4. DISCUSSION

4.1 Vezf1 is a widely expressed transcription factor

The major goal of this thesis was to elucidate the function of Vezf1 during the development of the murine vascular system. The original premise of this study was that Vezf1 is a zinc finger transcription factor whose expression during embryogenesis is restricted to endothelial cells and their precursors, as reported in (Xiong et al., 1999). However, it is demonstrated here that the published Vezf1 cDNA represents a chimeric cDNA clone, which contains two open reading frames corresponding to two independent genes, Vezf1 and Zneu1. Furthermore, it is shown that Zneu1 is expressed in an endothelial cell-restricted fashion, whereas Vezf1 shows widespread expression during mouse embryonic development.

Chimeric cDNA clones, which harbor intermolecularly ligated cDNA inserts, represent a highly undesirable artifact in the construction of cDNA libraries, and their relative abundance critically determines the quality of a given cDNA library (Ohara et al., 1997; Sambrook et al., 1989). The frequency of chimeric clones in the mouse E7.5 cDNA library used to clone the endogenous gene corresponding to the 1-13 retroviral insertion is not known (J. Gerhart, Columbia University, personal communication). Accordingly, statements about the quality of this library cannot be made.

Apart from being a chimera, the published Vezf1 cDNA displays two additional artifactual features that were introduced during library construction. The first one is the presence of a 175 bp stretch of intronic sequence (nt 2300-2475 of the published Vezf1 sequence), indicating that an incompletely spliced transcript was used as template for the reverse transcription reaction of the Zneu1 portion of cDNA clone 10-1. Secondly, database searches identified a mouse E11 embryo head cDNA clone (AK018086) that overlaps with 3’ UTR sequences of Vezf1 (nt 2118-2330) and extends the Vezf1 cDNA sequence by an additional 1523 bp to a total length of 3853 bp. This finding indicates that the poly(A)-like stretch between nucleotide position 2303 and 2330 of the Vezf1 cDNA sequence functioned as internal priming site in the poly(dT) primed first strand cDNA synthesis reaction of the 5’ Vezf1 portion of cDNA clone 10-1.
4. Discussion

The total length of the reconstructed \textit{Vezf1} cDNA is 3853 bp with the 3’ UTR being 2256 bp in length. A homologous 3’ UTR region was identified by database search for the human \textit{Vezf1} ortholog ZNF161. This extended ZNF161 cDNA sequence presumably represents the L (long 3’ UTR) clone mentioned in (Koyano-Nakagawa \textit{et al.}, 1994), which contains an additional 1.3 kb of 3’ UTR sequence compared to the published ZNF161 sequence. Only the sequence of the S (short 3’ UTR), internally primed version of ZNF161 had been submitted to GeneBank. The mouse \textit{Vezf1} and human ZNF161 cDNAs share an overall sequence identity of 90% and are still 86.5% identical in the 3’ UTR region. This high degree of sequence homology extends into the proximal promoter region, which is 91% identical, and is suggestive of a high extent of functional conservation in the 3’ UTR and promoter sequences between the mouse and the human genes. The high degree of homology between \textit{Vezf1} and ZNF161 is also reflected in the genomic organization, which is identical with respect to the number, the position and the size of the exons, as well as the nucleotide sequences at the exon-intron boundaries for these two genes.

