggest that *c-myc* is required for vasculogenesis (Baudino et al., 2002) and downregulation *of c-myc* in cultured ECs leads to cellular senescence (Guney and Sedivy, 2006). By contrast, we show here that vasculogenesis occurs in the absence of *c-myc*. Our method of gene excision is efficient, leading to the deletion of the entire coding region for c-Myc (Trumpp et al., 2001). We therefore respectfully disagree with this earlier conclusion. Gene disruption in the two studies was achieved by targeting a similar region of *c-myc*, so the reason for the discrepancy between our observations and those of Baudino et al. are currently unclear. However, we confirmed our results in three independent *c-myc*-deficient mutants (*c-myc* null, *Sox2-Cre* and *Tie2-Cre* CKs), and the presence of ECs in *c-myc* deleted embryos was also verified in a different laboratory (Dubois et al., 2008). We conclude that *c-myc* is not required for vasculogenesis in embryos.

At the cellular level, *c-myc*-deficient ECs did not exhibit detectable defects in cell proliferation, survival, migration or even capillary morphogenesis. These results are in contrast to the report that c-Myc is essential for EC proliferation in culture (Guney and Sedivy, 2006). We performed proliferation assays using primary ECs to closely mimic in vivo conditions. In addition, neither N- nor L-Myc compensated for the loss of c-Myc. We also show that about one third of *Tie1-Cre* CKs, in which c-Myc was deleted in the majority of ECs, survived into adulthood without apparent abnormalities. This result suggests that widespread deletion of *c-myc* in the endothelium is compatible with survival. Taken together, our observations suggest that abrogating c-Myc in ECs may not disrupt angiogenesis, and c-Myc likely regulates angiogenesis through a non-cell-autonomous fashion.

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Hematopoietic abnormalities caused by *c-myc* deletion lead to defects in angiogenesis

Although a primitive vascular network formed in the absence of *c-myc*, its angiogenic remodeling into a complex vascular tree was abnormal. Because c-Myc-deficient ECs appear to function normally, we propose that defective HCs cause the vascular defects observed in our *c-myc* CK embryos. Supporting this notion, vascular defects in the *Vav-iCre* CKs, where *c-myc* is deleted specifically in the hematopoietic lineage, resembled those in the *Tie2-Cre* CK embryos.

HCs are known to affect angiogenesis through hemodynamic influence (Lucitti et al., 2007) and oxidative stress, such as hypoxia (Jones et al., 2004; Ramirez-Bergeron et al., 2006). The reduced hematocrit in the mutants likely changes the viscosity of the blood, and hence alters the hemodynamic forces required for growth and maintenance of vessel size (Lucitti et al., 2007). Moreover, both *Vav-iCre* and *Tie2-Cre* CK embryos were anemic by 11.5 dpc. Hypoxia was evident by elevated VEGF levels, a common consequence of embryos in hypoxic conditions. Hypoxia causes pan-tissue damage via apoptosis (Graven et al., 1993). Therefore, both low hematocrit and the hypoxia-mediated apoptosis could contribute to the vascular defects observed in the *c-myc* mutants.

However, lack of hemodynamic force and increased hypoxic stress are not the only explanation for the absence of vascular remodeling in *c-myc* mutants. HCs secrete factors capable of promoting angiogenesis in a paracrine manner (Okamoto et al., 2005; Okuda et al., 1996). Lack of proangiogenic factors from HCs are responsible for angiogenic defects in *Arnt* (Ramirez-Bergeron et al., 2006) and *Aml1* (Takakura et al., 2000) mutants. As *Tie-2Cre;c-myc*$^{flox/-}$ embryos have hematopoietic defects, they might also lack HC-derived proangiogenic factors, which could explain their angiogenesis defects.

