Transcriptional Regulation of Vascular Development

Peter Oettgen

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circres.ahajournals.org/cgi/content/full/89/5/380

Transcriptional Regulation of Vascular Development

Peter Oettgen

Abstract—Vascular development is a highly organized sequence of events that requires the correct spatial and temporal expression of specific sets of genes leading to the development of a primary vascular network. The first step in this process is the differentiation of pluripotent stem cells into endothelial cells. This is followed by endothelial proliferation, migration, and eventual formation of endothelial tubes. Maturation of these primitive tubes into fully developed blood vessels requires the recruitment of surrounding pericytes and their differentiation into vascular smooth muscle cells. Many of the events that occur during vasculogenesis are recapitulated during angiogenesis. Transcription factors have been shown to serve as master switches for regulating a number of developmental processes. Using a candidate gene approach, the genomic regulatory regions required to direct vascular-specific gene expression of several receptor tyrosine kinases that are critical for vasculogenesis have been characterized and some of the transcription factors that are involved in the regulation of these genes have recently been identified. Many of these factors are also involved in the regulation of hematopoiesis and may have overlapping functions in determining hematopoietic and endothelial differentiation. Targeted disruption of other transcription factors that were not previously thought to be involved in vascular development have also been recently shown to play a role in blood vessel development. The purpose of this review is to provide an update on the progress that has been made in our understanding of the transcriptional regulation of vascular development over the past few years. (*Circ Res*. 2001;89:380-388.)

Key Words: vasculogenesis ■ angiogenesis ■ gene regulation ■ transcription

Until recently the transcription factors that are necessary for regulating vascular development were largely unknown. This is in sharp contrast to other developmental processes such as hematopoiesis and myogenesis where several cell- or tissue-specific transcription factors have been identified. Vascular development requires the differentiation of endothelial cells from pluripotent stem cells. Progress in identifying the molecular mechanisms underlying vascular development has lagged considerably, in large part because the model systems for studying vascular blood vessel development are more limited. The identification of several vascular-specific genes involved in vasculogenesis and the genomic regulatory regions required for directing their expression over the past decade has facilitated the identification of the transcriptional mechanisms required for vascular-specific gene expression. Targeted disruption of additional transcription factors that have been associated with vascular defects led to the elucidation of a role for these factors in vascular development. Angiogenesis, the development of additional blood vessels from a primary vascular network, may recapitulate many of the molecular events occurring during vascular development. The purpose of this review is to summarize the recent advances made in our understanding of the transcriptional regulation of vascular development and the potential clinical implications this may have for other related processes such as endothelial differentiation and angiogenesis.

Candidate Gene Approach

One common approach to identifying the transcription factors required for a particular process is to examine the transcriptional regulation of specific genes that are known to be critical for the process. This approach has been successful in identifying several of the transcription factors required for T- and B-cell differentiation and has similarly been used to facilitate the identification of the transcription factors required for vascular-specific gene expression. Several receptor tyrosine kinases, including the Flk-1, Flt-1, Tie1, and Tie2 genes, are known to be critical mediators of endothelial differentiation and vascular development. Targeted disruption of all of these genes leads to defects in vascular development and early embryonal lethality.[1–3] The genomic regulatory regions required for directing vascular-specific gene expression have been identified for several of these genes, which has been verified in vivo by their ability to direct the expression of the β-galactosidase (lacZ) gene in transgenic experiments. Interestingly for the Tie1 gene, the promoter alone is sufficient to direct lacZ expression in an endothelial cell–specific fashion.[4] In contrast, for both the Tie2 and Flk-1 genes an intronic enhancer is required to direct complete vascular-specific gene expression.[5,6] Comparison of the mouse and human DNA sequences within the regulatory regions of these genes has facilitated the identification of conserved binding sites for different classes of transcription

factors. Conservation of these sites suggests that the binding of family members of these transcription factors may be required to direct vascular-specific gene expression. Further support for this concept comes from the fact that point mutations in these sites lead to marked reductions in the vascular-specific gene expression of lacZ in transgenic animals. The Tie1 gene promoter contains conserved binding sites for Ets factors and AP-2.[4] Mutations in most of these conserved binding sites leads to marked reductions in the ability of the Tie1 promoter to direct lacZ gene expression in transgenic animals. A similar approach has been used to identify the transcription factor binding sites that are necessary for directing the vascular-specific gene expression of the Flk-1 gene.[6] Conserved binding sites for the Ets factors, SCL/tal-1 factor, and GATA factors were identified. Point mutations in some of these binding sites also lead to marked reductions in the vascular-specific expression directed by the Flk-1 regulatory regions in transgenic studies. Conserved Ets binding sites exist in the Tie2 and Flt-1 genes.[7,8] The results of these studies strongly support that these transcription factors are involved in the regulation of vascular development.

