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Placental rescue reveals a sole requirement for c-Myc in embryonic erythroblast survival and hematopoietic stem cell function

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The c-Myc protein has been implicated in playing a pivotal role in regulating the expression of a large number of genes involved in many aspects of cellular function. Consistent with this view, embryos lacking the *c-myc* gene exhibit severe developmental defects and die before midgestation. Here, we show that Sox2Cre-mediated deletion of the conditional *c-myc*^{flox} allele specifically in the epiblast (hence trophoectoderm and primitive endoderm structures are wild type) rescues the majority of developmental abnormalities previously characterized in *c-myc* knockout embryos, indicating that they are secondary defects and arise as a result of placental insufficiency. Epiblast-restricted c-Myc-null embryos appear morphologically normal and do not exhibit any obvious proliferation defects. Nonetheless, these embryos are severely anemic and die before E12. c-Myc-deficient embryos exhibit fetal liver hypoplasia, apoptosis of erythrocyte precursors and functionally defective definitive hematopoietic stem/progenitor cells. Specific deletion of *c-myc*^{flox} in hemogenic or hepatocytic lineages validate the hematopoietic-specific requirement of c-Myc in the embryo proper and provide in vivo evidence to support a synergism between hematopoietic and liver development. Our results reveal for the first time that physiological levels of c-Myc are essential for cell survival and demonstrate that, in contrast to most other embryonic lineages, erythroblasts and hematopoietic stem/progenitor cells are particularly dependent on c-Myc function.

KEY WORDS: c-Myc, Hematopoietic stem cell, Placenta, Embryonic hematopoiesis, Fetal liver, Survival, Mouse

INTRODUCTION

During the development of the vertebrate 'conceptus' (comprising all the products of conception, including the embryo, placenta and all extra-embryonic membranes), hematopoiesis initiates after gastrulation, when a subset of specialized mesodermal precursor cells become committed to the hematopoietic lineage. The establishment of the hematopoietic system occurs in two apparently independent waves: primitive yolk sac (YS) hematopoiesis followed by intra-embryonic definitive hematopoiesis. In the mouse, primitive blood formation begins at embryonic day (E) 7.0-7.5 in the YS where mesodermal clusters form morphological identifiable 'blood islands', whereby cells at the periphery differentiate into early endothelial cells and the cells in the centre form the first erythroid cells (Cumano and Godin, 2007; Ferkowicz and Yoder, 2005). Primitive pro-erythroblasts, characteristically large nucleated cells, enter the circulation at E9 where they undergo further maturation. Primitive YS hematopoiesis exists only transiently in the conceptus and although it does not contribute to the hematopoietic system long term, it ensures the rapid production of differentiated blood cells (primitive erythrocytes, macrophages, megakaryocytes) for the immediate needs of the embryo before the definitive system is sufficiently developed (Cumano and Godin, 2007; Palis and Yoder, 2001).

The first definitive hematopoietic cells to emerge in the conceptus are hematopoietic stem cells (HSCs). The main site of HSC emergence occurs autonomously within the aorta-gonad-mesonephros (AGM) region of the embryo proper, where hematopoietic clusters are observed along the ventral wall of the dorsal aorta from E10.0 (Dzierzak and Speck, 2008; Medvinsky and Dzierzak, 1996). Extra-embryonic tissues, including the YS (Lux et al., 2008; Samokhvalov et al., 2007), vitelline and umbilical arteries (de Bruijn et al., 2000) and placenta (Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Zeigler et al., 2006), have also been attributed with definitive hematopoietic potential. AGM derived HSCs isolated at E10.0-11.5 bear long-term repopulating HSC activity and can be identified by a combination of cell-surface markers, including the pan-hematopoietic marker CD45, AA4.1, Kit and CD34 (Bertrand et al., 2005; Kumaravelu et al., 2002; Sanchez et al., 1996; Taoudi et al., 2005).

Newly generated HSCs are subsequently thought to migrate to the placenta, which has recently been identified to act as a major (although temporary) embryonic HSC niche, where nascent HSCs from the AGM or umbilical arteries mature and expand (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). HSCs are then thought to migrate from the placenta via the umbilical arteries to the second major hematopoietic organ, the fetal liver, where they further mature, expand and undergo differentiation along erythroid, myeloid and lymphoid lineages. Just before birth, definitive HSCs begin to migrate to the bone marrow where they populate niches located at the endosteum and from where adult hematopoiesis is controlled and maintained during adulthood (Wilson and Trumpp, 2006). A number of genes have been shown to be important for primitive and/or definitive hematopoiesis. Many genes affecting primitive YS hematopoiesis encode transcription factors such as Scl, Lmo2 and

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Gata1, and embryos lacking primitive hematopoiesis die before midgestation (E10.5). By contrast, embryos containing mutations in genes that affect the proliferation and/or differentiation of early definitive stem/progenitor cells or more mature myeloid lineages, such as Myb, Rel, Aml/Runx1, Cbfβ and Lhx2, survive significantly longer and die between E11.5 and E16 (Godin and Cumano, 2002).

The proto-oncogene *c-myc* plays an important role during normal adult hematopoiesis and is overexpressed in numerous hematopoietic malignancies. It encodes a nuclear basic helix-loop-helix leucine zipper protein c-Myc, which coordinates expression of a large number of diverse genes involved in processes necessary for cell expansion, cell growth, metabolism, ribosome biogenesis, proliferation, differentiation and stem cell function (Adhikary and Eilers, 2005; Hurlin and Dezfouli, 2004). Recent data also suggest an involvement of c-Myc in the control of various stem/progenitor populations, including those found in the epidermis, intestine and bone marrow (Arnold and Watt, 2001; Benitah et al., 2005; Bettess et al., 2005; Muncan et al., 2006; Murphy et al., 2005; Oskarsson et al., 2006; Waikel et al., 2001; Wilson et al., 2004). In addition, c-Myc is a member of a group of four genes sufficient to convert both mouse tail tip fibroblasts and human fibroblasts into cells with pluripotent stem cell like activity, indicating that c-Myc may be a component to supply cells with self-renewal activity (Knoepfler, 2008; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007). Many primary and established cell lines require c-Myc to maintain proliferation, suggesting that Myc activity is indispensable for cell cycle progression (Grandori et al., 2000). This assumption appeared to hold true in vivo, as embryos in which the *c-myc* gene has been eliminated by gene targeting in ES cells (*c-myc*$^{\Delta ORF/\Delta ORF}$) fail to thrive and die before midgestation. c-Myc-deficient embryos are much smaller and are often delayed in development. They exhibit multiple defects, including an enlarged pericardial sac, failure to turn, a wavy neural tube, hypoplastic branchial arches and lack of blood cells (Davis et al., 1993; Trumpp et al., 2001). However, in this report, we show that embryos derived from epiblast-restricted deletion of c-Myc appear morphologically normal and exhibit no obvious proliferation defects, and we attribute most of the developmental defects of *c-myc*$^{\Delta ORF/\Delta ORF}$ embryos to placental insufficiency. Most interestingly, the only epiblast-derived lineage that absolutely requires c-Myc activity through to E12 is the hematopoietic system.

MATERIALS AND METHODS

Generation of mice and embryos

c-myc$^{flox/flox}$ mice as previously described (Trumpp et al., 2001) were crossed with *Sox2Cre* transgenic mice (Vincent and Robertson, 2003), *Tie2Cre* transgenic mice (Braren et al., 2006) or *HNF4αCre* transgenic mice (Vincent and Robertson, 2004) to generate epiblast-, vascular and hematopoietic-, and liver-specific *c-myc*-deficient mice, respectively. Controls are defined as *Sox2Cre;c-myc*$^{flox/+}$, *c-myc*$^{flox/+}$ or *c-myc*$^{\Delta ORFrec/+}$. No difference was observed between these embryos in any of the experiments described. For embryo analysis, timed matings were set up and the morning of the vaginal plug was considered as embryonic day 0.5 (E0.5). Analysis of the Cre-mediated deletion efficiency at the *c-myc*^{flox} locus was assessed by Southern blot analysis as previously described (Trumpp et al., 2001).

Histology

Freshly isolated embryos were fixed in 10% buffered formaldehyde overnight at 4°C, then dehydrated to 70% ethanol. After embedding in paraffin, 4 μm sections were cut by using a microtome. For routine histology, sections were stained with Hematoxylin and Eosin.

