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Dosage-dependent requirement for mouse *Vezf1* in vascular system development

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Abstract

Vezf1 is an early development gene that encodes a zinc finger transcription factor. In the developing embryo, *Vezf1* is expressed in the yolk sac mesoderm and the endothelium of the developing vasculature and, in addition, in mesodermal and neuronal tissues. Targeted inactivation of *Vezf1* in mice reveals that it acts in a closely regulated, dose-dependent fashion on the development of the blood vascular and lymphatic system. Homozygous mutant embryos display vascular remodeling defects and loss of vascular integrity leading to localized hemorrhaging. Ultrastructural analysis shows defective endothelial cell adhesion and tight junction formation in the mutant vessels. Moreover, in heterozygous embryos, haploinsufficiency is observed that is characterized by lymphatic hypervascularization associated with hemorrhaging and edema in the jugular region; a phenotype reminiscent of the human congenital lymphatic malformation syndrome cystic hygroma.

Keywords: Vezf1 function; ES cells; Knockout mice; Endothelial cells; Vascular development; Angiogenesis; Lymphangiogenesis

Introduction

Mammals have two structurally different circulatory systems, the closed blood vasculature and the open lymphatic system, that are functionally connected and act in concert to maintain tissue homeostasis. The blood vascular system, consisting of arteries, capillaries, and veins, efficiently carries nutrients, gases, and waste products to and from distant actively metabolizing tissues. The lymphatic system regulates tissue fluid balance by returning interstitial fluid and macromolecules from the tissue spaces of most organs back into the venous circulation and serves as a conduit for trafficking immune cells, thus complementing the function of the blood vascular system.

During embryonic development, the blood vascular system is formed via two distinct processes. Vasculogenesis describes the initial differentiation of mesodermally derived endothelial precursor cells, angioblasts, and their coalescence into a primitive vascular network. Angiogenesis refers to the subsequent growth, remodeling, and maturation processes of the primary vascular plexus to give rise to the mature blood vasculature (Carmeliet, 2000; Risau, 1997). The lymphatic system develops through sprouting from the venous system, a process that becomes first

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apparent in the jugular region of developing embryos at midgestation (Wigle and Oliver, 1999).

The vascular endothelial growth factor (VEGF) signaling pathway plays a critical role in the regulation of both blood vascular and lymphatic development. VEGF-A signaling, through binding to its blood endothelial cell-specific receptors VEGFR-1 and VEGFR-2, is essential for the early stages of blood vascular development and the initiation of vascular sprouting (Carmeliet et al., 1996a; Ferrara et al., 1996). In contrast, selective activation of VEGFR-3 signaling using receptor-specific mutants of VEGF-C and VEGF-D induces lymphangiogenesis in the skin of transgenic mice (Veikkola et al., 2001). The importance of VEGFR-3 signaling for lymphatic development is underscored by the findings that lymphatic vessels in *VegfC*-null embryos fail to sprout (Karkkainen et al., 2004), overexpression of soluble VEGFR-3 leads to inhibition of lymphangiogenesis (Makinen et al., 2001), and mutations in the tyrosine kinase domain of VEGFR-3 are linked to human hereditary primary lymphedema (Karkkainen et al., 2000). Recently, angiopoietin signaling, in addition to its well-established function during blood vascular remodeling and vessel stabilization (Gale and Yancopoulos, 1999), has also been implicated in the regulation of lymphatic development (Gale et al., 2002).

At the level of transcriptional regulation, Prox1 activity is required for maintaining lymphatic endothelial cell sprouting, and loss of Prox1 function results in arrested lymphatic development without affecting blood vessel formation (Wigle et al., 1999, 2002). Moreover, misexpression of Prox1 in blood endothelial cells confers a lymphatic endothelial phenotype, indicating that Prox1 is a master regulator of the lymphatic endothelial cell fate (Hong et al., 2002; Petrova et al., 2002). Using gene inactivation approaches, several transcription factors have been implicated in blood vascular development (for review, see Oettgen, 2001). For instance, genetic ablation of the bHLH-PAS protein hypoxia inducible factor 1α (HIF-1α) leads to defective yolk sac and cephalic vascularization (Iyer et al., 1998; Ryan et al., 1998), while the zinc finger lung Krüppel-like factor (LKLF) is required for vascular smooth muscle cell and pericyte recruitment during vessel stabilization (Kuo et al., 1997).

