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Insufficient VEGFA activity in yolk sac endoderm compromises haematopoietic and endothelial differentiation

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SUMMARY

Vascular endothelial growth factor A (VEGFA) plays a pivotal role in the first steps of endothelial and haematopoietic development in the yolk sac, as well as in the establishment of the cardiovascular system of the embryo. At the onset of gastrulation, VEGFA is primarily expressed in the yolk sac visceral endoderm and in the yolk sac mesothelium. We report the generation and analysis of a *Vegf* hypomorphic allele, *Vegf*^{lo}. Animals heterozygous for the targeted mutation are viable. Homozygous embryos, however, die at 9.0 dpc because of severe abnormalities in the yolk sac vasculature and deficiencies in the development of the dorsal aortae. We find that providing '*Vegf* wild-type' visceral endoderm to the hypomorphic embryos restores normal blood and endothelial differentiation in the yolk sac, but does not rescue the phenotype in the embryo proper. In the opposite situation, however, when *Vegf* hypomorphic visceral endoderm is provided to a wild-type embryo, the '*Vegf* wild-type' yolk sac mesoderm is not sufficient to support proper vessel formation and haematopoietic differentiation in this extra-embryonic membrane. These findings demonstrate that VEGFA expression in the visceral endoderm is absolutely required for the normal expansion and organisation of both the endothelial and haematopoietic lineages in the early sites of vessel and blood formation. However, normal VEGFA expression in the yolk sac mesoderm alone is not sufficient for supporting the proper development of the early vascular and haematopoietic system.

Key words: VEGFA, Vasculogenesis, Haematopoiesis, Visceral Endoderm, Mouse

INTRODUCTION

It has been demonstrated that VEGFA plays an important role in a variety of physiological and pathological conditions. To date there is a far better understanding of VEGFA function and regulation in pathological conditions – such as tumour growth and wound healing – than that of its role in embryogenesis or in normal adult tissues (for a review, see Neufeld et al., 1999). However, understanding the function of VEGFA in physiological conditions would be important for providing a better basis for the modulation of vessel formation or regression in pathological conditions such as ischaemia and cancer.

Previously performed, targeted gene inactivation studies clearly indicated an absolute requirement for VEGFA in early stages of embryonic development. These experiments showed that a 50% reduction in gene dose impairs vascular development and leads to embryonic lethality at mid-gestation (Carmeliet et al., 1996; Ferrara et al., 1996). The distinct expression pattern during organogenesis (Breier et al., 1992; Miquerol et al., 1999) suggests that VEGFA may be involved in a multitude of developmental processes, including postnatal growth and survival (Gerber et al., 1999).

Vasculogenesis, the main mechanism of vessel formation in early development, is based on the in situ formation of blood vessels from independently differentiating angioblasts. This process first takes place in the yolk sac (Risau and Flamme, 1995). The concomitant development of the first blood cells in this embryonic membrane facilitates nutrient supply to the embryo via the vitelline circulation. The simultaneous development of haematopoietic and endothelial cells, and the results of in vitro differentiation experiments (Choi et al., 1998) suggest the existence of a common precursor, the haemangioblast, for these two lineages. The precursors of the yolk sac mesodermal component are located in the proximal epiblast. From there they migrate to their final extra-embryonic position (Lawson et al., 1991) where they give rise to endothelial and haematopoietic cells. However, a crucial tissue transplantation experiment to detect such a bi-potential precursor population for these cells failed to confirm its

existence, and suggested a clear segregation of the two lineages following mesodermal cell migration through the primitive streak (Kinder et al., 1999). The two lineages, however, are connected through their dependence on VEGFR2, one of the VEGFA receptors. Targeted disruption of the *Vegfr2* gene (*Kdr* – Mouse Genome Informatics) resulted in a diverted migration of these cells to non-yolk sac mesodermal membranes such as the amnion (Shalaby et al., 1997). As a consequence, both endothelial and blood cells were missing in the mutant yolk sac. Interestingly, however, the authors showed that VEGFR2-deficient ES (embryonic stem) cells did have the potential to differentiate into blood cells in vitro. This finding has been confirmed by independent studies (Schuh et al., 1999; Hidaka et al., 1999), and extrapolation to in vivo development suggests that VEGFR2 might not be essential for the initiation of haematopoietic differentiation up to 7.5 days of embryonic development, but it is required for later lineage expansion.

After establishment of blood islands in the yolk sac, the primary function of VEGFA/VEGFR2 signalling appears to be anti-apoptotic. Although a survival factor function for VEGFA has previously been described (Alon et al., 1995), Carmeliet et al. have recently resolved a distinct antiapoptotic pathway in early vascular development involving VE-cadherin and VEGFA/VEGFR2 (Carmeliet et al., 1999a). Interference with this pathway is most probably the reason for the increased apoptosis of endothelial cells observed in the VEGFA loss-of-function mutation (Ferrara et al., 1996).

Owing to the lethal haploinsufficiency of the *Vegf* (previously *Vegfa*) knockout, it was not possible to carry out the study of the homozygous deficient phenotype with standard methods. We bypassed this obstacle by aggregating homozygous $Vegf^{-/-}$ ES cells with tetraploid wild-type embryos. This approach allowed the study of a completely ES cell-derived, VEGFA-deficient embryo proper (Carmeliet et al., 1996). These embryos were supported by wild-type extra-embryonic endoderm and trophoblast of the placenta. Therefore, the assay did not permit the investigation of the role of VEGFA in the extra-embryonic tissues. Interestingly, however, the extra-embryonic endoderm is one of the first tissues to express VEGFA during development (Miquerol et al., 1999), starting at least 3 days earlier than the expression in the embryo proper.

Already in the late 1960s Miura and Wilt noticed that the extra-embryonic endoderm, as secretory epithelium was absolutely required for proper blood island formation (Miura and Wilt, 1969). This observation was confirmed by a later study that investigated $Gata4^{-/-}$ embryoid bodies that could not form visceral endoderm. In this study, Bielinska et al. demonstrated that formation and organisation of blood islands was dependent on the presence of visceral endoderm (Bielinska et al., 1996). These findings were extended by a report by Belaoussoff et al. (Belaoussoff et al., 1998) showing that the visceral endoderm has an instructive function for haematopoietic and endothelial development during a narrow window of time. Only recently have the same group provided evidence that Indian hedgehog is one of the molecules derived from visceral endoderm that is responsible for the induction of endothelial and haematopoietic differentiation (Dyer et al., 2001).

We have recently reported the generation of a *lacZ* knock-in into the 3′untranslated region (UTR) of the mouse *Vegf* gene (Miquerol et al., 1999) and $Vegf^{lacZKI}$ (Fig. 1A). This allele facilitated the characterisation of VEGFA expression at the cellular level both during development and in the adult organism. Interestingly, the modification of the gene resulted in a slight (two- to threefold) overexpression of VEGFA in the developing organs. The increased VEGFA expression was incompatible with normal heart development and resulted in homozygous embryonic lethality (Miquerol et al., 2000). Together with the previously observed haploinsufficiency, this phenotype, which is due to slight overexpression, was a clear demonstration of minimal tolerance to alterations in VEGFA expression levels. The study of VEGFA expression in the yolk sac showed that both the visceral endoderm and the yolk sac mesodermal sheet express VEGFA (Miquerol et al., 1999). These two layers sandwich the first site of blood and vessel formation of the embryo. This finding has raised the question of whether VEGFA expression in both layers is necessary or whether one of them would be sufficient for proper vessel and haematopoietic differentiation in the yolk sac. We address this question by a novel hypomorphic allele generated by a slightly modified insertion of *lacZ* into the 3′ UTR of *Vegf*, distinct from the *lacZ* insertion reported previously (Miquerol et al., 1999). The new allele ($Vegf^{lo}$; Fig. 1A) allowed heterozygous survival but conferred homozygous embryonic lethality by 9.0 dpc. In the homozygous embryos, the formation of blood islands in the yolk sac, as well as the development of the dorsal aortae, were severely defective.