Immunohistochemistry

Histological slides were rehydrated and treated with 3% hydrogen peroxide for 20 minutes. Antigen retrieval was performed by boiling samples in Tris buffer (pH 9) for 20 minutes (c-Myc, BrdU, HNF4α) or proteinase K (laminin, cytokeratin). Blocking was carried out in 1% BSA in PBS for 30 minutes. Slides were incubated overnight at 4°C with the following primary antibodies: c-Myc (Upstate, 06-340) 1:250 or (Santa Cruz, N262) 1:250; laminin (Dako Cytomation, Z0097) 1:600; cytokeratin (Dako Cytomation, Z0622) 1:1200; BrdU (Oxford Biotechnology, OBT 0030) 1:100; PECAM (CD31) (Pharmingen, cat:01951D) 1:100; and HNF4α (Santa Cruz) 1:100. Slides were washed and incubated for 1 hour with secondary HRP antibodies (Dako, K4000/K4002 or Amersham, LA9350W) or Alexa488 (Molecular Probes, A11034).

Colony forming assay

CFU assays were performed as previously described (Delassus and Cumano, 1996). Single cell suspensions of E10.5 control and *Sox2Cre;c-myc*^{flox2} embryos (devoid of head and limbs) or YS were grown in methylcellulose containing a mixture of rIL3, rSCF, rGM-CSF (2ng/ml) and human rEpo (4 U/ml) (StemCell Technology, Vancouver, MethoCult M2231). Colonies were counted and analyzed after 8 days.

Flow cytometry analysis

Embryos were collected at E10.5-11.0 and dissected (to remove head and limbs) under a stereomicroscope. Tissues were mechanically disrupted to obtain single-cell suspensions by passing them through a 26 G needle. Cells were incubated for 30 minutes on ice with the following mAb conjugates: Ter119-APC-Cy7, Kit (CD117)-PE-Cy7, CD34-PE, CD71-PE (eBioscience) and BrdU-FITC (BD Bioscience). For the TUNEL assay the 'in situ cell death detection kit (fluorescein)' from Roche was used according to the manufacturer's instructions. Five- and six-color FACS analysis was performed using a FACS Canto Flow Cytometer (Becton Dickinson) and data were analyzed using FlowJo (Tree Star, USA) software. Cell sorting of CD45^{+} cells was performed on a FACS Aria Flow Cytometer (Becton Dickinson).

Analysis of Myc expression

RNA from control and *Sox2Cre;c-myc*^{flox2} embryos was isolated using the RNeasy mini kit (Qiagen). cDNA was generated using Stratascript Reverse Transcriptase (Stratagene) followed by gene-specific PCR with primers for *c-myc*, *N-myc* and *L-myc* as previously described (Wilson et al., 2004). All samples were normalized using β2-microglobulin.

RESULTS

Epiblast-specific deletion of *c-myc*^{flox} prevents all but one of the developmental defects observed in *c-myc*$^{\Delta ORF/\Delta ORF}$ embryos

Previous studies detailing the expression of Myc transcripts during gastrulation of the mouse conceptus revealed abundant levels of *c-myc* RNA in the following extra-embryonic structures: ectoplacental cone, extra-embryonic ectoderm, primary/secondary giant cells, allantois and chorion (Downs et al., 1989). In order to circumvent a putative requirement of c-Myc signaling activity in the extra-embryonic tissues of the conceptus and specifically dissect the role of c-Myc in the development of the embryo proper, we induced deletion of the conditional *c-myc*^{flox} allele (Fig. 1A) exclusively in the epiblast using a Sox2Cre mouse strain (Hayashi et al., 2002; Trumpp et al., 2001; Vincent and Robertson, 2003). *Sox2Cre;c-myc*$^{flox/flox}$ embryos were observed in the expected Mendelian ratio up to E11.5; however, no viable mutant embryos were observed beyond E12.5 (see Table S1 in the supplementary material). Analysis of the recombination efficiency revealed virtually complete deletion of the *c-myc*^{flox} allele within *Sox2Cre;c-myc*$^{flox/flox}$ embryo proper (Fig. 1B), whereas the extra-embryonic placental tissue comprised mostly of the unrecombined *c-myc*^{flox} allele (extra-embryonic ectoderm-derived chorionic trophoblast and spongiotrophoblast cells) and, as expected, few c-Myc-deficient epiblast-derived cells (chorionic mesoderm and allantois components) (Fig. 1B). The level of *c-myc* transcripts within the *Sox2Cre;c-myc*$^{flox/flox}$ embryo proper was decreased about 600-fold, although no significant change in the expression of *N-myc* (also known as *Mycn*) and *L-myc* was observed (Fig. 1C).

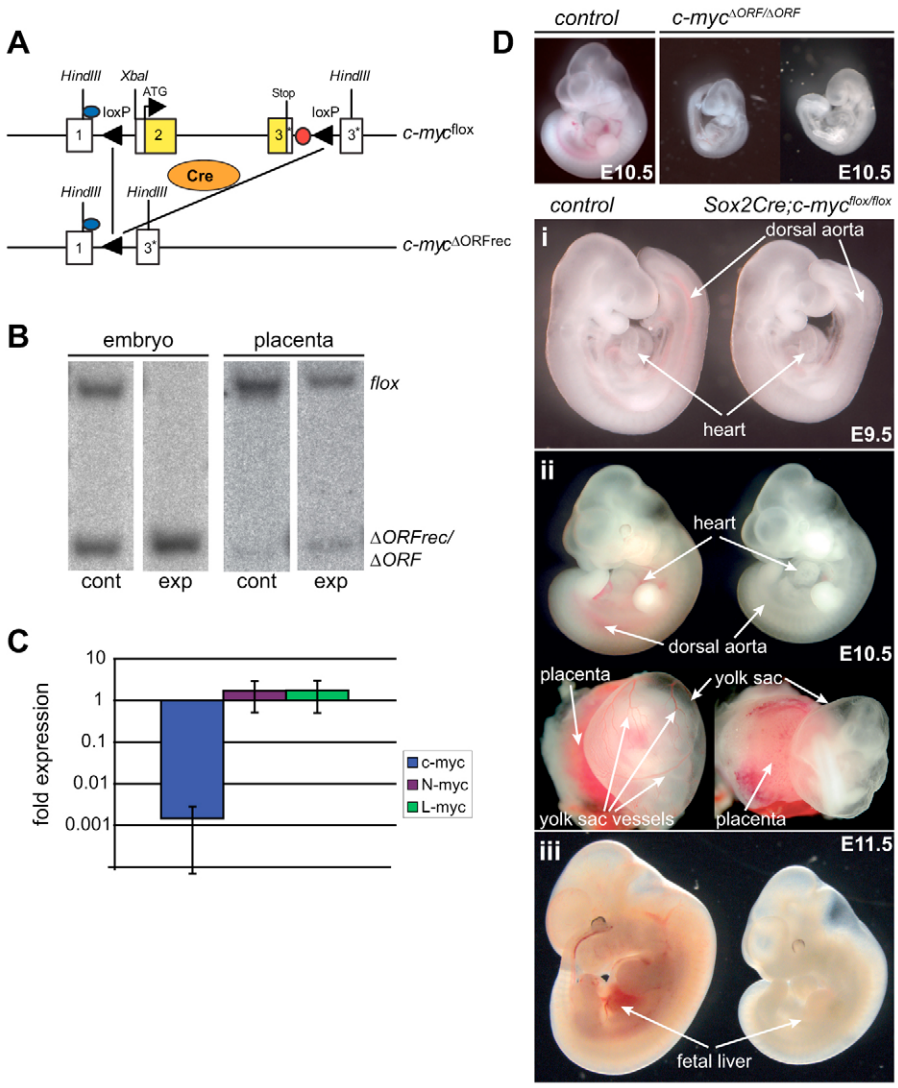

Fig. 1. Sox2-Cre mediated epiblast-specific deletion of *c-myc* reveals viable embryos up to E11.5. (**A**) Schematic diagram showing the conditional *c-myc*^{flox} allele (Trumpp et al., 2001). Exons are indicated as boxes 1-3. The open reading frame (ATG to Stop; yellow segments) is flanked by loxP sites (black triangles). Restriction sites and probe (blue ovals) used for Southern blot analysis are indicated. (**B**) Southern blot showing recombination at the *c-myc* locus in *c-myc*$^{flox/\Delta ORFrec}$ (cont) and *Sox2Cre;c-myc*$^{flox/flox}$ (exp) embryo and *c-myc*$^{flox/flox}$ (cont) and *Sox2Cre;c-myc*$^{flox/flox}$ (exp) placenta at E10.5. (**C**) Real-time RT-PCR analysis of *c-myc*, *N-myc* and *L-myc* expression in *Sox2Cre;c-myc*$^{flox/flox}$ embryos at E10.5. Expression levels in mutants are normalized to that in controls, which was set to 1. (**D**) Phenotypes of control (left) and *Sox2Cre;c-myc*$^{flox/flox}$ embryos (right) at (i) E9.5, (ii) E10.5 and YS, and (iii) E11.5.