It was demonstrated in this study, that the retroviral insertion 1-13, which displayed the reported endothelial restricted expression pattern and led to the isolation of the \textit{Vezf1} cDNA, corresponds to the \textit{Zneu1} gene. Therefore, the expression pattern of \textit{Vezf1} during mouse embryonic development was examined. Previously reported Northern blot analysis had shown that \textit{Vezf1} and also its human ortholog ZNF161 were expressed ubiquitously and detected in every tissue examined (Koyano-Nakagawa \textit{et al.}, 1994; Xiong \textit{et al.}, 1999). Employing RNA \textit{in situ} hybridization, \textit{Vezf1} was found, consistent with above mentioned Northern blot analysis, to be expressed in virtually every organ in mouse embryos. Expression levels varied between organs and appeared to be strongest in the brain, the neural tube, the liver, the branchial arches and the vertebral column. Furthermore, low levels of \textit{Vezf1} expression were found in the extraembryonic yolk sac, where it appeared to be concentrated around the yolk sac blood islands. Due to its strong, rather widespread expression, it was difficult to assess whether \textit{Vezf1} was expressed in blood vessels and whether expression was restricted to the endothelial cell layer. However, \textit{Vezf1} signal could be detected in transverse sections through embryonic vessels, suggesting that \textit{Vezf1} is expressed at least in a subset of the embryonic vasculature.
Studies in the zebrafish system support the notion of widespread expression of \textit{Vezf1}. The zebrafish ortholog of \textit{Vezf1} has been cloned in M. Fishman’s laboratory. Consistent with the data presented in this study, \textit{zfVezf1} showed overall widespread embryonic expression. Expression was high in the brain and appeared robust in the embryonic vasculature (J.-W. Xiong and M. Fishman, Mass. Gen. Hospital, personal communication).

A polyclonal anti-VEZF1 antibody was recently generated by L. Campagnolo in Dr. Stuhlmann’s laboratory. Preliminary analysis using this antibody found the VEZF1 protein to be expressed in a widespread yet less uniform manner than the \textit{Vezf1} mRNA during embryogenesis, being detected predominantly in neuronal and mesenchymal tissues. Moreover, robust staining of vascular structures in the embryo proper and the extraembryonic yolk sac was detected. The discrepancy between protein and RNA expression could be explained by post-transcriptional regulatory mechanisms, which are often mediated by sequences in the 5’ and 3’ UTRs. In this respect, it is tempting to speculate that the high degree of homology between the untranscribed regions of mouse \textit{Vezf1} and human ZNF161 may reflect a function in translational regulation of expression.

To study the mechanism of \textit{Vezf1} expression, the \textit{Vezf1} promoter was cloned and characterized in this study. It is shown here that the \textit{Vezf1} promoter is G/C-rich and lacks a typical CAAT and TATA box, features characteristic of promoters of genes that are thought to have housekeeping functions with respect to cell proliferation and usually display broad tissue distribution in their expression (Bird, 1986; Lavia et al., 1987). Thus, the architecture of the \textit{Vezf1} promoter correlates well with the observed widespread expression pattern of \textit{Vezf1 in vivo}.

RNA Ligase Mediated Rapid Amplification of cDNA ends (RLM-RACE) identified a single transcriptional start site 122 bp upstream of the ATG translation initiation codon, which is consistent with the 5’ end of a cDNA clone previously obtained by library screening. The transcriptional start site lies in a pyrimidine-rich sequence, CCATGTT, which is homologous to the pyrimidine (Py)-rich core sequence, PyPyAN(T/A)PyPy, of an initiator element (Javahery et al., 1994). An initiator element is capable of directing accurate transcription initiation in promoters that lack a TATA box (Smale, 1997). Interestingly, there are four potential Sp1-binding sites (Jones and Tjian, 1985) upstream
of the transcriptional start site of the \textit{Vezf1} gene. It has been reported that Sp1 can interact with initiator sequences and enhance the level of basic transcription (Smale, 1997). It remains to be determined whether the putative Sp1 binding sites in the 5’ proximal promoter region are involved in the control of \textit{Vezf1} transcription.