Although these studies have led to the identification of potential classes of transcription factors that may be required for the direction of vascular-specific gene expression, this has not resulted in the identification of the specific transcription factors within each of these classes. One approach toward identifying the specific transcription factors that are responsible for the regulation of these genes is to perform transactivation studies and compare the ability of the different members of a particular family of factors to transactivate the gene. This approach was used to test the ability of different members of the Ets factor family to transactivate the Flt-1, Tie1, and Tie2 genes. Whereas the Ets factors Ets-1 and Ets-2 most potently transactivated the Flt-1 gene promoter, they were only able to mildly transactivate the Tie1 and Tie2 gene promoters.[4,8] In contrast, the Ets factors NERF and ELF-1 more potently transactivated the Tie1 and Tie2 genes.[4,8] The ability of specific Ets factors to transactivate the specific gene targets also correlates with their ability to bind to specific conserved Ets binding sites. This suggests that different subsets of the Ets factors may regulate different vascular-specific genes.

Animal Models of Vascular Development

One of the major difficulties in identifying the specific transcription factors involved in regulating vascular-specific gene expression, particularly as it relates to blood vessel development, is the difficulty in isolating either embryonic or extraembryonic blood vessels during mouse embryogenesis. Because blood vessel development is a highly conserved process over evolution, the use of alternative model systems has permitted easier access to studying blood vessel development. Two animal models that have been particularly useful for these studies include the developing zebrafish and chicken. Both have the advantage of allowing direct visualization of blood vessels. Two genes that have been identified in zebrafish and seem to be critical early regulators for initiating vascular development are cloche and spade tail.[9]

Similarly, the stem cell leukemia transcription factor (SCL) was also shown to promote vasculogenesis, hematopoiesis, and endothelial differentiation when expressed ectopically in zebrafish mesoderm.[10] The Ets transcription factor Fli-1 has also been shown to be enriched in the developing blood vessels of zebrafish embryos.[11] As an alternative model of blood vessel development, several investigators have used the developing chicken as an easier model to study blood vessel development because of the easier access to developing blood vessels, particularly in the extraembryonic chorioallantoic membrane. These blood vessels can be microdissected at different stages of development facilitating the determination of whether specific genes are upregulated or enriched in developing blood vessels. This approach was used to identify which of the members of the Ets transcription factor family are upregulated during blood vessel development. A novel role for the Ets factor ELF-1 in vascular development was identified using this approach.[12] In situ hybridization and immunohistochemical experiments confirmed the enriched expression of this factor in extraembryonic and embryonic blood vessels of the developing chicken embryo.[12] The Ets factor Ets-1 has also been shown to be enriched in the developing blood vessels of the chicken and antisense oligonucleotides have been shown to inhibit angiogenesis when delivered to the chicken chorioallantoic membrane.[13]

Conservation of Transcription Factors Involved in Vascular Development

One potential criticism of using nonmammalian models to identify the transcription factors involved in regulating blood vessel development is that the same factors may not necessarily be evolutionarily conserved. Arguing against this is the fact that studies in the chicken and in zebrafish have demonstrated that not only are the factors conserved with regard to protein sequence, but also show a similar enriched expression pattern during vascular development. For example, the helix-loop-helix transcription factor SCL is expressed in developing blood vessels and in the vasculature of both the developing mouse and zebrafish.[10,14] The Ets factor ELF-1, which has previously been identified for its role for T cell–specific gene expression, has also been shown to be a strong transactivator of the Tie1 and Tie2 genes and is highly enriched in developing blood vessels of the developing chicken embryo. The overall homology between the chicken and human ELF-1 protein is 80%.[12] Similarly, the Ets factor Fli-1 has recently been shown to be a critical regulator of blood vessel development not only in zebrafish but also in the mouse.[11,15] In situ hybridization studies of the developing mouse have also demonstrated that Ets-1 is expressed in developing blood vessels associated with tumor angiogenesis.[16] Targeted disruption of Fli-1 in mice results in a loss of vascular integrity accompanied by bleeding and embryonic lethality at day 11.5.[15] Expression of the Tie2 gene is also downregulated in these mice. The expression of two of the GATA factors, GATA-2 and GATA-3, has recently been examined in human fetal tissues. Both factors are enriched in the developing dorsal aorta at 5 weeks old.[17]