Most noticeably, *Sox2Cre;c-myc*$^{flox/flox}$ mutant embryos exhibit an entirely different phenotype to that of *c-myc*$^{\Delta ORF/\Delta ORF}$-null embryos (Fig. 1D) (Trumpp et al., 2001). *Sox2Cre;c-myc*$^{flox/flox}$ mutants die during a very tight window between E11.25 and E11.75, and appear morphologically normal, with no apparent defects, delay in development or significant difference in overall embryo size up to E10.5 (Fig. 1D, parts i,ii). The only defect evident in both the embryo proper and YS was their pale appearance, indicative of a hematopoietic/erythroid deficiency (Fig. 1D, part ii). Close to their time of death at E11.5, mutant embryos appear slightly smaller, probably due to the severe anemia (see below) and consequently a failure to thrive (Fig. 1D, part iii). Although extra-embryonic rescue of c-Myc activity prolongs survival of the conceptus for an additional 2 days, loss of c-Myc activity within the embryo proper remains fatal.

c-Myc expression is required for branching morphogenesis of the chorioallantoic placenta

To address a putative role of c-Myc in extra-embryonic/placental tissues, IHC analysis of wild-type placentas from E9.5-E11.5 embryos was performed. This revealed c-Myc protein expression in all compartments of the chorioallantoic placenta, namely the trophoblast giant cells, spongiotrophoblast and chorionic/allantoic cells of the labyrinth layer (Fig. 2A), similar to what has previously been reported by RNA in situ hybridization (Downs et al., 1989). However, no significant c-Myc expression was detected in the deciduum, the maternal compartment of the placenta (Fig. 2A). Circulating hematopoietic cells of embryonic origin, as defined by their morphology (round cells with a high nucleus to cytoplasm ratio) and localization within the fetal blood spaces, also express high levels of c-Myc (Fig. 2A′).

Next, we examined the histology of the chorioallantoic placenta of *c-myc*$^{\Delta ORF/\Delta ORF}$ embryos. The chorioallantoic placenta is formed by the attachment of the epiblast-derived allantois to the extra-embryonic ectoderm derived chorionic trophoblast cells (at E8.5) and the subsequent invasion and branching of the fetal vessels into a highly vascularized structure referred to as the labyrinth (E9.5-E10.5) (Watson and Cross, 2005). Histological analysis of E10.5 *c-myc*$^{\Delta ORF/\Delta ORF}$ mutants revealed severe structural defects in the chorioallantoic placenta (Fig. 2B). Immunohistochemical (IHC) analysis further revealed a severe reduction in the number of epithelial derived (cytokeratin positive) chorionic-trophoblast cells (Fig. 2C) and a failure of the fetal blood vessels and allantoic mesenchyme (laminin positive) to invade the chorion compartment, resulting in a strongly hypoplastic labyrinth layer (Fig. 2C, right panel). Hence, *c-myc*$^{\Delta ORF/\Delta ORF}$ mutants appear to exhibit normal chorioallantoic attachment, but a failure in the initiation of branching

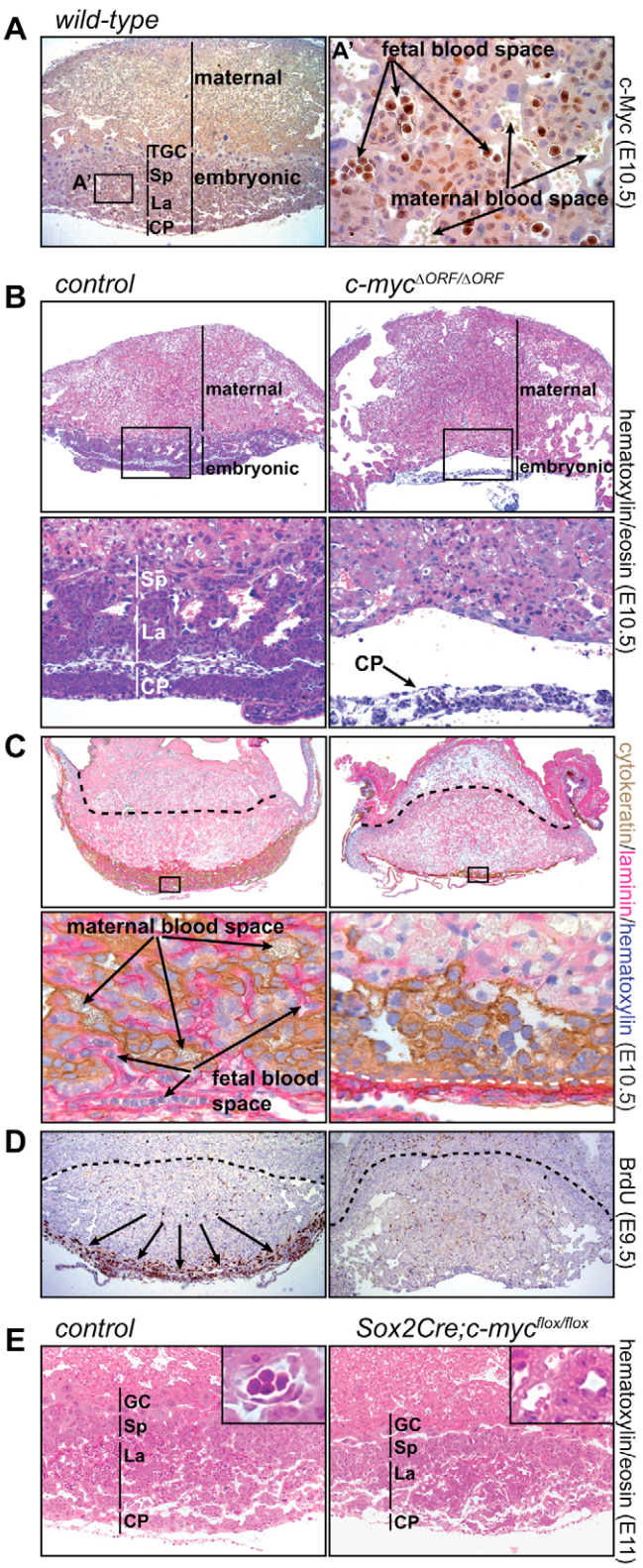

Fig. 2. Severe placental defects in *c-myc*$^{\Delta ORF/\Delta ORF}$ embryos. (**A**) Immunohistochemical analysis of c-Myc expression in the E10.5 wild-type placenta. c-Myc expression is detected in the embryonic parts of the placenta, including the chorionic plate (CP), labyrinth layer (La), spongiotrophoblast (Sp), trophoblast giant cells (TGC) and in hematopoietic cells derived from the embryo, which are located in the fetal blood spaces (inset A'). (B-D) Comparison of control (left) and c-Myc null (*c-myc*$^{\Delta ORF/\Delta ORF}$) embryos (right) at E10.5. (**B**) Hematoxylin and eosin (H&E) staining of control and c-Myc-deficient placenta at E10.5. The bottom panels show an enlargement of the boxed areas in the top panels. (**C**) Analysis of placental vascularization. Laminin (pink) is expressed on embryonic vessels and defines fetal blood spaces. Cytokeratin (brown) marks trophoblast cells and defines maternal blood spaces. The bottom panels show an enlargement of the boxed areas in the top panels. The white line demarcates the failure of vessels (pink) to invade into the trophoblast (brown) layer (**D**) Bromodeoxyuridine (BrdU) incorporation analysis of E9.5 control and c-Myc-deficient placenta. Highly proliferating (BrdU+; arrows) tissues are present in control (left) but not in *c-myc*$^{\Delta ORF/\Delta ORF}$ (right) placentas. (**E**) Hematoxylin and eosin (H&E) staining of control and *Sox2Cre;c-myc*$^{flox/flox}$ placenta at E10.5. Placental developmental defects are rescued with respect to *c-myc*$^{\Delta ORF/\Delta ORF}$ in B.

morphogenesis. In addition, c-Myc deficient placentas as a whole are severely hypoplastic and exhibit a dramatic loss in the number of proliferating cells, as assayed by 5-bromodeoxyuridine (BrdU) incorporation (Fig. 2D). These results therefore indicate that c-Myc activity within the extra-embryonic chorionic ectoderm or trophoblast cells is crucial for correct chorioallantoic placentation.