The functional analysis of the promoter and first intron sequences identified a 700 bp proximal promoter fragment that conferred robust expression in both the fibroblast and endothelial cell line. This finding correlates well with the widespread expression pattern of \textit{Vezf1 in vivo}. The inclusion of distal promoter and first intron sequences in the reporter constructs lead to a decrease in promoter activity in both cell lines, suggestive of negative regulatory elements within these sequences. Interestingly, the cotransfection of c-Ets1 and, to a lesser extent, also Egr-1 reverse the negative regulatory effects mediated by these sequences exclusively in the endothelial cell line. A single consensus binding site for c-Ets1 and four sites for Egr-1/Krox24 were detected in the proximal promoter region. Moreover, putative c-Ets binding sites were identified in the first intron sequence. Putative Ets binding sites are involved in the endothelium-specific expression of the \textit{Tie1} and \textit{Tie2} genes (Iljin \textit{et al}., 1999; Schläeger \textit{et al}., 1997). Moreover, c-Ets1-binding has been shown to be required for endothelial-specific expression and activation of the \textit{Flk-1} promoter \textit{in vivo} (Kappel \textit{et al}., 1999; Kappel \textit{et al}., 2000). Thus, it is tempting to speculate that c-Ets is involved in regulating the endothelial expression of \textit{Vezf1}. Electrophoretic mobility shift assays should elucidate whether c-Ets1 or Ets family members (Lelievre \textit{et al}., 2001) can functionally interact with the consensus binding sites. Moreover, mutational analysis of the c-Ets1 site \textit{in vitro} and \textit{in vivo} should reveal whether the binding sites are functionally significant in the regulation of \textit{Vezf1} expression.

Egr-1 (early growth response factor-1), a zinc-finger transcription factor is inducibly expressed (e.g. in response to vascular injury) in endothelial cells and acts as a transcriptional activator of the expression of several endothelial genes including PDGF (Silverman and Collins, 1999). In a model proposed by Khachigian and Collins, activation of PDGF expression by Egr-1 is mediated by displacing Sp1, which is required for basal levels of transcription, from the G/C-rich region of the core promoter (Khachigian and Collins, 1997). The Egr-1 binding sites at positions –65 and –95 of the \textit{Vezf1} promoter partially overlap with Sp1 sites. D. Lemons in Dr. Stuhlmann’s laboratory has been able to
demonstrate specific binding of Egr-1 to these two putative binding sites via electrophoretic mobility shift assays (EMSA), suggesting a similar mechanism of Egr-1 mediated Vezf1 activation as for PDGF. Mutational analysis of these binding sites in vivo should elucidate whether Egr-1 binding mediates constitutive endothelial expression or, alternatively, specific upregulation of Vezf1 during vascular injury and phases of endothelial proliferation.

The analysis for the presence of consensus transcription factor binding sites within the first intron revealed putative sites for GATA factors (Orkin, 1992), Ets proteins (Lelievre et al., 2001), LMO2 (Yamada et al., 2000), ARNT (Abbott and Buckalew, 2000), SCL/tal-1 (Visvader et al., 1998) and COUP (Zhou et al., 2000). Interestingly, embryos lacking the latter four show angiogenic remodeling defects of the early embryonic vasculature reminiscent of those observed in Vezf1 KO embryos, making them potential candidates as upstream regulators of VEZF1. In addition, binding sites for SCL/tal-1, GATA and Ets transcription factors have been identified as critical elements for the endothelium specific expression of several genes in transgenic embryos, including Tie2 and Flk-1 (Kappel et al., 2000; Schlaeger et al., 1997). In order to elucidate, if any of the identified consensus binding sites are functionally important for endothelial Vezf1 expression, a deletion analysis of the first intron is currently ongoing in Dr. Stuhlmann’s laboratory. Furthermore, the generation of transient transgenic embryos will reveal whether the identified promoter and first intron elements are sufficient to direct transgene expression in the developing embryo in vivo.

4.2 Zneu1

It was demonstrated in this study that Zneu1 is an endothelial-cell restricted gene. Thus, Zneu1 is a marker for vascular endothelial cells and their precursors. The only publicly available information regarding Zneu1 is its cDNA sequence, which was directly submitted to GenBank (AF184973)). The human ortholog, ZNEU1, has been submitted under accession # AF186111. Zneu1 is also referred to as Notch4-like protein. This name reflects the chromosomal localization of Zneu1 within the NOTCH4-containig HLA class III region, as well as well as a shared protein motif, the EGF-like domain. Zneu1 and NOTCH4 do not share any homology apart from the EGF-like domain. No additional
structural motifs were identified in the Zneu1 protein sequence. Future functional studies on Zneu1 will provide insights into its role during the development of the vascular system.