Vascular endothelial zinc finger 1 (*Vezf1*) was originally identified as a gene specifically expressed in vascular endothelial cells during early embryonic development (Xiong et al., 1999), although our subsequent analysis indicated expression in mesodermal and neuronal tissues as well (Lemons et al., 2005). *Vezf1* encodes a 518 amino acid nuclear protein that contains six zinc finger motifs of the C2H2 (Krüppel-like)-type and a proline-rich transcriptional transactivation domain at its C-terminus (Lemons et al., 2005). Consistent with the hypothesis that VEZF1 is a bona fide transcription factor, the human ortholog ZNF161/DB1 has been shown to selectively transactivate the endothelial cell-specific human endothelin-1 promoter in vitro (Aitsebaomo et al., 2001). In addition, *Vezf1* has been implicated in the regulation of endothelial cell proliferation, migration, and network formation in vitro (Miyashita et al., 2004). To investigate the role of *Vezf1* in vivo, we have generated a null allele by gene targeting. Here we report that inactivation of *Vezf1* results in lethality caused by angiogenic remodeling defects and loss of vascular integrity in homozygous mutant embryos. Furthermore, loss of a single *Vezf1* allele leads to an incompletely penetrant phenotype characterized by lymphatic hypervascularization that is associated with hemorrhaging and edema in the jugular region. This haploinsufficient phenotype is reminiscent of the human congenital malformation syndrome, cystic hygroma (Gallagher et al., 1999). Our studies show that *Vezf1* is a crucial regulator of blood vessel and lymphatic development that acts in a tightly regulated dose-dependent fashion during embryonic development.

Materials and methods

Construction of the Vezf1 targeting construct and ES cell manipulations

A 5′ *Vezf1* cDNA fragment (nt 3–776 of the published mouse *Vezf1* cDNA sequence, GenBank accession no. AF104410; Xiong et al., 1999) was used as a probe to screen a mouse 129/Sv lambda genomic library (provided by K. Andrikopoulous and F. Ramirez, Mount Sinai School of Medicine). One positive phage clone contained the first and second exon separated by 5 kb of intronic sequence and 15 kb of genomic sequence upstream of exon 1. For construction of the targeting vector, an 8-kb *Eco*RV/*Xho*I fragment containing the first exon, 6 kb of upstream genomic sequences and 2 kb of 3′ intronic sequence, was subcloned into pBluescriptIISK (Stratagene). A 390-bp *Eag*I fragment including the ATG translation initiation codon was replaced with an IRES-*lacZ*gt1.2*neo* cassette (Wang and Lufkin, 2000) via *Not*I linkers. Subsequent analysis of the *Vezf1* promoter revealed that the deletion included 91 bp upstream of the transcriptional start site, resulting in a non-functional *lacZ* allele.

R1 ES cells (Nagy and Rossant, 1993) were maintained in DMEM (high glucose) containing 15% heat-inactivated fetal calf serum (FCS), 0.1 mM β-mercaptoethanol, 20 mM HEPES, pH 7.3, 0.1 mM non-essential amino acids, and 1000 U/ml LIF on γ-irradiated primary MEFs as a feeder layer. The targeting vector was linearized with *Xho*I and introduced into R1 cells by electroporation (400 V, 125 μF; Bio-Rad Gene Pulser). ES cell clones were selected at G418 concentrations of 200–350 μg/ml for 10 days. ES cell clones homozygous for the targeted *Vezf1* allele were obtained by hyperselection (Mortensen et al., 1992) at concentrations between 400 and 1000 μg/ml of G418 for 14