Importantly, *Sox2Cre;c-myc*$^{flox/flox}$ embryos exhibit no defects in the formation of the chorioallantoic placenta (Fig. 2E). The only obvious difference between control and *Sox2Cre;c-myc*$^{flox/flox}$ placentas was a lack of hematopoietic cells within the placental vessels of mutants (Fig. 2E, insets). Hence, the extra-embryonic derivatives of the epiblast that contribute to placentation (allantois and chorionic mesoderm) remain functional in the absence of c-Myc activity. In conclusion, these data strongly suggest that the majority of developmental abnormalities observed in *c-myc*$^{\Delta ORF/\Delta ORF}$-null embryos are secondary defects owing to the failure to develop a functional placenta.

c-Myc deficient primitive erythroblasts undergo cell death

The pale appearance of *Sox2Cre;c-myc*$^{flox/flox}$ embryos suggests a defect in the generation and/or maintenance of the embryonic hematopoietic system. Histological analysis of E11.0 *Sox2Cre;c-myc*$^{flox/flox}$ embryos and YS revealed an almost complete absence of hematopoietic cells (Fig. 3A; data not shown). The majority of blood cells present during midgestation consist of primitive nucleated erythroblasts, generated predominately by YS derived precursors, which enter the embryo at the onset of circulation. Primitive erythroblasts express high levels of the transferrin receptor CD71 and increasing levels of the glycophorin A-associated TER119 during maturation (Fraser et al., 2007). FACS analysis of the erythroid lineage of E11.0 *Sox2Cre;c-myc*$^{flox/flox}$ embryos revealed a 11.5-fold reduction (wild type 51,089±10,951, mutant 4430±1797) in the number of more mature CD71^{+}Ter119^{+} early basophilic erythroblasts (PE, Fig. 3B,C). Even more apparent was the striking loss of erythroblast precursor cells (CD71^{+}Ter119^{-} primitive proerythroblasts; 1500-fold decrease, wild type 141,439±7844, mutant 899±123) (Fig. 3B,C).

In order to address whether the extremely low numbers of erythroid cells present in *Sox2Cre;c-myc*$^{flox/flox}$ embryos is due to aberrant cell death, we assayed TUNEL reactivity via FACS, which revealed a dramatic increase (19-fold) in the apoptosis rate of E9.5

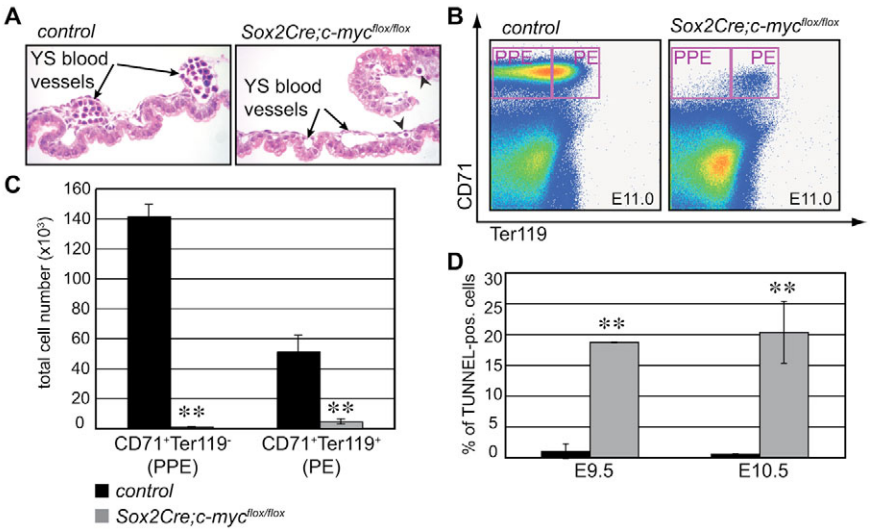

Fig. 3. c-Myc deficiency causes apoptotic loss of primitive erythroblasts. (**A**) H&E staining of control and *Sox2Cre;c-myc*$^{flox/flox}$ YS at E10.5. Control YS vessels contain numerous hematopoietic cells (arrows), whereas mutant vessels are mostly devoid of hematopoietic cells (arrows), although individual cells are sometimes observed (arrowheads). (**B**) FACS analysis of the primitive embryonic system at E11.0. CD71 and Ter119 are used to define primitive proerythroblasts (PPE; CD71^{-}Ter119^{+}) and primitive erythroblasts (PE; CD71^{+}Ter119^{+}). (**C**) Quantitative analysis of total PPE and PE number in control and c-Myc-deficient embryos at E11.0 (**$P\leqslant0.001$). (**D**) Quantitative analysis of TUNEL-positive CD71^{+} cells in E9.5 and E10.5 embryos (**$P\leqslant0.001$).

Sox2Cre;c-myc$^{flox/flox}$ CD71^{+} cells compared with controls (wild type 0.98±1.2%, mutant 18.65±0.07%) (Fig. 3D). This apoptotic phenotype became even more pronounced at E10.5, whereby the CD71^{+} population of *Sox2Cre;c-myc*$^{flox/flox}$ embryos exhibit a 41-fold increase in apoptosis (wild type 0.49±0.08%, mutant 20.28±5.03%) (Fig. 3D). This strong increase in the apoptosis rate was specific to the cells of the erythroid lineage, as there was no significant overall increase in cell death within *Sox2Cre;c-myc*$^{flox/flox}$ embryos (E9.5 wild type 6.35±1.55%, mutant 10.05±0.21%; E10.5 wild type 2.08±0.15%, mutant 4.51±0.28%). The existence of erythroblasts in *Sox2Cre;c-myc*$^{flox/flox}$ embryos (albeit decreased numbers) suggests the initial presence of proerythroblast precursor cells. However, our data show that these cells undergo massive apoptosis and are no longer present in sufficient numbers to maintain hematopoiesis, probably leading to the embryonic death of *Sox2Cre;c-myc*$^{flox/flox}$ embryos.

Definitive hematopoiesis is severely impaired in the absence of c-Myc activity

At E11.0 of gestation, the pan-hematopoietic marker CD45 detects both YS-derived primitive macrophages and hematopoietic progeny derived from definitive HSCs (Bertrand et al., 2005; Taoudi et al., 2005). We therefore examined the expression of CD45 and observed a striking reduction in the number of CD45^{+} blood cells in both the dorsal aorta and fetal liver of E11.0 *Sox2Cre;c-myc*$^{flox/flox}$ embryos (Fig. 4A). Quantification by FACS analysis revealed a sixfold decrease in the absolute number of CD45^{+} hematopoietic cells (Fig. 4B and C). In order to determine whether the *c-myc*^{flox} allele was indeed recombined in the few remaining CD45^{+} cells, these cells were isolated from *Sox2Cre;c-myc*$^{flox/flox}$ embryos by flow cytometry and their RNA analyzed by real-time RT-PCR. This analysis revealed a greater than 1000-fold downregulation of *c-myc* transcripts compared with control cells, demonstrating efficient elimination of *c-myc* mRNA within the small remaining CD45^{+} hematopoietic population.

Early definitive HSCs in the mid-gestation embryo express the cell-surface markers AA4.1 (CD93), Kit (CD117) and CD34 (Cumano and Godin, 2007; Dzierzak and Speck, 2008; Mikkola and Orkin, 2006; Sanchez et al., 1996). FACS analysis of E11.0 *Sox2Cre;c-myc*$^{flox/flox}$ embryos revealed an increase in the percentage of c-Kit^{+}AA4.1^{+} cells within the total CD45^{+} population (Fig. 4D and E). However, the absolute number of CD45^{+}c-kit^{+}AA4.1^{+} cells per mutant embryo was similar to that of control embryos (Fig. 4F). This was further confirmed using the additional stem cell marker CD34 (see Fig. S1 in the supplementary material). By contrast, *Sox2Cre;c-myc*$^{flox/flox}$ embryos exhibit a dramatic decrease in the number of all other definitive hematopoietic cells (CD45^{+}cKit^{+}AA4.1^{-}, CD45^{+}AA4.1^{+}cKit^{-} and CD45^{+}cKit^{-}AA4.1^{-}) (Fig. 4D, Fig. 3). In summary, these data suggest that although c-Myc is not required for the generation of CD45^{+}AA4.1^{+}c-kit^{+}CD34^{+} cells, which are highly enriched for definitive HSCs, c-Myc activity is essential to efficiently generate differentiated progeny from HSCs.