4.3 *Vezf1* is a critical regulator of vascular development

To determine the biological function of VEZF1, the *Vezf1* gene was disrupted by homologous recombination in ES cells. As *Vezf1* does not appear to be a member of a large family of related genes, functional redundancy was not expected for the inactivation of *Vezf1*. The absence of functional *Vezf1* transcripts in *Vezf1*−/− ES cells and embryos was demonstrated by Northern blot and RT-PCR analysis using different probes covering almost the entire *Vezf1* coding sequence, indicating that targeting of the *Vezf1* locus resulted in a null allele. The analysis of the effects of the inactivation of *Vezf1* in mouse embryos revealed that Vezf1 acts in a tightly regulated dose-dependent as well as in a strain dependent fashion during vascular development. Thus, in a mixed and outbred genetic background an incompletely penetrant, haplo-insufficient (autosomal), embryonic lethal phenotype with death associated with hemorrhaging and vascular malformations occurring between E12.5 and birth is observed. In the C57BL/6 background, an earlier onset of the mutant vascular phenotype was found. Preliminary data for the 129/Sv strain point to an even earlier defect, possibly at the level of hemangioblast differentiation. Strain (genetic background) specific phenotypes have been demonstrated in several gene inactivation studies. Targeted inactivation of the epidermal growth factor receptor (EGFR), for instance, resulted in peri-implantation death due to degeneration of the inner cell mass on a CF-1 background. On a 129/Sv background, homozygous mutants died at mid-gestation due to placental defects; on a CD-1 background, the mutants lived for up to 3 weeks and showed abnormalities in several organs (Threadgill *et al*., 1995). Similarly, targeted deletion of the bHLH-repressor protein I-mfa in a C57Bl/6 background resulted in embryonic lethality around E10.5 due to placental defects. I-mfa-null embryos on a 129/Sv background had no placental defect, generally survived to adulthood, and exhibited delayed caudal neural tube closure and skeletal patterning defects (Kraut *et al*., 1998).

4.3.1 *Vezf1*, a function in hemangioblast differentiation?

The analysis of the *Vezf1* KO phenotype in the 129/Sv background is hampered by the poor breeding efficiency of this strain. So far only a limited number of *Vezf1* mutant embryos generated by intercrossing F2 heterozygous animals has been analyzed. However
it is noteworthy, that E9.5 \textit{Vezf1}-- embryos were isolated, whose development appeared to be arrested at the late primitive stage (E7.5). This early phenotype is consistent with the early differentiation defects observed in the \textit{in vitro} analysis. Moreover, the fact that resorbed heterozygous embryos were detected at E10.5 and E12.5 supports the notion of an earlier phenotype in the 129/Sv than the C57BL/6 background. Further analysis of 129/Sv KO embryos should elucidate whether strain dependency is operative for the targeted mutation of the \textit{Vezf1} gene. To expedite this analysis, chimeric mice have recently been rederived by blastocyst injection of heterozygous ES cells and male chimeras are currently mated to 129/Sv females to generate a 129/Sv \textit{Vezf1 KO} congenic mouse line.

The analysis of the 129/Sv-derived mutant ES cells in the \textit{in vitro} differentiation system suggests that VEZF1 is required for the development of both the endothelial and hematopoietic lineages and is consistent with a function of VEZF1 at the level of hemangioblast differentiation or even earlier. Semi-quantitative RT-PCR analysis revealed that the endothelial markers, \textit{Flt-1} and \textit{Tie2}, as well as the hematopoietic marker \textit{SCL/tal-1} are downregulated in \textit{Vezf1}-- EBs. In contrast, no differences between wild type and homozygous mutant EBs are detected for the expression of \textit{Rex-1}, a marker for undifferentiated ES cells, demonstrating that the inactivation of \textit{Vezf1} does not lead to a general block of differentiation. Moreover, hematopoietic progenitor assays show that the hematopoietic differentiation potential of \textit{Vezf1}-- ES cell derived EBs is severely impaired. In future studies, to assess whether the inactivation of \textit{Vezf1} affects the differentiation of other cell lineages, it will be useful to further define the defects in \textit{Vezf1}-- EBs by analyzing the expression of an extended panel of marker genes. This analysis could include the pan-mesodermal marker \textit{Brachyury (T)} (Herrmann, 1991) and the following marker genes for mesodermally derived cell lineages: \textit{Nkx-2.5} (cardiomyocytes) (Lints \textit{et al}., 1993), \textit{myf5} (myocytes) (Montarras \textit{et al}., 1991), \textit{H1 globin} (primitive erythrocytes) (Palis \textit{et al}., 1999), \textit{c-fms} (macrophages) (Palis \textit{et al}., 1999) and \textit{c-myb} (definitive hematopoiesis) (Mucenski \textit{et al}., 1991), as well as markers for ectodermal (\textit{Fgf-5}) (Haub and Goldfarb, 1991) and endodermal (\textit{GATA 4}) (Heikinheimo \textit{et al}., 1994) differentiation. It is worth noting that although two different approaches, G418 hyperselection and retargeting of the \textit{Vezf1} locus, were used to generate \textit{Vezf1}-- ES cell clones, only one homozygous mutant ES cell clone was obtained after analyzing a total of 800 clones.
4. Discussion