A combination of chimaera studies showed that VEGFA expression in the yolk sac mesoderm sheet was not sufficient for normal vascular and haematopoietic development in this extra-embryonic membrane. However, expression in the visceral endoderm was necessary and sufficient for normal development of the yolk sac vasculature.

MATERIALS AND METHODS

Construction of the targeting vector

Vegf genomic sequences were derived from a λ genomic clone described earlier (Carmeliet et al., 1996). The 5′ homology arm, comprising exons 4-7 and the coding region of exon 8, was generated by fusing a *Sma*I-*Nco*I fragment (extending into intron 7) with a PCR amplified fragment terminating at the VEGFA stop codon. A PGK neomycin cassette flanked by loxP sites (Takeuchi et al., 1995) was inserted directly downstream of the stop codon followed by *IRES-lacZ* (Jang et al., 1988; Takeuchi et al., 1995) fused to the *Vegf* 3′UTR (Damert et al., 1997). All construction steps and exon sequences were verified by dideoxy sequencing.

Generation of $Vegf^{lo+neo/+}$ ES cells and $Vegf^{lo+neo/+}$ and $Vegf^{lo/+}$ mice

The targeting vector was linearised with *Not*I and electroporated into R1 ES cells (Nagy et al., 1993). G418-resistant ES cell clones were screened by Southern blot using *Bgl*II digestion of the genomic DNA and a 400 bp *Bgl*II-*Eco*RI probe encompassing *Vegf* exon 3. Correct targeting was further confirmed by *Eco*RV digestion and hybridisation with a 1 kb *Hin*dIII probe derived from the *Vegf* 3′ flanking region. Several independent clones were aggregated with CD-1 morulae as described before (Nagy and Rossant, 2000). Germline transmission was obtained for two clones (B7 and C7). F_1 progeny was subsequently bred to a deletor Cre recombinase transgenic mouse to delete the selectable marker cassette (Nagy et al., 1998) and obtain $Vegf^{lo/+}$ mice. All further analysis was performed on a mixed CD-1/129 background.

Genotyping of mice carrying the targeted allele

Vegf^{lo} offspring were PCR genotyped using the following oligonucleotides: 5′ stop, 5′-GAGCATTTATTCCCATGTCTG-3′; lacPCR, 5′-CATTACCAGTTGGTCTGGTG-3′; and fusionAS, 5′-CAGGCTTTCTGGATTAAGGAC-3′. These yielded fragments of 388 bp (wild type) and 163 bp (targeted allele). To distinguish the *Vegf*^{lo} and *Vegf*$^{lo+neo}$ alleles, a sense oligonucleotide from the PGK pA (Neo, 5′-CCACATACACTTCATTCTCAG-3′) and an antisense from *lacZ* (lacZ-276R, 5′-GATGGGCGCATCGTAACCGTGC-3′) were used, resulting in amplification of a 1000 bp fragment from the *Vegf*$^{lo+neo}$ allele.

Histological analysis, immunohistochemistry and in situ hybridisation

Embryos at different stages of development were dissected in cold PBS, fixed in 4% paraformaldehyde, paraffin-embedded and sectioned at 5 μm. Sections were stained with Haematoxylin/Eosin. Whole-mount immunohistochemistry using an antibody against PECAM-1 was performed as described (Davis, 1993). Procedures employed for detection of β-galactosidase activity are described elsewhere (Miquerol et al., 1999). Whole-mount in situ hybridisation and detection with AP-conjugated antibody was carried out according to the protocol published by Wilkinson and Nieto (Wilkinson and Nieto, 1993). Probes were kindly provided by J. J. Bieker (EKLF); H. Stuhlmann (VEZF) and J. Rossant (VEGFR1 and VEGFR2).

RNA and protein analysis

Total RNA from embryos was prepared using Trizol Reagent (Life Technologies) according to the manufacturers instructions. Reverse transcription was performed on 1 μg of RNA using the oligonucleotide *lacZ*-276R 5′-GATGGGCGCATCGTAACCGTGC-3′ and Superscript™ (Life Technologies) Reverse transcriptase at 55°C. The resulting cDNA was amplified using the oligonucleotides lacZ-276R and VEGF-D, 5′-GCCCTGGAGTGCGTG CCCACGTCAGA-GAGCA-3′. The PCR product was gel purified and cloned into pGEM-T-easy (Promega) for subsequent dideoxy sequencing. Protein extracts were obtained by homogenising embryos in 250 mM Tris-HCl pH 7.8 and three cycles of freezing and thawing. Protein concentration was determined using the BioRad reagent. VEGF immunoreactive material was quantified with the Quantikine ELISA kit (R&D) according to the manufacturers instructions.

Embryo aggregations

Tetraploid embryos for aggregation experiments were generated as described (Nagy et al., 1993). Aggregation of diploid embryos or GFP+ ES cells (Hadjantonakis et al., 1998) with tetraploid embryos was performed according to the protocol given elsewhere (Nagy and Rossant, 2000). The resulting chimaeras were dissected at 8.5-9.5 dpc for subsequent analysis. For all chimaera experiments, small pieces of the embryo proper were removed for genotyping using the oligonucleotides given above.

RESULTS

Knock-in of IRES-*lacZ* into the VEGFA 3′ untranslated region results in homozygous embryonic lethality

An IRES (internal ribosomal entry site)-*lacZ* cassette was inserted into the *Vegf* locus by homologous recombination in ES cells, with the primary aim of generating a *lacZ* tagged allele where expression of the reporter gene would exactly mimic the pattern of VEGFA expression. In contrast to our recently described *Vegf*^{lacZKI} allele (Miquerol et al., 1999) the IRES-*lacZ* cassette was inserted directly adjacent to the STOP codon located in exon 8. The *lacZ*-coding sequence was followed by the *Vegf* 3′ UTR including the endogenous polyadenylation signals (Fig. 1A,B). We anticipated that after a Cre recombinase-mediated removal of the loxP flanked neomycin selectable marker, the targeted allele should facilitate *lacZ* expression under the control of all known *Vegf* regulatory elements, including the 3′UTR (Levy et al., 1996; Shima et al., 1996).