Although definitive stem/progenitor cells are generated in the absence of c-Myc, they are non-functional

In order to address whether the phenotypic *Sox2Cre;c-myc*$^{flox/flox}$ HSCs are functional, we determined the number of cells with colony-forming unit (CFU) activity in E10.5 mutant embryos. In control embryos, BFU-E (blast-forming unit-erythroid) and CFU-G/M (colony-forming unit-granulocyte/macrophage) were found at expected frequencies (93±17.9) (Fig. 4G,H) (Trumpp et al., 2001). By contrast, cells derived from the embryo proper of *Sox2Cre;c-myc*$^{flox/flox}$ mutants formed no or only very few colonies (2.3±3.3) (Fig. 4G,H), with abnormal morphology (composed of extremely small cells), probably representing aberrantly differentiated cells (Fig. 4G, bottom right panel). Very similar results were also obtained in CFU assays derived from YS cells (data not shown). Thus, although the absolute number of phenotypic HSCs is maintained in the absence of c-Myc activity (Fig. 4F), c-Myc-deficient HSCs are defective and unable to generate a significant number of differentiated progeny.

Following the emergence of definitive HSCs within the AGM, hematopoietic cells are thought to migrate via the placenta (recently shown to contribute to HSC expansion) en route to the fetal liver, which serves as the major intra-embryonic hematopoietic organ to support HSC expansion and maturation. Although scarce, the presence of CD45^{+}Kit^{+} cells in the fetal liver of *Sox2Cre;c-myc*$^{flox/flox}$ mutants suggests migration of mutant stem/progenitor cells to the fetal liver is unimpaired (data not shown). This is further supported by the fact that the expression of a number of adhesion molecules overexpressed in c-Myc-deficient adult HSCs (Wilson et al., 2004) such as CD29 (β1 integrin), CD18

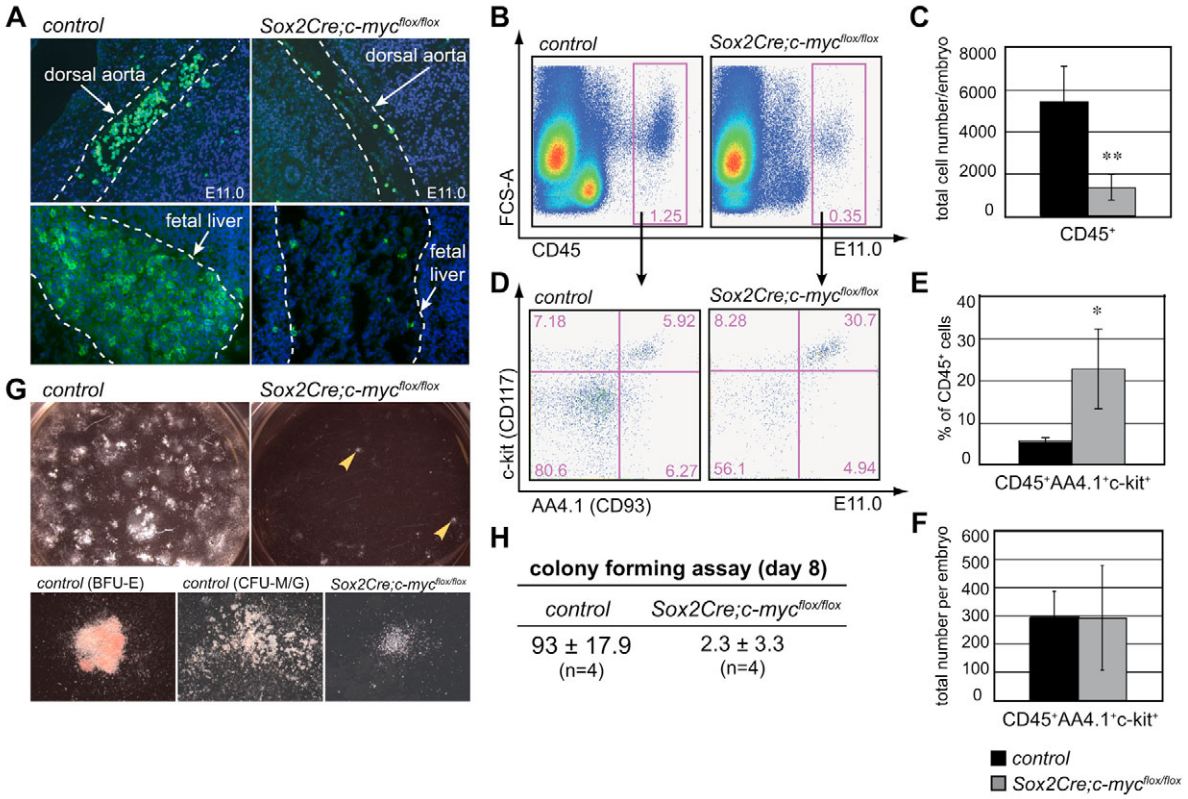

Fig. 4. Impaired definitive hematopoiesis in c-Myc-deficient embryos. (**A**) Expression of the pan-hematopoietic marker CD45 in control and c-Myc-deficient dorsal aorta (top panels) and fetal liver (bottom panels) at E11.0. (**B**) FACS analysis showing CD45 expression in E11.0 embryos. Numbers in the CD45$^+$ gates represent percentage of cells per embryo. (**C**) Quantitative analysis of total CD45$^+$ cells per E11.0 embryos (as defined in B) (**$P \leqslant 0.001$). (**D**) FACS analysis showing the expression of the stem cell markers Kit (CD117) and AA4.1 (CD93) in CD45$^+$ hematopoietic cells of control (left) and c-Myc-deficient (right) embryos at E11.0. Numbers represent percentages in the CD45$^+$ population. (**E**) Quantitative analysis showing the percentage of Kit$^+$AA4.1$^+$ (stem/progenitor) cells within the CD45$^+$ population in E11.0 control and *Sox2Cre;c-myc*$^{flox/flox}$ embryos (*$P \leqslant 0.01$). (**F**) Quantitative analysis showing the number of CD45$^+$Kit$^+$AA4.1$^+$ cells per E11.0 control and *Sox2Cre;c-myc*$^{flox/flox}$ embryos. (**G**) CFU-assay at E10.5. Examples of blast-forming unit-erythroid (BFU-E) and colony-forming unit-macrophage/granulocyte (CFU-M/G) are shown for control embryos. Cells derived from *Sox2Cre;c-myc*$^{flox/flox}$ embryos failed to give rise to typical colonies, but only produced very rare colonies composed of very small cells (bottom right). (**H**) Quantitative analysis of total colony number per embryo after 8 days in culture.

(β2 integrin), CD49b (α2 integrin), CD49e (α5 integrin) and CD11a (αL integrin), are not significantly changed in *Sox2Cre;c-myc*$^{flox/flox}$ mutant stem/progenitor cells (data not shown). Consistent with the observation that c-Myc-deficient HSCs are unable to generate colonies in vitro, all CD45$^+$ cells reside in the fetal liver of *Sox2Cre;c-myc*$^{flox/flox}$ mutants exclusively as single cells, suggesting that c-Myc deficient HSCs (which constitute ~25% of the CD45$^+$ population, Fig. 4E) are unable to expand and generate progeny in the mutant fetal liver micro-environment (Fig. 4A bottom right panels). Thus, despite the fact that HSC generation is unaffected in the absence of c-Myc activity, these cells are functionally defective in vitro and in vivo. Together these results show that c-Myc is an essential factor necessary for embryonic HSC function.

In order to elucidate the molecular mechanism responsible for the observed hematopoietic defect, we determined the expression of the c-Myc target p21^{CIP1} (Oskarsson et al., 2006) and critical regulators of embryonic hematopoiesis, including *Scl* (Shivdasani et al., 1995), *Runx1* (Castilla et al., 1996), *Gata1* (Pevny et al., 1991), *Gata2* (Tsai et al., 1994), *Klf4* and *Klf6* (Matsumoto et al., 2006) in CD45$^+$ cells by real-time RT PCR. As has been observed in other c-Myc-deficient cells (Oskarsson et al., 2006), p21^{CIP1} was upregulated (2.1-fold), but among the others, only Klf4 was statistically significantly upregulated (1.6-fold) in c-Myc-deficient CD45+ cells (see Fig. S4 in the supplementary material).