(based on the initial targeting frequency for the Vezf1 locus of 3%, 12 clones were expected). The one clone isolated was generally indistinguishable from wild type controls, displaying normal morphology and growth characteristics and differentiating efficiently in vitro, as judged by morphological appearance and down-regulation of Rex-1 expression. However, for future studies, a rescue experiment, the reexpression of Vezf1 during EB formation, should be designed to verify that the observed phenotype in Vezf1−/− EBs was indeed a consequence of the absence of Vezf1 expression and not a clonal artifact.

4.3.2 Vezf1 function in angiogenic remodeling and the maintenance of vascular integrity

The analysis of Vezf1−/− embryos in the F5 C57BL/6 background revealed that VEZF1 function is neither required for the early stages of vascular development, angioblast differentiation and vasculogenesis, nor for embryonic hematopoiesis. Instead, loss of Vezf1 leads to an incompletely penetrant mutant vascular phenotype affecting angiogenesis, vascular hemostasis and lymphatic development. Vascular defects become apparent at day 9.5 of gestation in homozygous mutant embryos. E9.5 Vezf1−/− embryos display specific defects in the angiogenic remodeling process of the primary vascular plexus in the vasculature of the aortic arch system, the head, the neck and the dorsal part of the intersomitic vessels. In addition, several sites of hemorrhaging, typically in the head and trunk, are detected, indicating that the integrity of the vasculature is compromised in E9.5 KO embryos. Interestingly, no vascular abnormalities in E9.5 Vezf1−/− embryonic yolk sacs were detected, suggesting that Vezf1 function is not required for yolk sac vascular development. This is a rather unexpected finding as Vezf1 is expressed in the yolk sac mesoderm, and remodeling defects in the embryo proper are usually mirrored in the yolk sac vasculature (Adams et al., 1999; Dumont et al., 1994; Wang et al., 1998).

At later stages of embryonic development, Vezf1−/− embryos that obviously survive this early crisis were detected. These embryos displayed normal morphology of the vascular system and all organ system examined, including heart and placenta, but showed distinct sites of internal bleeding, most prominently in the jugular region and the head.

Vezf1 shows widespread expression during embryonic development, which is especially high in the CNS. The gross morphological and histological analysis of Vezf1 KO embryos, however, did not reveal any defects in organ systems other than the vasculature. Although subtle defects in other tissues cannot be excluded, this finding suggests that Vezf1 is only
required for the formation and function of the vascular system. Alternatively, since the vascular system is the first organ system to evolve during development, the embryonic lethality associated with the vascular defects might therefore obscure additional later phenotypes in other organ systems. A conditional *Vezf1* KO approach, allowing the inactivation of *Vezf1* in particular tissues and/or in adult mice, should be able to elucidate possible additional *Vezf1* functions.