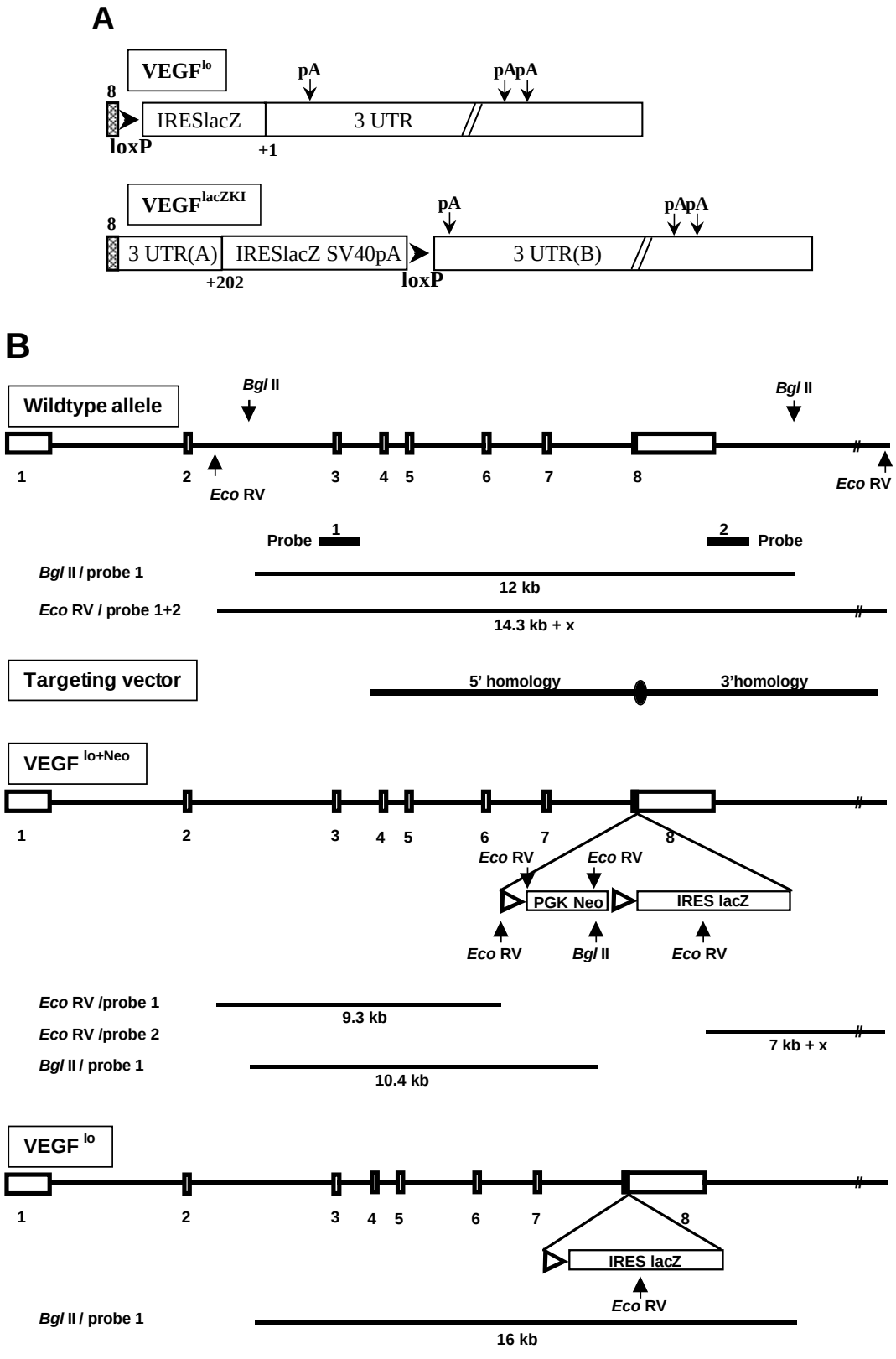
Electroporation into R1 ES cells (Nagy et al., 1993) yielded 12 correctly targeted clones (Southern blot analysis is shown in Fig. 1C). Germline transmission after ES cell/morula aggregation (Nagy and Rossant, 2000) was achieved for two independent ES cell clones. Heterozygous animals were viable, fertile and did not show any obvious phenotypic alterations. As the selectable marker cassette was still present in these animals, they were subsequently bred to a general deletor strain of Cre mice (Nagy et al., 1998), yielding viable offspring heterozygous for the neomycin-excised allele. Breeding these heterozygous mice with each other did not result in any live homozygous newborns. Subsequent analysis at different stages of embryonic development revealed that the homozygous embryos died between 8.5 and 9.0 dpc. Morphological evaluation of mutant embryos at 8.5-9.0 dpc showed the same defects as observed in *Vegf*$^{+/-}$ embryos: disorganised yolk sac vasculature with large ‘lacunae’ and reduced lumina of the dorsal aortae (Fig. 2A-H). Sectioning demonstrated few and largely empty blood islands (Fig. 2I,J) and a reduction in the number of blood cells in the embryo proper, particularly noticeable in the sinus venosus (Fig. 2O,P). Development of the cardinal veins seems to proceed normally (Fig. 2M-P) and development of the heart is delayed; however, the endocardium is formed (Fig. 2M,N). To examine whether the impaired vascular development at the time of death results from regression of blood islands or defective recruitment of endothelial/haematopoietic precursors, we examined embryos at an earlier stage of development. Sections of mutant embryos at 8.0 dpc showed the same reduction in the number of blood islands devoid of primitive erythrocytes as observed at later stages, indicating that failure of precursor cell recruitment is the major cause for the defects in yolk sac vasculature (Fig. 2Q-T). The similarity to the *Vegf*$^{+/-}$ phenotype implicates a hypomorphic function of the knock-in allele that we therefore termed Vegf low (*Vegf*^{lo}).

Analysis of marker molecules indicative for endothelial [VEGF-R1 (Fong et al., 1996), VEGFR2 (Yamaguchi et al., 1993) and VEZF (Xiong et al., 1999)] and haematopoietic [EKLF (Southwood et al., 1996)] differentiation revealed a severely reduced expression of VEGF-R1 and EKLF indicating a lack of mature endothelial and haematopoietic cells (Fig. 3). In the 8.5 dpc *Vegf*$^{lo/lo}$ mutants, VEGFR2 expression was found in the cardinal veins and endocardium, albeit at a much lower level then in wild-type littermates (Fig. 4A,B). In the yolk sac, VEGFR2-expressing cells were found at the periphery of the rare blood islands (Fig. 4C,D). At 9.0 dpc, low level expression of VEGFR2 and VEZF was observed in the head region and in the intersomitic vessels that arise through angiogenesis (Fig. 3). This finding indicates that once vessels are formed by vasculogenesis, angiogenesis can proceed at low levels of VEGFA activity.

Taken together, these data suggest that the functionally hypomorphic *Vegf* allele affects differentiation of both the endothelial and haematopoietic lineages.

Altered *Vegf* RNA production from the knock-in allele

In order to understand the molecular mechanisms of the embryonic lethality observed, the analysis of both the RNA and protein resulting from the targeted allele was performed. RT-PCR analysis using oligo dT-primed cDNA from homozygous mutant embryos and oligonucleotides bridging *Vegf*- and *lacZ*-coding sequences yielded a 616 bp instead of the expected 1217 bp product (Fig. 5C). Furthermore, we could not detect any RT-PCR product when a reverse primer in the exon 8-



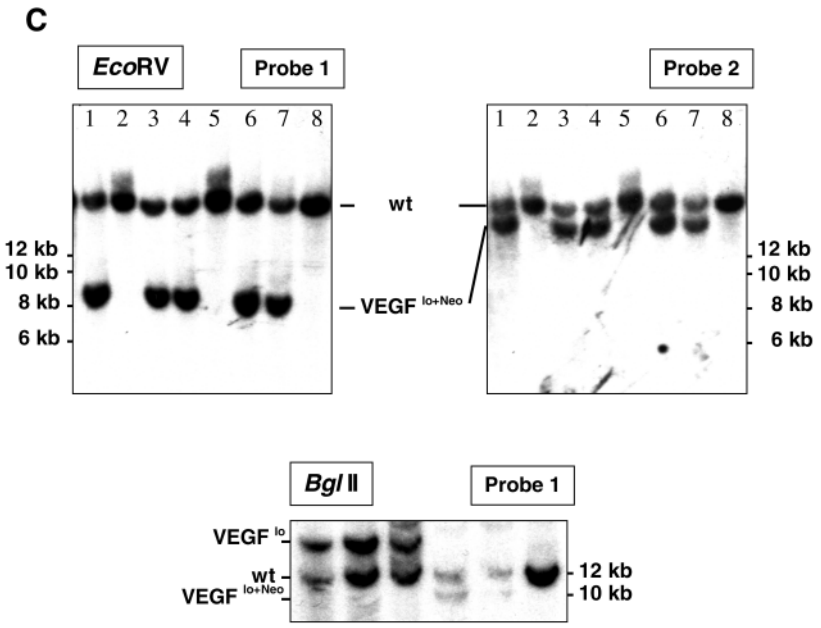


Fig. 1. Knock-in of IRES-*lacZ* into the VEGFA locus. (A) *Vegf* IRES-*lacZ* knock-in alleles with insertion of the reporter cassette at different positions of the *Vegf* 3′UTR. LoxP sites remaining after excision of the selectable marker are indicated by arrowheads. Sequences are not drawn to scale. (B) Schematic representation of the wild-type, targeted ($Vegf^{lo+Neo}$) and Neo-excised ($Vegf^{lo}$) allele. Exons are depicted as boxes. Probes used in Southern blot analysis and expected fragment lengths are indicated. (C) Southern blot analysis with Probes 1 and 2 on *Eco*RV digested genomic DNA showed proper homologous recombination both at the 5′ and 3′ homology arms of the target vector, respectively. The Southern blot analysis with Probe 1 on *Bgl*II digested genomic DNA showed successful removal of the loxP flanked neo selectable marker.