Non-hematopoietic tissues proliferate and develop normally even in the absence of c-Myc

In search of non-hematopoietic defects in *Sox2Cre;c-myc*$^{flox/flox}$ embryos, a detailed analysis of E10.5-E11.5 mutant embryos was performed. For tissues difficult to distinguish by standard histological analysis alone, specific markers such as Pdx1 (pancreas) and alkaline phosphatase (primordial germ cells) were included (Kim and MacDonald, 2002; Lawson et al., 1999). Surprisingly, these studies revealed no obvious developmental abnormalities (see Fig. S2 in the supplementary material) apart from fetal liver hypoplasia (discussed in more detail below). In addition, BrdU incorporation analyses revealed no significant difference in the proliferative capacity of c-Myc-deficient organs/tissues (Fig. 5A). Similarly, no major change in the rate of apoptosis (caspase 3 and TUNEL) was observed in mutant embryos (data not shown and see above). Moreover, PECAM (CD31) staining of *Sox2Cre;c-myc*$^{flox/flox}$ and *c-myc*$^{\Delta ORF/\Delta ORF}$ embryos revealed the presence of an extensive vascular network, suggesting that c-Myc is not required for the establishment of the

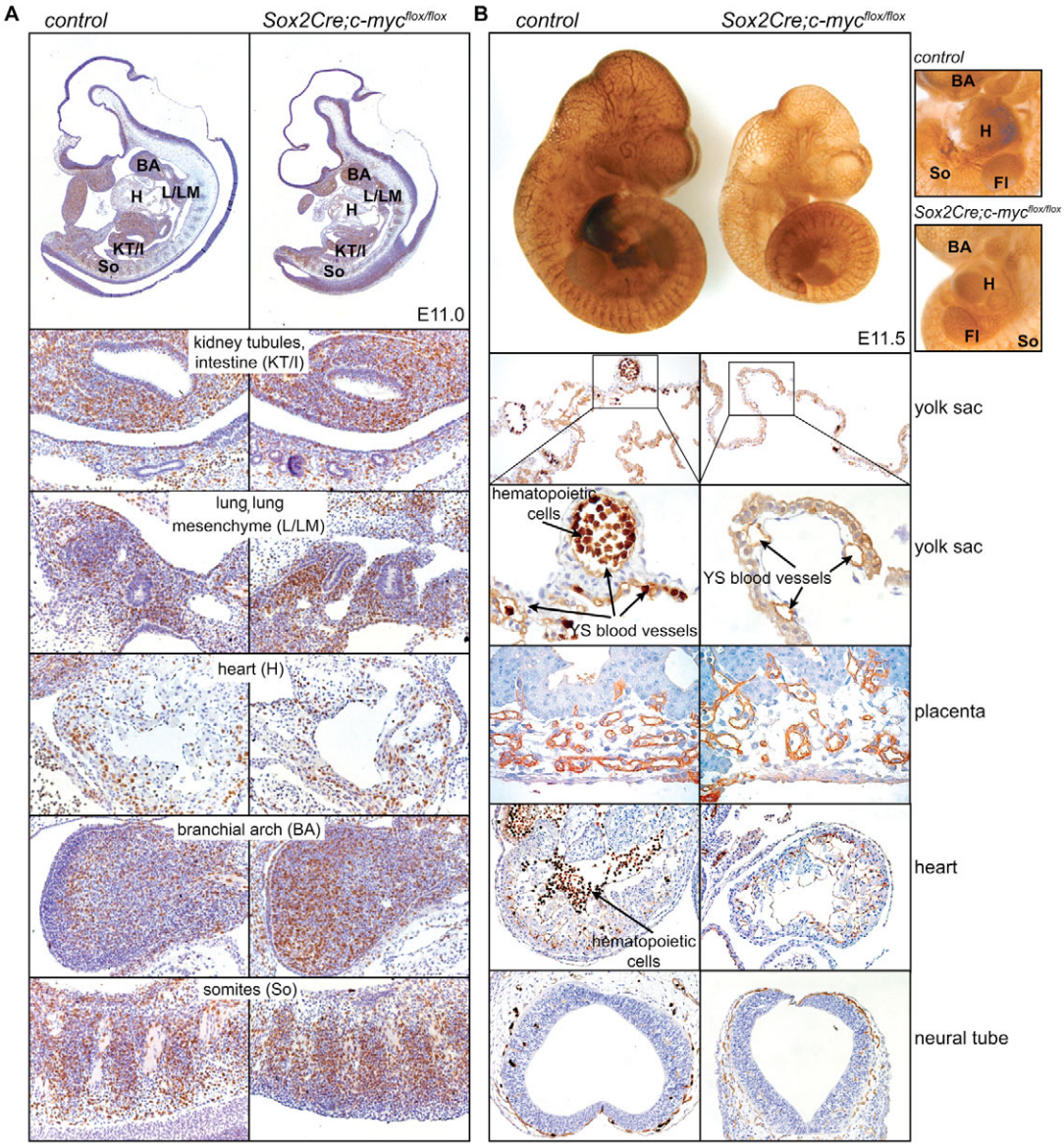

Fig. 5. Proliferation and development of non-hematopoietic tissues, including the vascular system, are normal in c-Myc-deficient embryos. (**A**) Immunohistochemical analysis of BrdU incorporation in control (left) and c-Myc-deficient (right) embryos at E11.0. (**B**) PECAM (CD31) staining in wholemounts (top) and histological sections (bottom) show normal development of the vascular system in the embryo, the placenta and the YS of *Sox2Cre;c-myc*$^{flox/flox}$ embryos at E11.0.

vascular system as has recently been reported (Fig. 5B; data not shown) (Baudino et al., 2002). These results indicate that c-Myc is not directly required to maintain cell proliferation and survival outside the hematopoietic system in vivo.

Genetic confirmation for the unique requirement for c-Myc during hematopoietic development

The results presented thus far indicate that the only epiblast-derived tissue perturbed in the absence of c-Myc activity is the hematopoietic system. In order to genetically prove this hypothesis, we generated *Tie2Cre;c-myc*$^{flox/flox}$ embryos, whereby c-Myc is specifically deleted in the hemogenic precursor of hematopoietic and endothelial lineages (Braren et al., 2006). As expected, *Tie2Cre;c-myc*$^{flox/flox}$ embryos are lethal at E11.5 and exhibit a hematopoietic phenotype identical to that described above for *Sox2Cre;c-myc*$^{flox/flox}$ embryos (Fig. 6A; data not shown). FACS analysis of E11.0 *Tie2Cre;c-myc*$^{flox/flox}$ embryos revealed a severe decrease in the total number of CD45^{+} hematopoietic cells (Fig. 6B), and the typical increase in the relative percentage of CD45^{+}c-Kit^{+}AA4.1^{+} stem/progenitors and concomitant decrease in progenitors and mature cells (Fig. 6C). Similarly, we observed no major vascular defects in E11.0 *Tie2Cre;c-myc*$^{flox/flox}$ YS or embryos (data not shown). The virtually identical phenotypes of *Tie2Cre;c-myc*$^{flox/flox}$ and *Sox2Cre;c-myc*$^{flox/flox}$ embryos provide strong genetic evidence that in the embryo proper, c-Myc is uniquely required for the development of the hematopoietic lineage.

Fetal liver hypoplasia develops as a result of hematopoietic cell deficiency

Although the location of fetal liver niches, the cell types they are comprised of or the molecules required for its activity are not known, it is likely that hepatoblastic cells are at least in part involved in the

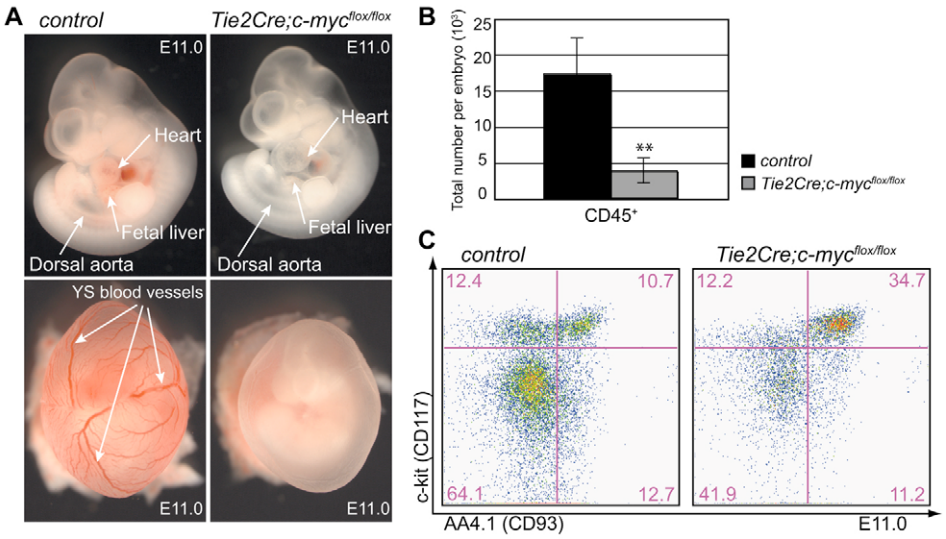

Fig. 6. Hematopoietic- and endothelial-specific elimination of c-Myc via Tie2Cre. (**A**) Phenotypes of control and *Tie2Cre;c-myc*$^{flox/flox}$ embryos and YS at E11.0. (**B**) Quantitative analysis of total CD45$^+$ cells per E11.0 embryos (**$P \leqslant 0.001$). (**C**) FACS analysis showing the expression of the stem cell markers Kit (CD117) and AA4.1 (CD93) in CD45$^+$ hematopoietic cells of control (left) and *Tie2Cre;c-myc*$^{flox/flox}$ (right) embryos at E11.0. Numbers represent percentages in the CD45$^+$ population.