The observed hemorrhaging in E9.5 *Vezf1* KO embryos might be a consequence of the vascular remodeling defects, or, alternatively, it may constitute a primary defect. The analysis presented in this thesis does not allow distinguishing between these two possibilities. The occurrence of vascular leakage in later stage KO embryos without the association with vascular dismorphogenesis is consistent with a primary defect. Moreover, the fact that angiogenic remodeling defects similar to those found in E9.5 *Vezf1* KO embryos have been reported for the inactivation of several genes, including *angiopoietin1*, *Tie2*, *VEGFR-3*, *ephrinB2*, *EphB4*, *EphB2/B3* and *Notch1/Notch4*, which are not accompanied by hemorrhaging, further supports this notion (Adams *et al.*, 1999; Dumont *et al.*, 1998; Gerety *et al.*, 1999; Krebs *et al.*, 2000; Sato *et al.*, 1995; Suri *et al.*, 1996; Wang *et al.*, 1998). The mechanism underlying the compromised vascular integrity in *Vezf1* KO remains to be resolved. Two possible causes were addressed in this study, namely defects in VSMC differentiation and/or recruitment (Kuo *et al.*, 1997; Liu *et al.*, 2000) and defective megakaryocyte differentiation (Spyropoulos *et al.*, 2000). However, both processes were found to be normal in homozygous mutant embryos.

Another possible cause for the vascular fragility might be a defective coagulation system. Interestingly, emerging evidence implicates the clotting system in the process of angiogenesis during embryonic development. In a recent report, thrombin signaling through the protease-activated G protein-coupled receptor PAR1 has been demonstrated to directly regulate endothelial cell function during blood vessel formation (Griffin *et al.*, 2001). Moreover, tissue factor KO embryos show yolk sac vascular defects in addition to bleeding (Bugge *et al.*, 1996; Carmeliet *et al.*, 1996b). As vessel fragility and angiogenic remodeling defects are observed in *Vezf1* KO embryos, this raises the intriguing possibility that *Vezf1* plays a role in coagulation function. The analysis of expression and activity of
the coagulation factors in *Vezf1* KO embryos should determine whether *Vezf1* is indeed involved in the regulation of the coagulation system. Other possible mechanisms for the hemorrhaging in *Vezf1* KO mice are defects in the structural integrity of the endothelial cells themselves (Sato *et al*., 1995), defective inter-endothelial cell contacts or defective interactions between endothelial cells and the underlying basal membrane (Vestweber, 2000). Ultrastructural analysis might be able to elucidate whether any of these processes are affected in *Vezf1* KO embryos.

*Vezf1* is a zinc finger transcription factor, but its downstream target genes are not known. To identify genes that are regulated by *Vezf1*, the expression of a large canon of genes known to regulate endothelial cell proliferation, angiogenic remodeling and the maintenance of vascular integrity was examined. However, the loss of *Vezf1* function could not be correlated with changes in the expression levels of any of these genes. Thus, so far no downstream targets of *Vezf1* have been identified and the molecular mechanisms of VEZF1 function remain unclear. It is possible, of course, that *Vezf1* regulates the expression of yet unidentified genes. Expression profiling using cDNA micorarray chips should be instrumental in identifying the *Vezf1* target genes and thus provide an entry into the molecular mechanisms that underlie *Vezf1* function (Brown and Botstein, 1999). In a recent study, ZNF161 binding and transactivation of the human Endothelin-1 promoter *in vitro* was demonstrated (Aitsebaomo *et al*., 2001). Interestingly, the analysis of mice deficient in Endothelin-1 (ET-1) revealed an incompletely penetrant phenotype that is complementary to that of *Vezf1*−/− embryos with respect to the aortic arch system, as the first and second aortic arch artery, which normally regress between E10.5 and E11.5, persist throughout embryonic development (Kurihara *et al*., 1995; Kurihara *et al*., 1994). ET-1 is expressed in the endocardium of the outflow tract the heart, the endothelium of the aortic arch arteries, the dorsal aorta and the epithelium of the pharyngeal arches in E10 embryos (Kurihara *et al*., 1995). This finding makes *ET-1* a likely VEZF1 target gene. Furthermore, due to the reciprocal KO phenotypes one would postulate negative regulation of *ET-1* by VEZF1 and thus upregulation of *ET-1* in *Vezf1*−/− embryos. However, no differences in the levels of *ET-1* expression between E10.5 *Vezf1* KO and wild type embryos were detected by semi-quantitative RT-PCR. Future analysis of the spatial expression of *ET-1* by RNA *in situ* hybridization or immunohistochemical antibody staining should clarify whether *ET-1* expression is altered in *Vezf1* KO embryos.
4. Discussion