Targeted Disruption and Overexpression Studies

An alternative approach that has resulted in the identification of other transcription factors that are required for blood vessel development is through targeted disruption. In many cases this has unexpectedly resulted in determining a novel role for a particular factor in blood vessel development. An example is targeted disruption of the AP-1 transcription factor family member Fra1, which leads to abnormalities in extraembryonic vascularization.[33] The zinc finger transcription factor LKLF is expressed in a variety of vascular and nonvascular cell types. However, targeted disruption of this transcription factor leads to abnormalities in later stages of blood vessel development.[34] Although the early events of both angiogenesis and vasculogenesis were normal in LKLF-deficient mice, they develop abnormalities in the smooth muscle architecture of the tunica media, leading to aneurysmal dilatation of the blood vessels with eventual blood vessel rupture. Diminished numbers of endothelial cells, pericytes, and extracellular matrix deposition are also seen. The transcription factor Tfeb, a bHLH transcription factor, was recently shown to be required for vascularization of the placenta.[35] The homeobox gene Hox D3 is induced in endothelial cells in response to basic fibroblast growth factor (bFGF), and antisense oligonucleotides to Hox D3 block the ability of bFGF to induce the expression of urokinase plasminogen activator. Overexpression of Hox D3 increases integrin $\alpha_v\beta_3$ expression in endothelial cells.[36] Another homeobox transcription factor that may contribute to both hematopoeisis and endothelial differentiation is hhex. Overexpression of this factor in zebrafish embryos leads to enhanced endothelial and erythroid differentiation.[37]

Overlapping Transcriptional Mechanisms Between the Hematopoietic and Endothelial Lineages

Perhaps one of the most interesting recent findings regarding the transcriptional regulation of vascular development has been the determination that the transcription factor SCL/tal-1, which was originally thought to play a role strictly in hematopoiesis, also seems to be critical for embryonic blood vessel development. Targeted disruption of this gene leads to embryonic lethality by day 9.5, due to an absence of yolk sac erythropoiesis.[52] However, it was unclear whether this gene might also contribute to nonhematopoietic pathways at later stages of development. By performing transgenic experiments in which the GATA-1 promoter is used to restore SCL gene expression in hematopoietic lineages in SCL$^{-/-}$ mice, the mice develop striking abnormalities in yolk sac angiogenesis.[14] This suggests that certain transcription factors may be critical for the normal development of both hematopoietic cells and blood vessels, and that there may be a common stem cell precursor for both lineages. The most striking defects were a disorganized array of capillaries and absence of normal vitelline blood vessel formation. Although the larger vitelline blood vessels were not present, a smaller network of interconnecting vessels did exist. The architecture of these vessels revealed normal-appearing endothelial cells as well as the smooth muscle cells or pericytes that constituted the outer lining of the blood vessels. The expression of a number of vascular-specific genes including Tie-1, Tie-2, Flk-1, and Flt-1 also appeared normal. Members of the Ets transcription factor family that were originally described for their role in lymphoid development have now also been shown to regulate vascular-specific genes. The Ets factor NERF was originally identified for its role in regulating the expression of B cell–specific genes such as the tyrosine kinase blk.[53] The NERF gene is expressed as at least three isoforms, NERF1a, NERF1b, and NERF2. Whereas NERF2 is a potent transactivator, the NERF1 isoforms have a truncated transactivation domain and act as natural dominant-negative forms of NERF2. These isoforms are differentially expressed in different cell types. Whereas NERF1a and 1b are expressed in B cells, NERF2 is highly expressed in endothelial cells and is a strong transactivator of the endothelial-specific Tie1 and Tie2 genes.[8] Similarly, the related Ets factor ELF-1, which was originally shown to regulate T cell–specific genes, was also shown to be enriched in developing blood vessels of the chicken.[12] The Ets factor Tel was originally identified for its role as a proto-oncogene in the development of human leukemias. Interestingly, targeted disruption of this factor led not only to defects in hematopoiesis but also to defects in extraembryonic angiogenesis.[54]

Endothelial Differentiation

One of the first steps during vascular development is the differentiation of endothelial cells from pluripotent stem cells. This process initially involves the expression of other endothelial-specific markers such as CD31(PECAM-1). VE-cadherin is associated with the differentiation of these cells into mature endothelial cells. The specific transcription factors that mediate these events have not yet been identified. However, the presence of conserved binding sites in the regulatory regions of vascular-specific genes for several of the transcription factors involved in hematopoiesis suggests that members of the same transcription factor families are also involved in the process of endothelial differentiation. Several studies have recently suggested the existence of a common precursor for both endothelial cells and cells of hematopoietic origin. The possible existence of a common precursor was originally suggested because of the close association of hematopoietic cells and endothelial cells in the developing embryos in the so-called blood islands. Hematopoietic and endothelial cells coexpress a number of genes. One of the earliest markers expressed on cells of endothelial and hematopoietic origin is the VEGF receptor flk-1. Further support for the existence of the hemangioblast comes from differentiation of pluripotent embryonic stem cells along endothelial and hematopoietic lineages.[38,39] When individual blast colonies are allowed to differentiate further, they form adherent cells that express more endothelial-specific markers such as PECAM-1 and Tie2, whereas many of the nonadherent cells presumed to be of hematopoietic origin expressed such genes as β-H1 and βmajor, consistent with cells derived from the erythroid lineage. Furthermore, when Flk-1–positive cells were isolated from embryonic stem cells and allowed to differentiate in vitro, they could be sorted into cells of both endothelial and hematopoietic origin by flow cytometry using surface markers specific for endothelial or hematopoietic