fetal liver niche (Cumano and Godin, 2007). To address whether c-Myc activity is important for the generation, maintenance or function of hepatoblasts and putative HSC micro-environment, E10.5 *Sox2Cre;c-myc*$^{flox/flox}$ fetal livers were examined and observed to be dramatically reduced in size compared with control liver samples (a decrease which is numerically larger than the loss of hematopoietic cells alone). Histological analysis revealed severe morphological defects, whereby mutant livers exhibited a mesenchymal rather than typical epithelial organization of this organ (Fig. 7A,B). Nevertheless, *Sox2Cre;c-myc*$^{flox/flox}$ liver cells expressed normal levels of the hepatocyte nuclear factor 4α (HNF4α), confirming their identity as hepatoblasts (Fig. 7C). To address genetically whether c-Myc is directly required for early liver development, the *c-myc*^{flox} allele was specifically deleted in fetal liver hepatoblasts from E9.0 using the *Hnf4a-Cre* mouse line (Vincent and Robertson, 2004). Unexpectedly, *Hnf4aCre;c-myc*$^{flox/flox}$ embryos were viable and histological analysis of E10.5-E16.5 embryos revealed no apparent difference in liver size or morphology (Fig. S3). Moreover, detailed analysis of all major hematopoietic cell types including HSCs were present in normal numbers in hepatoblast-restricted c-Myc null embryos (data not shown). These data suggest that c-Myc is neither directly required for embryonic liver development nor for the establishment or maintenance of the hematopoietic liver micro-environment. Moreover, these genetic results confirm that the severe liver phenotype observed following epiblast-restricted deletion of c-Myc (*Sox2Cre;c-myc*$^{flox/flox}$ embryos) is not due to loss of c-Myc within the hepatic cells, but rather represents a secondary defect due to defective hematopoiesis. This hypothesis is further supported by the observation that the fetal livers of *Tie2Cre;c-myc*$^{flox/flox}$ embryos, the hepatoblasts of which continue to express c-Myc, show the same reduction in fetal liver size and hematopoietic defects observed in *Sox2Cre;c-myc*$^{flox/flox}$ embryos (Fig. 7D). These data therefore show that in the absence of hematopoietic cells, hepatoblasts fail to expand and are unable to establish a normal liver morphology. To our knowledge, these results provide the first genetic evidence that hematopoietic cells are crucial for the early expansion and structural establishment of the embryonic liver.

DISCUSSION

The results of this genetic study have revealed several unexpected roles for c-Myc function during early development of the mouse embryo (see model in Fig. 7E). The first is the importance of c-Myc activity in extra-embryonic tissues, given the severe defects in chorioallantoic placentation that arise in *c-myc*$^{\Delta ORF/\Delta ORF}$ null embryos. Moreover, we genetically demonstrate that most of the developmental defects present in *c-myc*$^{\Delta ORF/\Delta ORF}$-null embryos, including abnormalities of the heart, pericardium, neural tube, delay in embryo turning and embryo size (Davis et al., 1993; Trumpp et al., 2001) are virtually absent in c-Myc-deficient embryos that develop with a wild-type extra-embryonic support system (*Sox2Cre;c-myc*$^{flox/flox}$ and *Tie2Cre;c-myc*$^{flox/flox}$ embryos). We have therefore shown that c-Myc, initially believed to be crucial for the development of many embryonic structures, is indeed primarily important for placental development, and that this causes severe secondary defects and early lethality. Future studies detailing the spatio-temporal expression of c-Myc in the individual components/structures necessary to generate a functional chorio-vitelline and chorio-allantoic placenta should reveal the exact role of this protein in placentation. However, this initial assessment suggests that c-Myc plays an important role in the chorionic trophoblast cells. c-Myc expression is controlled by a large number of signaling pathways, including the canonical Wnt-β-catenin, Notch and Ras-MAPK pathway or signaling occurring downstream of many receptor tyrosine kinases such as PdgfR, Kit or TpoR (Barone and Courtneidge, 1995; Chanprasert et al., 2006; Grandori et al., 2000; Sansom et al., 2007; Sharma et al., 2007). Given that our study has uncovered c-Myc as a crucial component of placental development, it is likely that other knockout mouse models of genes influencing c-Myc expression may also exhibit thus far undocumented placental defects.

Second, via extensive analysis of *Sox2Cre;c-myc*$^{flox/flox}$ and *Tie2Cre;c-myc*$^{flox/flox}$ embryos, we have shown that c-Myc activity is dispensable for the development of most embryonic organs/tissues until E11.5. Given the large body of evidence indicating that c-Myc is crucial to maintain proliferation (Adhikary and Eilers, 2005; Grandori et al., 2000; Prathapam et al., 2006; Trumpp et al., 2001; de Alboran, 2001), it is surprising that we did not observe multiorgan hypoplasia as previously characterized in *c-myc*$^{\Delta ORF/\Delta ORF}$ embryos (Fig. 1D) (Davis et al., 1993; Trumpp et al., 2001). In addition to other genetic studies reporting c-Myc independent proliferation in a subset of cells in adult tissues (Baena et al., 2005; Bettess et al., 2005; Oskarsson et al., 2006; Wilson et al., 2004), it therefore appears that c-Myc activity is not required to maintain at least some highly self-renewing adult tissues nor sustain the high proliferative

capacity of most embryonic cell types in vivo. These observations are puzzling given the large quantity of data indicating that c-Myc function is essential for cell cycle progression and cell growth of diverse cell types in vitro (de Alboran et al., 2001; Douglas et al., 2001; Mateyak et al., 1997; Oskarsson et al., 2006; Prathapam et al., 2006; Trumpp et al., 2001). One possible explanation for the continued proliferation of c-myc-deficient cells in vivo is functional redundancy with other Myc family members (N-Myc and L-Myc). Although neither *N-* nor *L-myc* transcripts are significantly upregulated in c-Myc-deficient embryos, the phenotype of epiblast-specific deletion of both c-Myc and N-Myc is more severe than ablation of either gene alone (N.C.D., Robert N. Eisenman and A.T., unpublished). Hence, it remains plausible that in cell types predominantly expressing c-Myc, the remaining low levels of endogenous N-Myc may be sufficient to maintain the proliferation of non-hematopoietic cell types in *Sox2Cre;c-myc*$^{flox/flox}$ mid-gestation embryos. Such functional redundancy does not appear to occur in most cultured cells, however, given that cell cycle progression of many cell types arrest upon inactivation of c-Myc alone. The only approach to address whether a completely Myc independent proliferation program exists is to eliminate all three Myc family members genetically (c-Myc, N-Myc and L-Myc) in an inducible fashion in vivo. In any case, it is important to note that elimination of c-Myc alone may not necessarily affect the proliferation and survival of cells in vivo. This conclusion has important consequences regarding the use of c-Myc inhibitors designed as anti-cancer drugs in a clinical setting as they may have less severe side effects than so far anticipated.

Previous studies have shown that c-Myc plays an essential role in the de novo formation of the vascular system (Baudino et al., 2002), and both endothelial cells and hematopoietic cells are thought to arise from a common progenitor (the hemangioblast) (Lacaud et al., 2001). These data would suggest that the hematopoietic phenotype we observe in *Sox2Cre;c-myc*$^{flox/flox}$ embryos may arise due to defective vasculogenesis or hemangioblast development. However, in contrast to the study performed by Baudino and colleagues, we observed no major vascular defects in the YS or embryo proper of three genetically distinct c-Myc-deficient mouse models (conventional knockout, *c-myc*$^{\Delta ORF/\Delta ORF}$; epiblast-specific deletion, *Sox2Cre;c-myc*$^{flox/flox}$; and endothelial/hematopoietic specific deletion, *Tie2Cre;c-myc*$^{flox/flox}$). The reason for this apparent discrepancy remains unclear; however, we have found that *c-myc*$^{\Delta ORF/\Delta ORF}$-null embryos exhibit variability in their time of death (E9.0-E10.5) and it is likely that endothelial markers are no longer informative in dying embryos. Nevertheless, we provide strong genetic evidence that c-Myc is not directly required for the formation of the embryonic vascular system. As described in the accompanying paper by He et al. (He et al., 2008) minor vascular