coding region was used (data not shown). Cloning and sequencing the 616 bp fragment revealed the absence of the exon 8-coding region as well as of the entire IRES (Fig. 5A, lowest panel). Therefore, the mRNA produced from the *Vegf* knock-in allele should be translated into a modified VEGFA protein, in which the exon 8 encoded six C-terminal amino acids are replaced by 66 amino acids derived from the *lacZ*-coding region (Fig. 5B). As the *lacZ*-coding sequence present in the predicted fusion protein is not in frame with VEGFA, this protein cannot confer β-galactosidase activity. Surprisingly, although the entire IRES sequence is skipped in the process of splicing, β-galactosidase activity is detectable in the knock-in embryos and adult mice, the expression pattern corresponds to that recently described for the $Vegf^{lacZKI}$ allele (data not shown) (Miquerol et al., 1999). Compared with measurements in wild-type littermates, a threefold increase in VEGFA immunoreactivity could be detected in the mutant embryos by ELISA (Fig. 5D). These results suggest that two different proteins can be translated from the chimaeric mRNA: a highly abundant C-terminally modified VEGFA and a functional β-galactosidase, probably supported by the strong *lacZ* Kozak sequence (Kozak, 1999).

In addition to skipping of the exon 8-coding sequence, disturbance in VEGFA alternative splicing involving exons 6 and 7 (Breier et al., 1992) was observed. Only a single mRNA containing exons 5 and 7 and thus corresponding to the VEGFA 164 isoform could be detected from the $Vegf^{lo}$ allele (Fig. 5A,C).

Wild-type VEGFA expression in the visceral endoderm is sufficient for normal vessel formation and haematopoietic differentiation in the yolk sac

Previously, we have demonstrated that VEGFA is expressed both in the visceral endoderm and the yolk sac mesoderm at the time when vasculogenesis occurs (Miquerol et al., 1999). Using chimaera approaches, we have addressed the requirement for both sources of this growth factor for normal yolk sac development. Green fluorescent protein (GFP)-expressing (Hadjantonakis et al., 1998) *Vegf* wild-type tetraploid embryos were aggregated with diploid $Vegf^{lo/lo}$ embryos from the segregating F_2 generation (Fig. 6A). In these chimaeras, the tetraploid component contributed only to the yolk sac visceral endoderm and the trophoblast lineages, while the diploid component contributed to all lineages, including the embryo proper. However, we often observed a few ‘contaminating’ tetraploid cells in the gut of otherwise totally diploid embryos (Fig. 6B). Therefore, to establish the genotype of the diploid component of the chimaeras from each embryo proper recovered, we carefully removed a small part lacking GFP-positive cells for PCR analysis.

Yolk sac endoderm is one of the few lineages where the two chimaera components do not intermingle extensively (Gardner, 1984). Instead, large patches are formed, each consisting of one of the components. In the chimaeras presented here the tetraploid *Vegf* wild-type patches are distinguishable by GFP expression (Fig. 6B,C), whereas areas of diploid-derived $Vegf^{lo/lo}$ visceral endoderm can be stained for β-galactosidase activity (Fig. 6E,I,L).

Rescue of blood island formation and vascular development was found in areas of the tetraploid (*Vegf* wild type) visceral endoderm (Fig. 6F-H,I,L). As the yolk sac mesoderm is $Vegf^{lo/lo}$ in these chimaeras, this rescue indicates that the *Vegf* wild-type visceral endoderm is sufficient for normal blood and vessel formation in this membrane. In $Vegf^{lo/lo}$ (β-galactosidase positive) areas of visceral endoderm, the formation of blood islands is severely compromised (Fig. 6F-I,L).

The sections show that the range of rescue by VEGFA secreted from wild-type tetraploid areas is very short, reaching a distance of only a few cell diameters (marked by an asterisk in Fig. 6L). This notion was further supported by the observation that even extensive tetraploid contribution was not sufficient to rescue the intra-embryonic phenotype. All embryos showed the same small dorsal aortae lumen (arrows in Fig. 6J) and delayed heart development (Fig. 6K) as did unmanipulated $Vegf^{lo/lo}$ embryos. Interestingly, however, the haematopoietic cell population in the embryo proper increased