Transcription Factors Critical for Smooth Muscle Differentiation

Transcription Factor	Family	Role	References
AML-1	CBF	Angiogenesis	65
ELF-1	Ets (wHTH)	Tie2 gene regulation	12
Ets-1	Ets (wHTH)	Angiogenesis	13
Fli-1	Ets (wHTH)	Vascular development Tie2 gene regulation	15
NERF2	Ets (wHTH)	Tie2 gene regulation	8
TEL	Ets (wHTH)	Yolk sac angiogenesis	54
MEF2C	MADS box	Vascular development Smooth muscle cell differentiation	48
SMAD5	MADS box	Smooth muscle differentiation Angiogenesis	49
SCL/tal-1	bHLH	Vascular development	14
dHAND	bHLH	Vascular smooth muscle differentiation	51
Tfeb	bHLH-Zip	Placental vascularization	35
HESR1, gridlock	bHLH	Aorta development Endothelial tube formation	42, 43
EPAS	PAS-bHLH	Angiogenesis	26, 27
HIF-1α	PAS-bHLH	Angiogenesis	24
ARNT	PAS-bHLH	Angiogenesis	21
Fra1	bZip	Endothelial differentiation Flk-1 gene regulation	33
Vezf1	Zinc finger	Endothelial differentiation	41
LKLF	Zinc finger	Vascular smooth muscle differentiation	34
HOXD3	Homeobox	Endothelial response to angiogenic factors	36
COUP-TFII	Nuclear receptor	Yolk sac angiogenesis Regulation of angiopoietin-1	66

cells.[40] Some of the specific transcription factors that may be required for endothelial differentiation have recently been identified. The vascular defects seen in mice with targeted disruption of the immediate-early gene Fra1 were partially attributed to a marked reduction in the number of endothelial cells. The defects were mainly seen in the placenta with severely impaired vascular development leading to embryonic lethality between E10.0 and E10.5.[33] The zinc finger transcription factor Vezf1 is expressed solely in vascular endothelial cells and their precursors.[41] Endothelial-specific expression of Vezf1 was also observed in endothelial cells of the developing dorsal aorta, the branchial arch artery, and endocardium and colocalized with Flk-1 expression.

Endothelial Tube Formation

Following their differentiation from pluripotent stem cells, endothelial cells migrate and form primitive tubes. The basic HLH transcription factor HESR1 has recently been shown to be upregulated during endothelial tube formation.[42] Overexpression of this gene in endothelial cells results in downregulation of Flk-1 which may result in inhibiting endothelial cell proliferation by diminishing endothelial responsiveness to VEGF. Antisense oligonucleotides directed against HESR1 were able to block the formation of capillary tubes. The homologue of this factor in zebrafish is called gridlock and is a critical mediator of the development of arteries such as the aorta but not of veins.[43] The homeobox gene HOX B3 has

recently been shown to be involved in facilitating capillary morphogenesis.[44] Overexpression of this factor in the chicken chorioallantoic membrane leads to increased capillary vascular density, and antisense oligonucleotides inhibit endothelial tube formation of microvascular endothelial cells cultured on extracellular matrix. Another transcription factor involved in endothelial tube formation is nuclear receptor PPAR-γ. In contrast to HESR1 and HOX B3, ligands that activate this transcription factor block endothelial tube formation and endothelial proliferation.[45]

Smooth Muscle Cell Differentiation

After initial endothelial tube formation, vessel maturation requires the subsequent recruitment of surrounding mesenchymal cells and their differentiation into vascular smooth muscle cells. This process has been shown to involve the interaction of endothelial cells with mesenchymal cells and the release of specific growth factors such as platelet-derived growth factor.[46,47] A number of transcription factors have also recently been shown to be critical for smooth muscle differentiation (Table). One family of transcription factors that is crucial for muscle development, in general, is the MADS-box transcription factor family. Two members of this family, SMAD5 and MEF2C, have recently been shown to be important in vascular development and in smooth muscle cell differentiation.[48,49] Targeted disruption of SMAD5 leads to vascular defects resulting in embryonal lethality at day 10.5