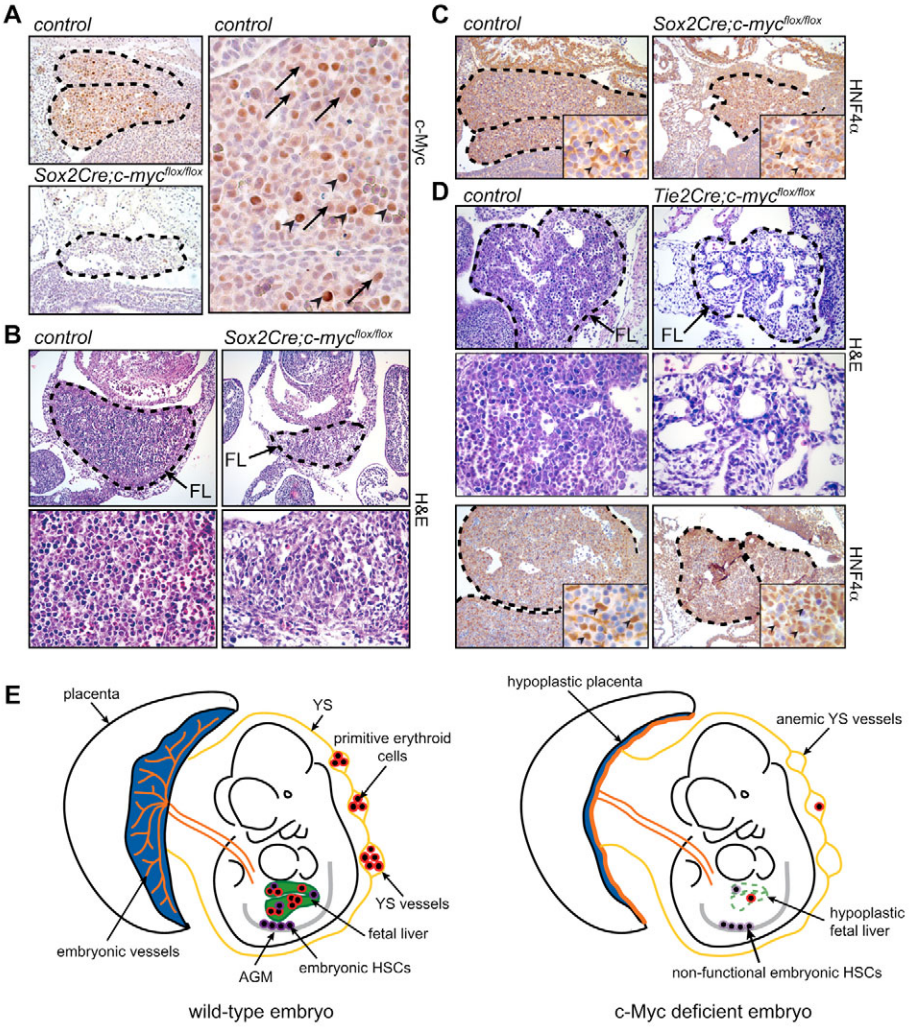

Fig. 7. Fetal liver development requires hematopoietic cells but not c-Myc. (**A**) c-Myc expression in the fetal liver of E10.5 control (upper panel; higher magnification is shown in the right panel) and *Sox2Cre;c-myc*$^{flox/flox}$ (bottom left) embryos. The broken line outlines the fetal liver. c-Myc is expressed in hepatoblasts (arrows) and blood cells (arrowheads). (**B**) Hematoxylin and Eosin (H&E) staining of control and *Sox2Cre;c-myc*$^{flox/flox}$ fetal livers at E10.5. The top panels show low magnification, whereas the bottom panels show a high magnification. (**C**) HNF4α expression in control and *Sox2Cre;c-myc*$^{flox/flox}$ embryos at E11.0. (**D**) H&E staining (top panels) and HNF4α expression (bottom panels) in E11.0 control and *Tie2Cre;c-myc*$^{flox/flox}$ embryos. (**E**) Model illustrating the different direct roles for c-Myc in the midgestation embryo and the placenta. First, c-Myc is required to generate a functional embryonic placenta. Second, only in the presence of a normal placenta are the direct roles of c-Myc in the embryo proper revealed. Primitive YS erythropoiesis is drastically decreased. Although definitive AGM derived HSCs are generated in normal numbers, these mutant HSCs are non-functional and only very few hematopoietic cells (of primitive and definitive origin) reach the fetal liver, which as a consequence remains hypoplastic and fails to expand. All other organs and tissues proliferate and develop normally.

abnormalities are present upon c-Myc deletion; however, they arise secondarily owing to the lack of hematopoietic cells (He et al., 2008).

Our genetic studies have revealed that during early embryogenesis, only primitive and definitive hematopoietic cells are dependent on c-Myc function (Fig. 7E). At time of death, *Sox2Cre;c-myc*$^{flox/flox}$ and *Tie2Cre;c-myc*$^{flox/flox}$ mutant embryos present with ~10% of the normal number of erythroblasts and exhibit an almost complete lack of erythroblast precursors, owing to vast apoptosis of CD71$^+$ cells. Thus, we provide the first genetic evidence that, in contrast to pathologically high Myc expression, as is often found in many tumors (Evan et al., 2005; Pelengaris et al., 2002), endogenous c-Myc activity has a pro-survival role in primitive erythroid cells. Although this is a novel finding, it is not completely unexpected given c-Myc is a downstream target of a number of receptor tyrosine kinases that have known pro-survival function, including Kit, Pdgf and Tpo (Kaushansky, 2005; Levitzki, 2004; Martelli et al., 2006). Future studies will need to determine whether the here uncovered pro-survival role of endogenous c-Myc is specific for hematopoietic cells or whether this is a general feature of endogenous Myc function in other cell types too.

E11.0 *Sox2Cre;c-myc*$^{flox/flox}$ embryos exhibit a striking decrease in the absolute number of CD45$^+$ hematopoietic cells. By contrast, the number of phenotypic definitive HSCs remains constant in the presence or absence of c-Myc, suggesting that the generation, specification and proliferation of HSCs is a c-Myc-independent process. Despite the generation of normal HSC numbers, c-Myc-deficient HSCs fail to give rise to a significant amount of differentiated progeny and lack CFU activity, strongly suggesting that HSCs lacking c-Myc activity are non-functional. These data are in agreement with what we observed following elimination of c-Myc in adult bone marrow HSCs (Murphy et al., 2005; Wilson et al., 2004; Wilson and Trumpp, 2006). Although our analysis of the hematopoietic regulators Scl, Runx1, Gata1 and Klf6 revealed no change in expression levels within the CD45$^+$ cell compartment of *Sox2Cre;c-myc*$^{flox/flox}$ embryos, it is important to note that the proportion of phenotypic HSCs within this population is five times higher than in control embryos. This may indicate that the expression of each transcription factor is indeed up to five times lower in mutant HSC/progenitors. Unfortunately, isolation of a significant number of highly pure HSC populations from mutant embryos have technical limitations and thus prohibited us from addressing this possibility directly.

In the embryo, HSCs emerge from the AGM and migrate in a β1 integrin-dependent manner to the fetal liver where they mature and expand (Cumano and Godin, 2007; Hirsch et al., 1996). c-Myc-deficient embryonic HSCs express normal levels of β1 integrin and CD45$^+$Kit$^+$ cells are found in the liver, suggesting that the migration of HSCs to the fetal liver is not c-Myc dependent. However, in contrast to normal embryonic HSCs, which upon reaching the fetal liver undergo massive expansion, c-Myc-deficient hematopoietic cells reside exclusively as single cells, further supporting the conclusion that they are unable to generate hematopoietic progeny. However, unlike in the adult, no accumulation of phenotypic HSCs is observed in c-Myc mutant embryos. This may be attributable to the lack of appropriate fetal liver stromal environment required to support hematopoietic activity, given the severely impaired development of the fetal liver in mutant embryos. Only Sox2Cre (but not Tie2Cre) induces recombination in the hepatoblast lineage, yet both mouse models exhibit hepatic disorganization and hypoplasia, thus it is likely that the liver phenotype arises as a direct result of hematopoietic insufficiency. This is further supported by the fact that restricted elimination of c-Myc in the hepatoblast lineage has no discernable effect on either fetal liver or hematopoietic development. At the onset of fetal liver development (at E9.5), only primitive hematopoietic cells are present, raising the possibility that primitive erythroblasts are required to promote fetal liver development in order to generate an appropriate microenvironment or 'niche' for incoming HSCs. In conclusion, these data provide first genetic evidence that proper fetal liver development requires the presence of hematopoietic cells and suggests that hepatic and hematopoietic progenitors develop in a symbiotic relationship in which hematopoietic and hepatic cells exchange signals required to promote the development of each other.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/14/2455/DC1

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