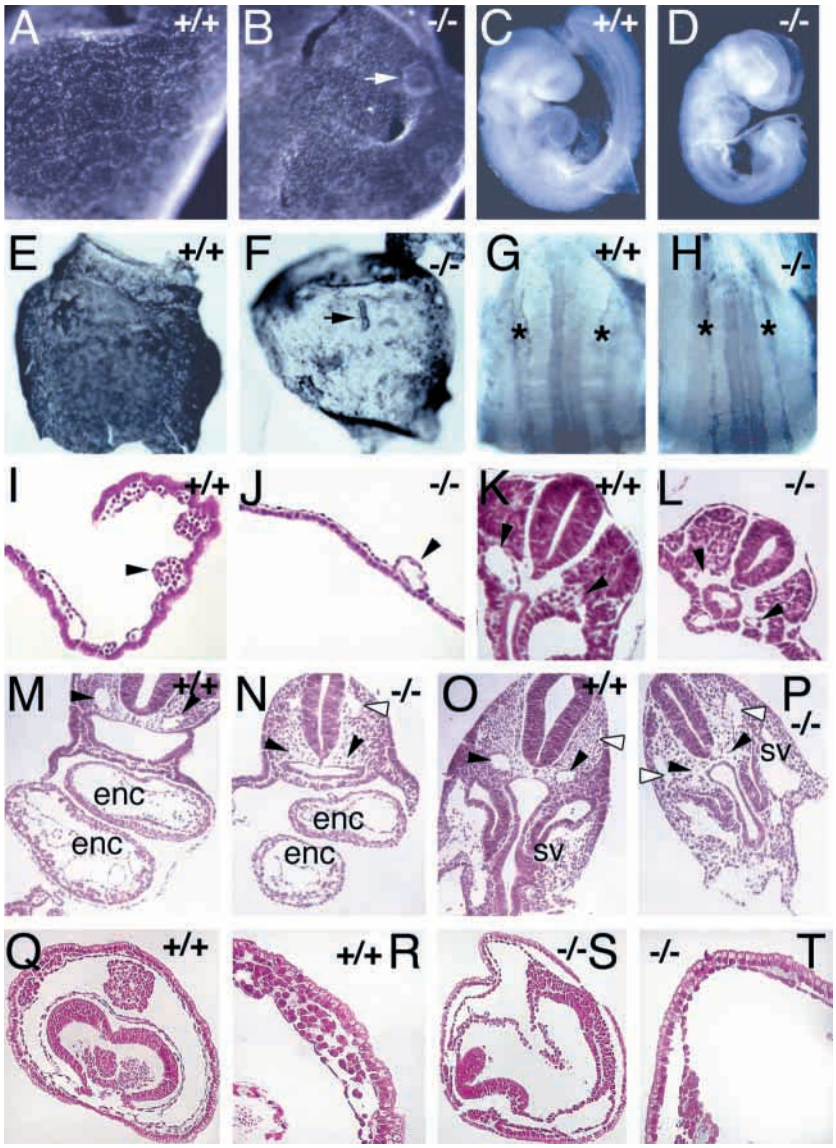


Fig. 2. Phenotypical consequences of homozygous IRES-*lacZ* knock-in into the *Vegf* locus. +/+, wild type; –/–, *Vegf*$^{lo/lo}$. (E-H) α-PECAM specific immunohistochemistry; (I-T) Haematoxylin/Eosin-stained sections. (A,B,E,F) In the 8.5 dpc yolk sac, defects in vascularisation (B,F) lead to formation of endothelial cell-lined 'lacunae' (arrows). (I,J) Few 'blood islands' (arrowheads) devoid of primitive erythrocytes are observed in *Vegf*$^{lo/lo}$ embryos (J) in comparison with wild-type littermates (I). (C,D) Growth retardation in *Vegf*$^{lo/lo}$ (D) becomes obvious shortly before death at 8.5-9.0 dpc (compare with wild type in C). (G,H,K,L) Lumen formation of the dorsal aortae is impaired (H,L) in the aorta-gonad-mesonephros region at 8.5 dpc when compared with wild type (G,K). Dorsal aortae are indicated with asterisks (G,H) and arrowheads (K,L). (M-P) Intra-embryonic defects with delayed heart development and lack of blood cells in the sinus venosus (sv) (N,P) compared with wild type (M,O). Endocardium (enc) and cardinal veins (white arrowheads) are formed properly. Dorsal aortae are indicated with black arrowgeads. (Q-T) Phenotypic alterations in the yolk sac are obvious as early as 8.0 dpc. Embryos show a reduction in the number of blood islands and those that are present are devoid of primitive erythrocytes.

with increasing contribution of *Vegf* wild-type tetraploid cells to the yolk sac visceral endoderm (red arrows in Fig. 6J,K). This finding is in accordance with the previously published notion that the yolk sac blood islands are a major source of early haematopoietic cells colonising the embryo proper.

VEGFA is required in the visceral endoderm for hemangioblast formation

To test whether endoderm expression of VEGFA is necessary for normal blood island formation and vessel development in the yolk sac, we performed a reverse chimaera experiment by aggregating *Vegf*$^{lo/lo}$ tetraploid embryos with *Vegf* wild-type, GFP-expressing ES cells (Fig. 7A). In this case, the yolk sac endoderm was completely derived from tetraploid *Vegf*$^{lo/lo}$ embryos (easily detectable by β-galactosidase staining, see Fig. 7C,G,H), whereas the entire extra-embryonic mesoderm, including endothelial and haematopoietic cells, was wild-type ES cell derived. As shown in Fig. 7B,C, the *Vegf* wild type, GFP-positive embryo proper developed normally until 9.5 dpc, the time of analysis. Histological sections revealed normal vessel formation along the entire embryo (arrowheads in Fig. 7F,I,J). However, only few blood cells could be observed in the AGM (aorta-gonad-mesonephros) region (Fig. 7F), the heart and the sinus venosus (Fig. 7I,J).

Comparison of yolk sacs derived from aggregations of tetraploid *Vegf*$^{lo/lo}$ (Fig. 7D) and *Vegf*$^{lo/+}$ (Fig. 7E) embryos revealed a more uniform and intense GFP fluorescence in the latter setup. This finding already indicated proper migration of GFP positive mesodermal precursors and expansion of the lineages when the tetraploid component was heterozygous *Vegf*$^{lo/+}$ (Fig. 7E) that is not observed with tetraploid *Vegf*$^{lo/lo}$ embryos (Fig. 7D). Sectioning as shown in Fig. 7G,H confirmed this notion. Appropriate yolk sac vascular development occurred only when the visceral endoderm was derived from a heterozygous *Vegf*$^{lo/+}$ embryo (Fig. 7G,H). In *Vegf*$^{lo/lo}$/wild-type ES chimaeric yolk sac only the embryo-derived vitelline vessels develop normally and show intense GFP fluorescence (Fig. 7D). We can therefore conclude that secretion of functional VEGFA from the visceral endoderm is not only sufficient, but is in fact necessary for proper blood

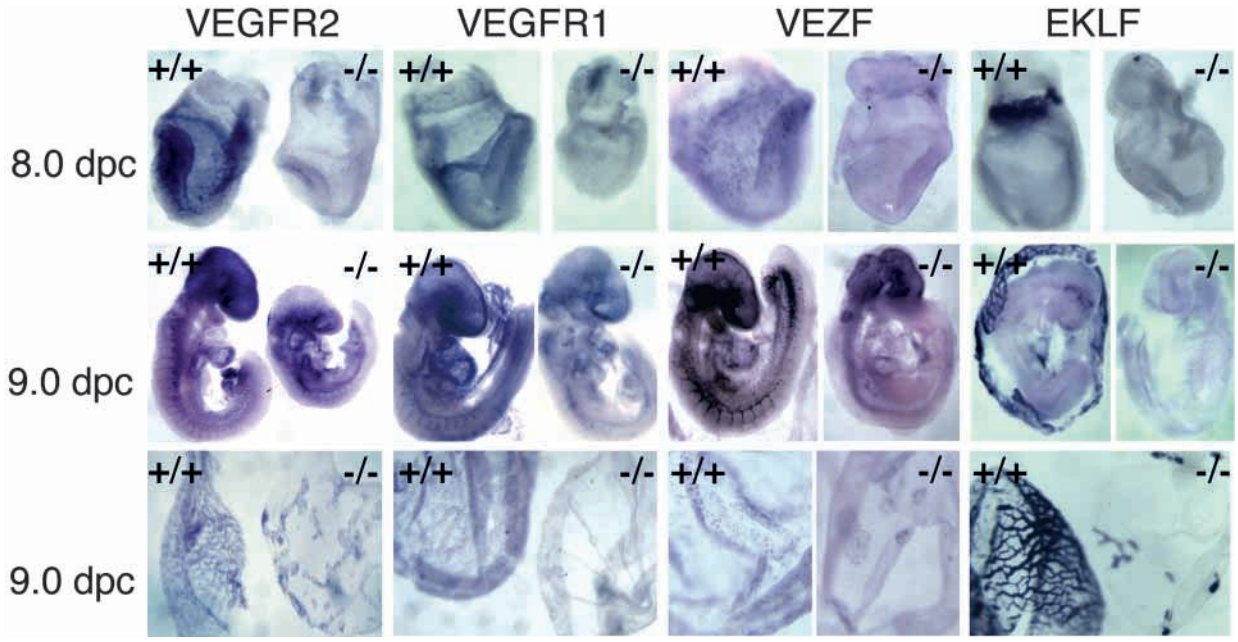


Fig. 3. Expression of endothelial (VEGFR1, VEGFR2 and VEZF) and haematopoietic (EKLF) markers is disturbed in *Vegf*$^{lo/lo}$ embryos. Whole-mount in situ hybridisation for the marker gene expression was performed on 8.0 and 9.0 dpc wild-type (+/+) and *Vegf*$^{lo/lo}$ (–/–) embryos. Note the presence of intersomitic vessels sprouting from the dorsal aorta in 9.0 dpc *Vegf*$^{lo/lo}$ (–/–) embryos that hybridised to the VEGFR2 probe.

island formation, and hence endothelial and haematopoietic differentiation in the yolk sac. The absence of blood cells in the rare blood islands corresponds well with the reduced number of blood cells in the embryo proper, highlighting the importance of the yolk sac as a source of haematopoietic cells at this developmental stage.