Consistent with this hypothesis, pale *Vav-iCre;c-myc*$^{flox/-}$ embryos exhibited a significant decrease of *Il1b* and *Mmp2* mRNA at 11.5 dpc. IL1β is secreted primarily from monocytes and macrophages. In the anemic *Vav-iCre;c-myc*$^{flox/-}$ embryos, the significant decrease of CD11b^{+} myeloid cells in fetal livers and peripheral blood likely explains the decrease of IL1β expression and secretion. IL1β and MMPs were recently found to form an axis to regulate the bioavailability of VEGF in angiogenesis (Shchors et al., 2006). IL1β mobilizes VEGF from the extracellular matrix (ECM) to ECs during active angiogenesis, via its ability to promote expression and proteolytic activation of stromal MMPs (Mountain et al., 2007; Shchors et al., 2006). MMPs not only modulate the ECM but also cleave the ECM binding domain of VEGF and release isoforms of VEGF to ECs (Bergers et al., 2000). Depletion of these and other paracrine factors originating from hematopoietic cells is likely to contribute to the impaired angiogenesis in the mutant, despite the elevated VEGF mRNA levels.

We therefore suggest that a combination of defects including reduction in hemodynamic stress and hypoxia-induced apoptosis with a shortage in proangiogenic factors contributes to the vascular defects in the mid-gestation *c-myc* mutant embryos. If these vascular defects resulted exclusively from the loss of HCs, then preserving *c-myc* in HCs while deleting it in ECs should allow the mutant embryos to survive beyond midgestation and develop a normal vasculature. Indeed, when we deleted *c-myc* in the majority of ECs but only a subset of HCs using the *Tie1-Cre* line, all of these mutants survived to late gestation, well past the lethality of *Vav-iCre;c-myc*$^{flox/-}$ embryos. These results suggest that c-Myc regulates angiogenesis through its control over hematopoiesis and the production of paracrine factors.

The physiological function of *c-myc* may be restricted to hematopoietic lineages in the embryo and placenta

Retention of *c-myc* in the visceral endoderm and the extra-embryonic ectoderm of *Sox2-cre* mutants prevented the gross organ abnormalities seen in *c-myc*$^{-/-}$ embryos and extended the embryo survival, demonstrating that *c-myc* plays an essential function in these tissues. In the embryo proper, the severe *Vav-iCre* CKs phenocopied the *Tie2-Cre* and *Sox2-Cre* CKs, suggesting the possibility that *c-myc* in the HCs is most crucial for the development and survival of the embryo at this stage.

Therefore, our genetic evidence suggests the possibility that *c-myc* functions restrictively in placenta and HCs but less so in other tissues. Supporting this notion, we found no significant cell autonomous requirement for *c-myc* in ECs. Other studies also indicate that c-Myc is dispensable for the homeostasis of the adult intestinal epithelium (Benitah et al., 2005; Oskarsson et al., 2006), postnatal hepatocyte proliferation (Baena et al., 2005) and liver regeneration (Li et al., 2006). Although further investigation is required to delineate the precise physiological function of c-Myc, our data and the published findings raise the hypothesis that *c-myc* may be uniquely required in the hematopoietic lineage and placenta, playing a less crucial role in other cell lineages in vivo.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/14/2467/DC1

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Table S1. Deletion of c-Myc by Tie2-Cre results in embryonic death at 11.5 dpc with anemia

Embryonic stage (dpc)	Litters	Mutant embryos			
		Mutant/control	Pale	Growth retarded	Alive
8.5	10	21/83 (25.3)	0/21 (0)	0/21 (0)	21/21 (100)
9.5	7	8/44 (15.4)	0/8 (0)	0/8 (0)	8/8 (100)
10.5	15	34/117 (22.5)	32/34 (94)	19/34 (56)	28/34 (18)
11.5	6	7/52 (11.9)	7/7 (100)	7/7 (100)	0/7 (0)
12.5	4	3/33 (9.1)	3/3 (100)	3/3 (100)	0/3 (0)

Numbers are given with percentage in parentheses.