to 11.5. The defects included enlarged blood vessels with diminished numbers of vascular smooth muscle cells. The absence of SMAD5 results in apoptosis of mesenchymal cells and marked reduction in the differentiation of mesenchymal cells into vascular smooth muscle cells.[49] Similarly, the targeted disruption of MEF2C leads to abnormalities in smooth muscle cell differentiation and the inability of endothelial cells to form into vascular structures.[48] LKLF is a member of the Krüppel-like family of zinc finger transcription factors. Targeted disruption of this gene leads to vascular defects. Most notably there is a reduction in the number of differentiated smooth muscle cells and pericytes. These defects result in aneurysmal dilatation of the large vessels and eventual rupture with intra-amniotic hemorrhage.[34] A similar phenotype was recently reported for mice lacking the cytoplasmic domain of ephrin B2, suggesting that signaling through ephrin B2 may involve activation of LKLF or similar transcription factors during later stages of blood vessel development.[50] The bHLH transcription factor dHAND has recently been shown to be crucial for yolk sac vascular development. In dHAND null mice, endothelial cell differentiation and recruitment of surrounding mesenchymal cells occurs normally. However, the mesenchymal cells fail to differentiate into vascular smooth muscle cells.[51] One of the genes that was shown to be downregulated in these mice was the $VEGF_{165}$ receptor neuropilin, suggesting that dHAND may be a critical mediator of the VEGF signaling pathway.

Transcriptionally Mediated Hypoxia Responses During Later Stages of Blood Vessel Development

After the development of a primary vascular network, the developing embryo requires the formation of additional blood vessels or angiogenesis. This process is largely driven by hypoxia, which serves as a stimulus for the release of angiogenic growth factors. One of the main classes of transcription factors that promote this process is the basic helix-loop-helix (bHLH)-PAS domain family. A prototype member of this family is the arylhydrocarbon-receptor nuclear translocator (ARNT).[18] ARNT forms a heterodimeric complex with another PAS transcription factor, hypoxia-inducible factor 1α(HIF-1α).[19] In response to oxygen deprivation, these transcription factors stimulate the expression of such angiogenic factors as vascular endothelial growth factor (VEGF).[20] Targeted disruption of the ARNT gene results in embryonic lethality by day 10.5.[21] Although a primary vascular network forms, the predominant defective angiogenesis occurs in the yolk sac and branchial arches, and overall growth of the embryos is stunted. These defects are similar to those observed in VEGF- or tissue factor–deficient mice.[22,23] Thus, although the primary vascular network developed, the angiogenic responses to hypoxia are severely impaired. Similar findings are observed in HIF-1α knockout mice in which embryonic lethality occurs by day 10.5 as a result of cardiac and vascular malformations.[24] Although neither of these transcription factors is expressed in a vascular-specific way, their role in angiogenesis and vascular development is primarily related to their ability to stimulate the production of angiogenic factors, such as VEGF, in response to hypoxia. A third member of this family of transcription factors, endothelial PAS domain protein 1 (EPAS1), was recently identified.[25] EPAS is predominantly expressed in endothelial cells and can also heterodimerize with ARNT. Targeted disruption of the EPAS gene has been evaluated by two different groups resulting in two different phenotypes.[26,27] Tian et al[26] detected abnormalities in catecholamine homeostasis in EPAS$^{-/-}$ mice and no distinct abnormalities in blood vessel formation, whereas Peng et al[27] identified vascular defects at later stages of embryogenesis during vascular remodeling in their EPAS$^{-/-}$ mice. The differences in the phenotype cannot be attributed to differences in targeting construct because both groups disrupted the expression of the bHLH domain, but are more likely attributable to differences in the strain of the mice or subtle differences in the embryonic stem cells used. Although the formation of a primary vascular network or vasculogenesis occurs, later defects in vascular remodeling are observed during large vessel formation associated with hemorrhage and inability of the vessels to fuse properly. This suggests that all three of these PAS family members play a similar role in facilitating later stages of vascular remodeling and angiogenesis in the developing embryo.

Modulation of the function of HIF-1α is also achieved by interaction with other proteins. The transcriptional adapter proteins p300 and CREB-binding protein (CBP) form a multiprotein/DNA complex together with HIF-1α on the promoters of the VEGF and erythropoietin genes to promote expression of these genes in response to hypoxia.[28] Interestingly, CBP-deficient mice exhibit abnormalities in both vasculogenesis and angiogenesis.[29] In contrast, the von Hippel-Lindau tumor suppressor protein (pVHL) has been shown to promote proteolysis of HIF-1α through ubiquitylation under normoxic conditions. Defective VHL function is associated with cancers that exhibit dysregulated angiogenesis and upregulation of hypoxia inducible genes.[30]