DISCUSSION

By insertion of an *IRES-lacZ* into the *Vegf* locus, we generated a new allele that, by comparison with the *Vegf*$^{-/-}$ phenotype, confers a hypomorphic function. Although the exact mechanism of action requires further investigation, this allele enabled us to dissect the role of VEGFA in early embryonic development, especially in yolk sac vasculogenesis and haematopoiesis. Chimaera analysis using a combination of *Vegf* wild-type tetraploid and *Vegf*$^{lo/lo}$ diploid embryos supported and extended the findings obtained from tetraploid embryo/*Vegf*$^{-/-}$ ES cell aggregations. In particular, providing wild-type extra-embryonic membranes did not rescue the intra-embryonic vascular phenotype of embryos with insufficient level of VEGFA expression (Carmeliet et al., 1996). This might be explained by the finding that the dorsal aortae are formed by intra-embryonic vasculogenesis (Hirakow and Hiruma, 1981), which cannot be properly supported by the *Vegf*$^{-/-}$ or *Vegf*$^{lo/lo}$ embryo proper in these chimaeras.

Going beyond the knockout tetraploid setting, however, we created chimaeric yolk sac visceral endoderm by using *Vegf*$^{lo/lo}$ embryos instead of ES cells as the diploid source. This approach allowed the study of wild-type and VEGFA-deficient essential lineages in the same embryo. Inspection of the chimaeric yolk sacs revealed that blood islands are only properly formed in areas where the visceral endoderm is composed of *Vegf* wild-type tetraploid cells, but not in *Vegf*$^{lo/lo}$ patches of visceral endoderm. This observation already indicated that VEGFA activity derived from the visceral endoderm is a crucial element in blood island formation. Interestingly, rescue of blood island formation by wild-type

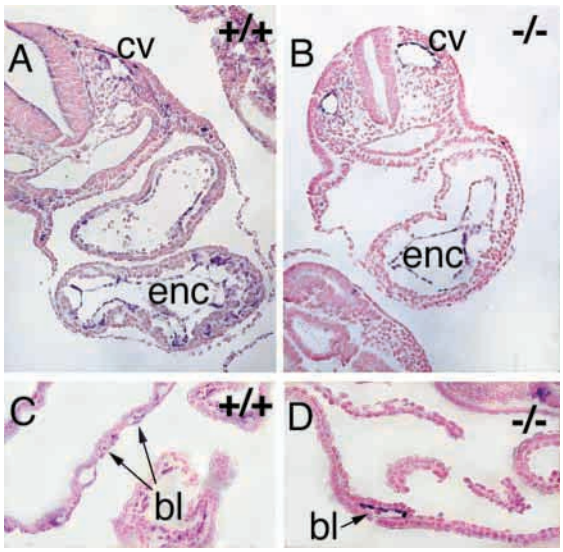


Fig. 4. Expression of VEGFR2 in wild-type (A,C) and *Vegf*$^{lo/lo}$ (B,D) embryos at 9.0 dpc. Sections are derived from whole-mount in situ hybridisation with a VEGFR2 probe. Transverse sections show VEGFR2 expression in the endocardium (enc) and the cardinal vein (cv) in *Vegf*$^{lo/lo}$ embryos (B) and in the endothelial cells lining one of the rare blood islands (bl; D).

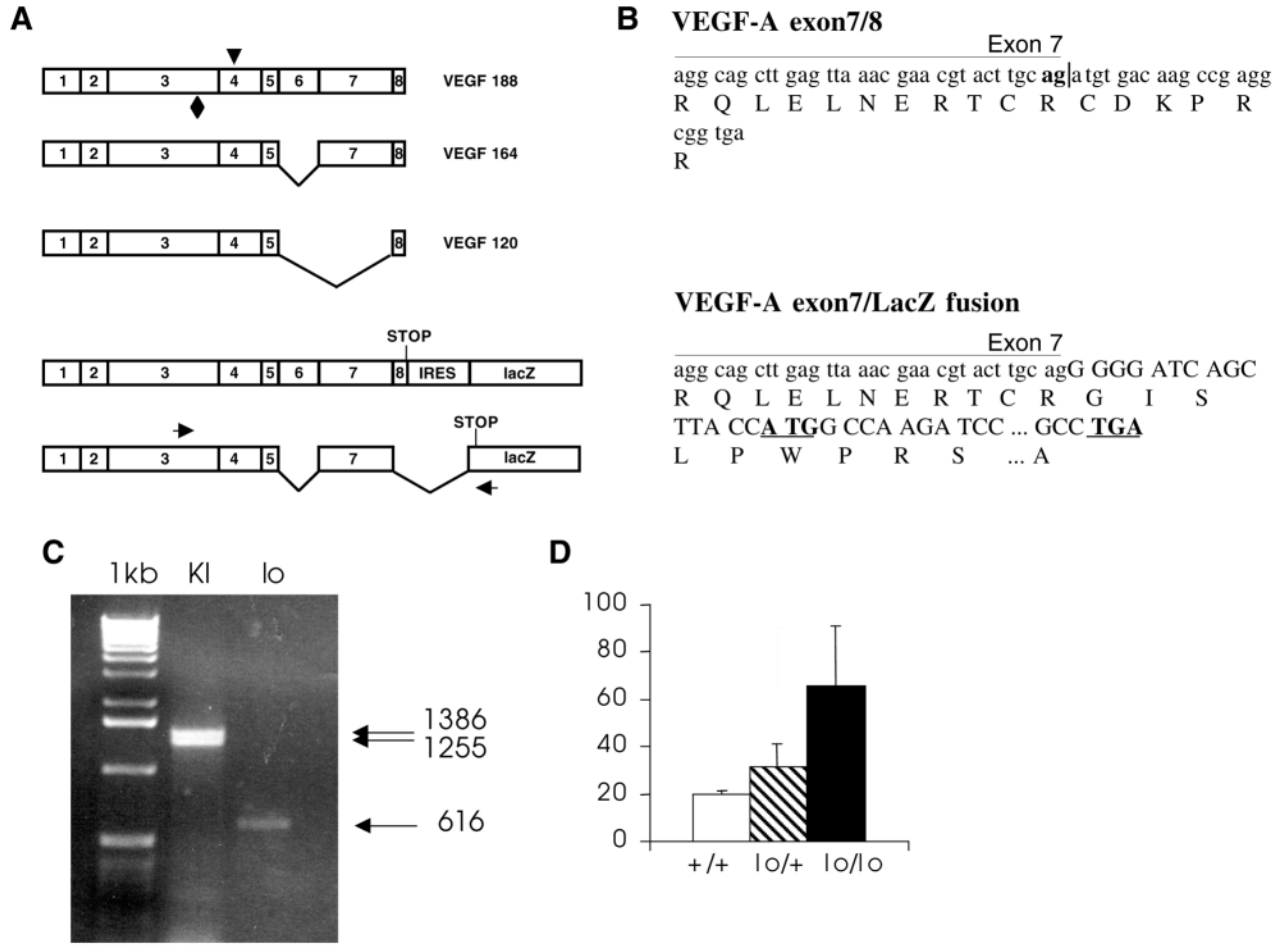


Fig. 5. Aberrant splicing of the *Vegf-lacZ* fusion RNA results in loss of exon 8-coding sequences and the complete IRES. (A) Schematic representation of mouse VEGFA isoforms (VEGF 188, VEGF 164 and VEGF 120) (◆, VEGFR1 binding site; ▼, VEGFR2 binding site) and the predicted *Vegf-lacZ* fusion RNA followed by the actual RNA generated by aberrant splicing (primers used in RT-PCR analysis are indicated as arrows). (B) Sequence comparison of the VEGFA exon7/8 junction (top) and the exon 7/lacZ splice junction of the $Vegf^{lo}$ RNA (bottom). The out-of-frame *lacZ* start codon and the stop codon, which terminates the translation of the fusion protein, are underlined. (C) RT-PCR analysis of the $Vegf^{lo}$ allele (lo, lane 3). Oligo dT-primed cDNA was amplified using the primers indicated in A, amplification from the $Vegf^{lacZKI}$-allele is shown for comparison (KI, lane 2). (D) Quantitative analysis of VEGFA-immunoreactive material in 8.5 dpc mouse embryos of different genotypes. Amounts of VEGFA immunoreactive material are given in pg/ml.