4.3.3 Vezf1, a negative regulator of lymphatic development

Approximately 20% of the Vezf1+/− embryos displayed hypervascularization associated with edema and hemorrhaging in the jugular region indicating that Vezf1 acts in a tightly regulated, dose-dependent manner during vascular development. This incompletely penetrant, haploinsufficient (autosomal) phenotype is caused neither by imprinting of the residual wildtype allele, nor by the generation of a dominant-negative VEZF1 mutant peptide. Only few incidents of haploinsufficiency with respect to vascular development have been reported. The only other transcription factor that acts in a gene dosage-dependent manner is the T-box factor Tbx-1, which is required for normal development of the pharyngeal arch arteries (Lindsay et al., 2001). In addition, the growth factors, VEGF and TGF-β, display dose dependency in their vascular function (Carmeliet et al., 1996a; Dickson et al., 1995; Ferrara et al., 1996).

Histological and quantitative morphometric analysis of E13.5 Vezf1+/− embryos suggests that endothelial hyperproliferation is the cause for the formation of aberrant and dysfunctional vessels specifically in the jugular region. The jugular region constitutes the first and major site of sprouting of the developing lymphatic vessels from venous system (Sabin, 1909), a process that is initiated around E12.5 in mouse embryos. Interestingly, the hyperplastic vessels in the jugular region stain positive for two lymphatic markers VEGFR-3 and LYVE-1, suggesting that they are of lymphatic nature (Banerji et al., 1999; Dumont et al., 1998; Kaipainen et al., 1995; Prevo et al., 2001). Furthermore, they do not express smooth muscle α-actin, a marker for the vascular smooth muscle cell layer, which is a hallmark of blood vascular structures. It has been reported that lymphatic capillaries may contain stagnant blood, which is removed into the venous circulation once the lymphatic system becomes functional (Clark, 1912; Lewis, 1905; Miller, 1913). This observation might explain the finding that the hyperplastic, lymphatic vessels, as they are dysfunctional, contain blood. Taken together, these data indicate that lack of a single Vezf1 allele due to increased lymphatic endothelial cell proliferation results in deregulated lymphatic outgrowth from the venous system and/or lymphatic expansion. Accordingly, Vezf1 would be a negative regulator of lymphatic development and/or lymphatic endothelial cell proliferation.

To gain a better understanding of the etiology of the lymphatic defect in heterozygous Vezf1 embryos, it will be informative to examine the expression of the homeobox gene
**Prox1.** Prox1 is a specific marker of a subpopulation of endothelial cells that gives rise to the lymphatic system (Oliver *et al*., 1993; Wigle and Oliver, 1999). As early as E10.5, Prox1-positive cells are detected in the wall of the anterior cardinal vein. As development proceeds, these Prox1-positive cells bud from the anterior cardinal vein and migrate dorsoanteriorly to form the lymphatic jugular sacs. At later stages of development Prox1 is expressed throughout the entire lymphatic system. In addition, podoplanin, a novel lymphatic mucoprotein (Breiteneder-Geleff *et al*., 1999) and collagen IV, a basal membrane component that is exclusively associated with the blood vascular system (Ezaki *et al*., 1990), should be useful markers to confirm the lymphatic nature of the hyperplastic vessels. Moreover, assays to measure cellular proliferation like BrdU-labeling of embryos (Wigle *et al*., 1999) should be useful in determining whether increased lymphatic endothelial cell proliferation is indeed the mechanism that leads to jugular hypervascularization in Vezf1+/− embryos. Finally, it will be interesting to examine whether Vezf1 is expressed in the recently described lymphangioblasts and, if so, whether it plays a functional role in lymphangioblast differentiation and/or proliferation.