The signaling mechanisms by which hypoxia activates HIF-1α are beginning to be elucidated. The catalytic subunit of phosphatidylinositol (PI) 3-kinase, p110, plays a pivotal role in the induction of HIF-1 activity in response to hypoxia.[31] Both induction of VEGF gene expression and HIF-1α activity in response to hypoxia could be blocked by the addition of a PI3-kinase inhibitor. Further support of this concept comes from experiments in which VEGF gene expression and HIF-1 activity is induced by cotransfection of p100. Other studies have recently demonstrated that HIF-1α activity may also be modulated by the mitogen-activated protein kinases p42 and p44.[32]

Temporal and Spatial Aspects of Vascular Development

One of most intriguing aspects of any developmental process is how differentiating cells migrate to the proper location in the correct spatial and temporal organization to form specific structures such as organs or tissues. Blood vessel development similarly involves the correct spatial organization of differentiating endothelial and vascular smooth muscle cells. Endothelial differentiation is an early event followed by the formation of primitive tubes. The subsequent recruitment of surrounding mesenchymal cells and their differentiation into
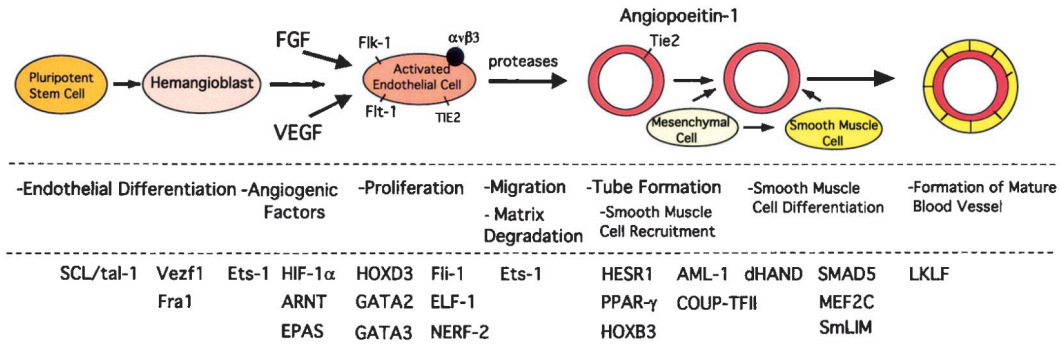
Role of transcription factors during different stages of vascular development.

vascular smooth muscle cells is a later event leading to the formation of stable blood vessels. Growth factors including platelet-derived growth factor, bFGF, VEGF, angiopoietin-1, and transforming growth factor-β (TGF-β) are key mediators of these events promoting the proliferation and migration of cells. Several of the transcription factors described above are either key regulators of the expression of either the growth factors or their receptors, or mediators of the cellular responses to these growth factors. A summary of the temporal role for these transcription factors is shown in the Figure. One of the earliest transcription factors required for the differentiation of a pluripotent stem cell into a hemangioblast is SCL/tal-1.[64] Knockout studies suggest that two transcription factors that may be required for differentiation or survival of endothelial cells early in development are Fra1 and Vezf1.[33,41] Another early step in the differentiation of endothelial cells is the expression of VEGF receptors that promote not only the differentiation, but also the proliferation, of endothelial cells. Regulation of the VEGF receptor gene expression is mediated by the Ets transcription factors, GATA factors, and bHLH factor dHAND.[6,7,51] The expression of VEGF is largely mediated by the PAS domain family of transcription factors, including HIF-1α, EPAS, and ARNT in response to hypoxia. The next stage of blood vessel development involves the proliferation and migration of the endothelial cells and their formation into primitive tubes. Endothelial tube formation is regulated at least in part by the transcription factors HESR1 and PPAR-γ.[42,45] Maturation of primitive endothelial tubes into mature blood vessels requires the recruitment of surrounding mesenchymal cells or pericytes and their differentiation into vascular smooth muscle cells. This process is largely mediated by the angiopoietins and the Tie2 receptor. Tie2 gene expression has been shown to be regulated by the Ets factors NERF, ELF-1, and Fli-1.[8,12,15] One of the possible transcriptional regulators of angiopoietin-1 expression is the transcription factor AML1. Targeted disruption of this factor led to abnormalities in angiogenesis that could be rescued by administration of angiopoietin-1.[65] Another transcription factor that also seems to regulate angiopoietin-1 levels is the nuclear receptor COUP-TFII.[66] Targeted disruption of this gene is associated with angiogenic defects and marked reductions in the level of angiopoietin-1. The differentiation of mesenchymal cells into vascular smooth muscle cells is also a highly orchestrated

process. Members of the MADS-box factors such as SMAD5 and MEF2C mediate the effects of TGF-β thereby promoting endothelial mesenchymal interactions and smooth muscle cell differentiation. Crucial gaps in our understanding of the role of specific transcription factors in this process include the identification of the transcriptional mediators that mediate endothelial responses to growth factors such as VEGF and angiopoietin-1. The factors listed above likely represent only a small subset of the factors required for vascular development. Several additional factors likely exist for the different stages of vascular development.