visceral endoderm patches does not extend beyond a few cell diameters. Recently, Cleaver and Krieg identified *Xenopus* VEGF 122 as the 'chemotactic' isoform that acts on dorsal aorta-forming angioblasts over a considerable distance (Cleaver and Krieg, 1998). From these data it could be hypothesised that the smallest VEGFA isoform mediates chemotaxis, whereas the heparin-bound VEGFA 164 and 188 act more locally and induce differentiation once the final destination in the yolk sac is reached. In our experimental set-up, diffusible VEGFA 120 secreted from wild-type visceral endoderm might be able to facilitate precursor migration even through $Vegf^{lo/lo}$ patches, resulting in an even distribution of cells over the whole yolk sac. The local action of the higher molecular weight isoforms in wild-type patches then allows further expansion of blood islands that is inhibited in areas of $Vegf^{lo/lo}$ visceral endoderm. If at least one VEGFA isoform could act over a distance of more than a few cell diameters (and therefore beyond $Vegf^{lo/lo}$ patches), blood island formation would be expected to be blocked close to the boundaries of the visceral endoderm. Certainly it would be interesting to see how blood island formation proceeded in a set-up where embryos that lacked a single VEGFA isoform (Carmeliet et al., 1999b) constitute the tetraploid component.

As yolk sac blood island formation was rescued by wild-type visceral endoderm, it was not surprising that the number of blood cells in the embryo proper was elevated with increasing contribution of wild-type visceral endoderm. Palis et al. have reported that definitive yolk sac derived erythroid and myeloid progenitors colonise the embryo with the onset of circulation (Palis et al., 1999). However, the still controversial issue of the relative contribution of yolk sac versus P-Sp (para-aortic splanchnopleura)/AGM-derived haematopoietic stem cells to foetal liver haematopoiesis (Keller et al., 1999) could not be addressed with this approach, as the embryos were not viable beyond 9.5 dpc because of intra-embryonic vascular malformations.

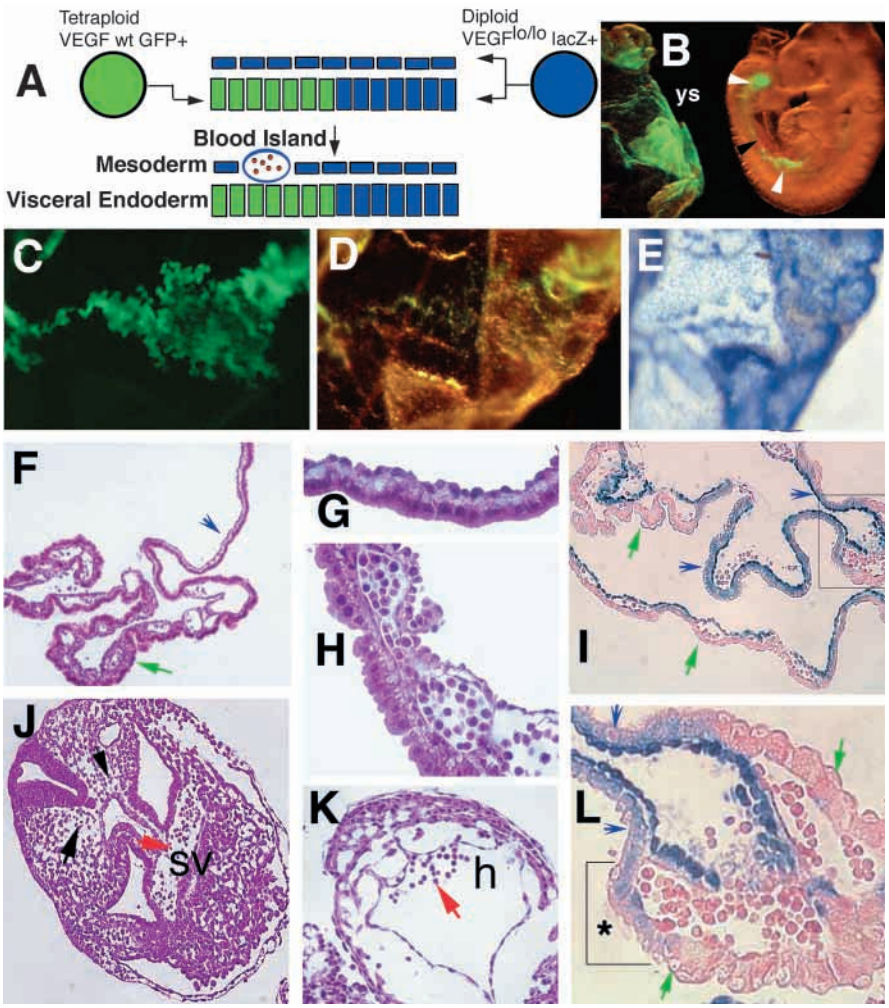


Fig. 6. Rescue of blood island formation by wild-type visceral endoderm. (A) Schematic representation of the aggregation setup: tetraploid *Vegf* wild-type (wt) *GFP+* embryos were aggregated with diploid $Vegf^{lo/lo}$ embryos (F_2), resulting in chimaeric visceral endoderm and $Vegf^{lo/lo}$ mesoderm. (B) Whole-mount photograph of a 9.0 dpc embryo and its chimaeric yolk sac (ys), which was taken under GFP visualising light conditions. The black arrowhead points to trace contribution of tetraploid cells to the gut epithelium. White arrowheads indicate the remains of the yolk sac attached to the embryo. (C) One of the GFP-positive patches in the yolk sac. (D) Photograph of the same area as in C using light conditions that allow the visualisation of GFP-negative cells as well. (E) The same area as in C,D after *lacZ* staining. (F) Appropriate (green arrow) and impaired (blue arrow) blood island formation in the chimaeric yolk sac. Higher magnifications of the respective areas are shown in G,H. (I) β-galactosidase staining reveals blood islands only in areas where the visceral endoderm is *Vegf* wild type (*lacZ*-negative cells, green arrows), whereas blood islands cannot be formed in opposition to $Vegf^{lo/lo}$ (*lacZ*-positive cells, blue arrows) endoderm. (L) Higher magnification of the boxed area in I. The short range rescue of blood island formation by VEGFA secreted from wild-type visceral endoderm is marked by an asterisk. (J,K) Extensive wild-type tetraploid contribution to the yolk sac visceral endoderm is not sufficient to rescue intra-embryonic vascular defects. Lumina of the dorsal aortae (black arrows) are barely detectable (J). An increase in the number of blood cells (in the heart in K and sinus venosus in J (red arrows) is observed with increasing wild-type tetraploid contribution. h, heart; sv, sinus venosus.

Although VEGFA was initially characterised as endothelial specific mitogen, it is now accepted that it affects haematopoietic development through VEGFR2 as well. F_1 $Vegf^{+/-}$ embryos display a severely reduced number of primitive erythrocytes in the yolk sac blood islands (Ferrara et al., 1996). Our analysis of the $Vegf^{lo/lo}$ embryos led to similar findings. In vitro differentiation experiments have suggested an important function for VEGFA in blood cell differentiation. According to a report by Nakayama et al., emergence of progenitors for both the erythroid and lymphoid lineages in embryoid bodies is stimulated by VEGFA (Nakayama et al., 1998). Choi et al. could demonstrate that blast colonies with haematopoietic and endothelial potential were formed from VEGFA-responsive embryonic precursors in vitro (Choi et al., 1998). Additional experiments to assess the haematopoietic potential of VEGFA deficient progenitors are under way.