The edematous phenotype observed in E13.5 Vezf1+/− embryos is reminiscent of a human malformation syndrome, called cystic hygroma. Cystic hygromas are congenital malformations of the lymphatic system that occur at sites of lymphatic-venous connection, most commonly in the posterior neck (Gallagher *et al*., 1999). Typically, cystic hygromas develop late in the first trimester either as a consequence of failure of the lymphatic vessels to connect to the venous system, which leads to the accumulation of fluid in dilated lymphatics and progressive lymphedema, or due to abnormal budding of lymphatic endothelium (Edwards and Graham, 1990). Rates as high as 1 in 100 unselected pregnancies have been reported for this syndrome. Although cystic hygromas are frequently associated with other malformation syndromes characterized by chromosomal abnormalities, for instance Turner syndrome or Trisomy 21, there are reports of fetal nuchal cystic hygromas with a normal karyotype (Marchese *et al*., 1985). It will be important to examine if haploinsufficiency of the human Vezf1 ortholog ZNF161 can be associated with this human developmental abnormality. Moreover, it will be of great interest to determine whether loss of VEZF1 function can be correlated with conditions, which are postulated to be the result of excessive proliferation of lymphatic endothelial
cells, such as lymphangioma, lymphangiosarcoma and Kaposi’s sarcoma (Witte et al., 1997).

**4.3.4 Vascular overexpression of Vezf1**

The preliminary analysis of Vezf1 function by overexpression in the blood vasculature appears to underscore the importance of Vezf1 as a regulator of vascular development. Three independent Tie2VIL transgene expressing lines were identified that displayed hypervascularization (more and larger vessels) in snout, ears, skin and hindlimbs. Vascular expression of the Tie2VIL transgene was monitored by β-galactosidase (encoded by the LacZ reporter gene) staining of E12.5 F1 transgenic embryos. The three transgenic lines 1, 14 and 17 showed only weak expression of the LacZ reporter gene, which was nevertheless, consistent with the findings of (Schlaeger et al., 1997), confined to the blood vasculature. Whether these low LacZ expression levels reflect the inherent weak promoter activity of the Tie2 promoter/enhancer elements or, alternatively, are a result of the low efficiency of translation initiation mediated by the IRES element in endothelial cells, as reported by (Peng et al., 2000), remains to be resolved. Hypervascularization was observed in about 30% of the transgenic animals derived from F1 (heterozygous transgenic) intercrosses. This suggests that homozygosity of the transgene is necessary to elicit the vascular phenotype. This dose-dependency of Vezf1 function in transgenic mice is consistent with findings for the inactivation of Vezf1. Careful quantitative expression analysis of the Vezf1 transgene will be required to establish a correlation between the vascular phenotype and the ectopic expression levels of Vezf1. The problem of differentiating between endogenous and ectopic Vezf1 in this analysis could be circumvented by utilizing a LacZ probe on a Northern blot, which would unequivocally detect the transgenic mRNA. Moreover, the generation of transgenic mice with regulatable vascular expression of Vezf1 should be useful in addressing the dose-dependency of Vezf1 function. In addition, detection of the ectopic VEZF1 protein by either Western blot or immunohistochemistry will be necessary to formally prove the overexpression of VEZF1.

As this analysis of vascular Vezf1 overexpression is preliminary, several open questions remain. The cause of death of the F2 Tie2VIL transgenic mice is unclear. It may due to the internal bleeding observed in these animals. Furthermore, the underlying mechanism of the hypervascularization needs to be elucidated. The identification of Vezf1 target genes
will be instrumental in this respect. Increased endothelial cell proliferation is one likely mechanism. This raises the intriguing possibility that \textit{Vezf1} acts as negative regulator of proliferation in lymphatic endothelium, while it promotes proliferation of arterial endothelial cells. Alternatively, \textit{Vezf1} may function in a regional or vascular bed-specific way, as hypervascularization is observed only in certain tissues or regions (provided that this is not a consequence of regional differences in expression levels). Future studies will hopefully address some of these important questions and improve our understanding of the function of \textit{Vezf1} during vascular development.