Determinants of Vascular Specificity in the Absence of Vascular-Specific Transcription Factors

Although several of the transcription factors that are important for blood vessel formation have been identified, many of them are not particularly endothelial or vascular cell specific. Several of the factors seem to be involved in both hematopoiesis and vasculogenesis. What ultimately determines the formation of blood vessels? Either additional vascular cell–specific proteins are required to promote the regulation of a vascular-specific phenomenon, or it is possible that the right combination of transcription factors in a particular cell type may be equally important. One such recently described additional protein is the zinc finger transcription factor Lmo2, a member of the LIM family of proteins.[55] Lmo2 serves as a bridging molecule between GATA factors and E-box proteins that does not require its direct binding to DNA.[56] "CASTing" experiments, in which double stranded DNA sequences with random internal sequences were coincubated with nuclear extracts from the erythroid lineage MEL cell, have been used to attempt to identify the DNA binding specificity of the Lmo2 protein. Antibodies directed against Lmo2 were used to isolate the oligonucleotides binding to Lmo2 protein. Evaluation of nucleotide sequence of these oligonucleotides demonstrated consensus binding sites for GATA and E-box factors. Further analysis suggested that Lmo2 was not directly binding to the oligonucleotides but was acting to bridge the two proteins. Interestingly, overexpression of Lmo2 inhibits erythroid differentiation.[57] Furthermore, targeted disruption of Lmo2 demonstrates a role for this transcription factor in vascular remodeling during blood vessel development.[58] This suggests that these bridging proteins may actually be just as

important in determining differentiation into the specific lineages. Lmo2 belongs to a subset of the LIM proteins called "LIM only" that lack a homeodomain and hence do not bind to DNA. A subset of LIM-only proteins, the cysteine-rich proteins (CRP), have been shown to be important regulators of muscle development. CRP1, for example, is expressed in gut smooth muscle and has been implicated in muscle differentiation.[59] CRP3/MLP is expressed in the heart and skeletal muscle. Overexpression of CRP3/MLP in cultured myoblasts augments differentiation. Further support of a role for CRP3 in muscle development comes from CRP3-deficient mice that exhibit profound defects in cardiac as well as skeletal muscle.[60] CRP2/SmLIM is principally expressed in vascular smooth muscle cells, with preferential expression in arterial versus venous blood vessels.[61] Another family of proteins that function primarily through protein-protein interactions with transcription factors are the ID proteins. These are HLH proteins that lack a DNA binding domain and function by forming heterodimers with bHLH transcription factors.[62] Mice deficient in Id1 and Id3 exhibit defects in endothelial differentiation and angiogenesis.[63] In summary, these data suggest that these adapter proteins may be just as important in determining tissue-specific expression of certain genes and directing developmental processes. Similar properties are exhibited by the SCL/tal-1 transcription factor that does not require DNA binding for its function.[64] Mutant forms of the SCL gene, lacking the putative DNA binding domain, were able to rescue hematopoietic defects from the phenotype of SCL$^{-/-}$ embryos, as well as the hematopoietic and vascular defects of the cloche zebrafish mutants, which are thought to be upstream of SCL.

Another mechanism for providing cell-type specificity, even though the particular factor may be expressed in several cell types, is through differential expression of functionally different isoforms of the transcription factor in different cell types. The Ets transcription factor NERF, for example, which was originally identified as being important in B-cell function by regulating the B cell–specific tyrosine kinase blk, has also subsequently been shown to regulate the Tie2 tyrosine kinase in endothelial cells.[8,53] The NERF gene has multiple isoforms that are differentially expressed in B cells compared with endothelial cells.[8]

Clinical Implications

The identification of the genomic regulatory regions and the specific transcription factors required for vascular-specific gene expression has several implications regarding the potential treatment of several diseases. First, the identification of vascular-specific fragments allows the possibility of delivering genes and their protein products specifically to blood vessels. The Tie1 promoter has been used not only to direct the expression of the β-galactosidase gene in an endothelial-specific fashion but has also been used to express growth hormone.[4] Although these experiments were performed in transgenic animals, they could similarly be used in viral vectors to direct endothelial specific gene expression. Potential therapeutic uses of these vectors include the expression of modulators of inflammation or cell growth in diseases such as the restenosis associated with angioplasty, the vasculopathy