Failure of blood island formation in $Vegf^{lo/lo}$ patches of chimaeric yolk sacs already indicated an important function of visceral endoderm-derived VEGFA. However, based on this first chimaeric approach, we could not exclude the possibility that VEGFA secreted by yolk sac mesoderm was involved in vasculogenesis in an autocrine manner. Until now $Vegf^{-/-}$ or hypomorphic conceptuses, which are necessary for addressing the consequences of VEGFA malfunction in extra-embryonic tissues by tetraploid aggregations, were not available. The use of tetraploid $Vegf^{lo/lo}$ embryos in combination with *Vegf* wild-type ES cells allowed us to investigate the effect of impaired VEGFA function in the visceral endoderm in a setting where the mesodermal target cell population was wild type. The failure of $Vegf^{lo/lo}$ visceral endoderm to induce blood island formation properly in wild-type mesoderm convincingly demonstrates the intrinsic requirement for VEGFA in the yolk sac visceral endoderm. The importance of the visceral endoderm layer for proper blood island formation was discovered in the 1960s. Using chick embryos as a model system, Miura and Wilt found that although blood cells can differentiate in the absence of visceral endoderm, no endothelial cells and blood islands form. Trans-filter experiments suggested the involvement of soluble factors (Miura and Wilt, 1969). Our data now identify VEGFA as one of these factors. Using $Gata4^{-/-}$ embryoid bodies that lack visceral endoderm, Bielinska et al. have reported that the visceral endoderm is essential for organisation of blood islands, but is not required for differentiation of primitive

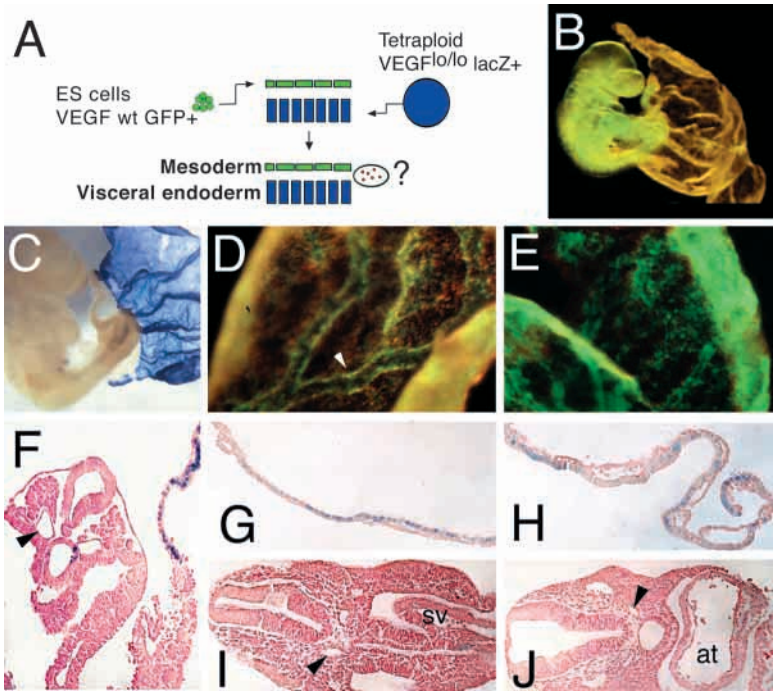


Fig. 7. *Vegf*$^{lo/lo}$ visceral endoderm cannot support blood island formation in wild-type mesoderm. (A) Schematic representation of the aggregation setup. Tetraploid *Vegf*$^{lo/lo}$ embryos were aggregated with *Vegf* wild-type (wt) *GFP*+ ES cells resulting in completely *Vegf*$^{lo/lo}$-derived visceral endoderm and wild-type mesoderm. (B-D) Whole-mount photographs of a 9.5 dpc chimaeric embryo and yolk sac derived from tetraploid *Vegf*$^{lo/lo}$/*Vegf* wild-type *GFP*+ ES cell aggregation. Embryo proper (B) and vitelline vessels (arrowhead in D) are wild-type *GFP*+ whereas *Vegf*$^{lo/lo}$-*lacZ* positive cells constitute the yolk sac visceral endoderm (C). (E) Uniform and intense GFP fluorescence in a 9.5 dpc chimaeric yolk sac derived from tetraploid *Vegf*$^{lo/+}$/*Vegf* wild-type *GFP*+ ES cell aggregation indicates proper expansion of mesoderm-derived cell lineages. (G,H) β-galactosidase staining of yolk sac derived from tetraploid *Vegf*$^{lo/lo}$/wild-type *GFP*+ ES cell (G) and *Vegf*$^{lo/+}$/wild-type *GFP*+ ES cell (H) aggregations. Only few blood islands devoid of primitive erythrocytes are seen in association with *Vegf*$^{lo/lo}$ visceral endoderm (G), whereas blood island formation proceeds normally when supported by *Vegf*$^{lo/+}$ visceral endoderm (H). (F,I,J) Normal vascular but not haematopoietic development in the embryo proper. Dorsal aortae (arrowheads) show appropriate lumen formation. The number of blood cells in the heart atrium (at) and sinus venosus (sv) is reduced compared with completely wild-type embryos.

erythroblasts and endothelial cells (Bielinska et al., 1996). More recently, Belaoussoff et al. have investigated the function of visceral endoderm in mouse embryo explant cultures. They showed a requirement for visceral endoderm signals in haematopoietic and vascular development. It also became clear that this process was restricted to the onset of gastrulation, a narrow time window between 6.0 and 6.5 dpc (Belaoussoff et al., 1998). In the meantime, Dyer et al. have identified Indian hedgehog as a visceral endoderm-secreted factor that, alone, is sufficient to induce endothelial and haematopoietic differentiation in these cultures (Dyer et al., 2001). However, to date there is no direct link between signalling leading to vascular and haematopoietic development and the hedgehog cascade. Thus, the nature of the interplay between factors secreted by the visceral endoderm remains to be determined. Interestingly, Indian hedgehog is expressed in a proximal-to-distal gradient in the visceral endoderm of gastrulation stage embryos (Dyer et al., 2001) – a pattern that might be consistent with findings that a spatial (as well temporal) order of recruitment of blood island progenitors is present (Kinder et al., 1999). No such pattern has been established for visceral endoderm expression of VEGFA. Differences in the expression pattern of the VEGFA isoforms would be an attractive way in which to explain the movement of mesodermal precursor populations during gastrulation.

Regarding haematopoiesis, our chimaera approach might in perspective serve as a useful model for dissecting the contribution of extra- and intra-embryonic sites for definitive haematopoiesis. In tetraploid *Vegf*$^{lo/lo}$/wild-type GFP+ chimaeras the yolk sac haematopoiesis is completely abolished. Thus, it would certainly be interesting to investigate whether the embryos are viable beyond 9.5dpc, solely depending on intra-embryonic sites of definitive haematopoiesis.

Based on the data currently available on developmental expression and function of VEGFA and its receptors, as well as reports investigating the role of other molecules and tissues in gastrulation, the following model of blood island formation and vasculogenesis can be proposed. Mesodermal cells pre-destined to give rise to haematopoietic and/or endothelial cells (Nishikawa et al., 1998) leave the primitive streak attracted by VEGFA secreted from the yolk sac visceral endoderm (Shalaby et al., 1995; Shalaby et al., 1997). In the permissive environment of the yolk sac, they can complete their differentiation program. The haematopoietic and endothelial lineages can expand in response to VEGFA (Schuh et al., 1999; Hidaka et al., 1999) and their organisation into blood islands can be accomplished. During this process, VEGFR1 acts to restrict the expansion of the endothelial lineage (Fong et al., 1999).

The availability of the VEGFA hypomorphic allele described here should now facilitate further dissection of the molecular mechanisms involved in migration and endothelial/haematopoietic differentiation processes during gastrulation. The use of specific developmental restrictions of components of chimaeric embryos, such as tetraploid conceptuses and ES cells, to direct mutant and wild-type cell towards defined allocations in chimaeras was found to be a powerful tool to uncover the intrinsic requirement for VEGFA expression in the visceral endoderm.

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