related to cardiac transplantation, and the chronic inflammation associated with atherosclerosis. An example of a protein that has been successfully used to treat restenosis is the Fas ligand, which promotes cell death.[67] However, if this gene was expressed in nonvascular cells, it could lead to significant adverse effects. In addition to inflammatory conditions, a vascular-specific promoter might also be used to block vascular growth during tumor growth since most endothelial cells are not actively proliferating. The identification of transcription factors that may serve as master switches of endothelial differentiation or angiogenesis may also allow the use of these factors to be used in a therapeutic manner or serve as a therapeutic target for blocking angiogenesis. The ability of two transcription factors to direct angiogenesis was recently shown in two studies. In the first study, gene delivery of the early growth response transcription factor egr-1 in a wound healing model enhances the degree of angiogenesis and promotes normal healing.[68] Similarly, the administration of a constitutively active form of the transcription factor HIF-1α augments the angiogenic response by expression of this transcription factor in vivo in a rabbit model of hind limb ischemia.[69] The fact that several other transcription factors have been shown to be enriched during blood vessel development, or that the targeted disruption of these genes is associated with significant vascular defects, as described above, suggests that these factors may also be used therapeutically to promote angiogenesis. Alternatively, several of these newly identified transcription factors could serve as targets for inhibiting blood vessel development or angiogenesis. Drugs used to augment or interfere with the function of these factors could enhance the development of angiogenesis in such diseases as ischemic heart disease, where the development of new blood vessels may be beneficial. Downregulation or blockade of the function of these factors might also be effective at inhibiting the angiogenesis that promotes such diseases as cancer, rheumatoid arthritis, or diabetic retinopathy.

References

1. Sato TN, Qin Y, Kozak CA, Audus KL. Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system [published erratum appears in *Proc Natl Acad Sci U S A*. 1993;90:12056]. *Proc Natl Acad Sci U S A*. 1993;90: 9355–9358.
2. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*. 1995;376:66–70.
3. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*. 1995;376:62–66.
4. Iljin K, Dube A, Kontusaari S, Korhonen J, Lahtinen I, Oettgen P, Alitalo K. Role of Ets factors in the activity and endothelial cell specificity of the mouse Tie gene promoter. *FASEB J*. 1999;13:377–386.
5. Schlaeger TM, Bartunkova S, Lawitts JA, Teichmann G, Risau W, Deutsch U, Sato TN. Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc Natl Acad Sci U S A*. 1997;94:3058–3063.
6. Kappel A, Schlaeger TM, Flamme I, Orkin SH, Risau W, Breier G. Role of SCL/Tal-1, GATA, and Ets transcription factor binding sites for the

regulation of flk-1 expression during murine vascular development. *Blood*. 2000;96:3078–3085.

7. Wakiya K, Begue A, Stehelin D, Shibuya M. A cAMP response element and an Ets motif are involved in the transcriptional regulation of flt-1 tyrosine kinase (vascular endothelial growth factor receptor 1) gene. *J Biol Chem*. 1996;271:30823–30828.

8. Dube A, Akbarali Y, Sato TN, Libermann TA, Oettgen P. Role of the Ets transcription factors in the regulation of the vascular- specific Tie2 gene. *Circ Res*. 1999;84:1177–1185.

9. Thompson MA, Ransom DG, Pratt SJ, MacLennan H, Kieran MW, Detrich HW III, Vail B, Huber TL, Paw B, Brownlie AJ, Oates AC, Fritz A, Gates MA, Amores A, Bahary N, Talbot WS, Her H, Beier DR, Postlethwait JH, Zon LI. The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Dev Biol*. 1998;197:248–269.

10. Gering M, Rodaway AR, Gottgens B, Patient RK, Green AR. The SCL gene specifies haemangioblast development from early mesoderm. *EMBO J*. 1998;17:4029–4045.

11. Brown LA, Rodaway AR, Schilling TF, Jowett T, Ingham PW, Patient RK, Sharrocks AD. Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. *Mech Dev*. 2000;90:237–252.

12. Dube A, Thai S, Gaspar J, Rudders S, Libermann TA, Iruela-Arispe L, Oettgen P. Elf-1 is a transcriptional regulator of the Tie2 gene during vascular development. *Circ Res*. 2001;88:237–244.

13. Wernert N, Stanjek A, Hugel A, Giannis A. [Inhibition of angiogenesis on the chicken chorioallantoic membrane by Ets 1 antisense oligodeoxyribonucleotides]. *Verh Dtsch Ges Pathol*. 1999;83:212–215.

14. Visvader JE, Fujiwara Y, Orkin SH. Unsuspected role for the T-cell leukemia protein SCL/tal-1 in vascular development. *Genes Dev*. 1998; 12:473–479.

15. Hart A, Melet F, Grossfeld P, Chien K, Jones C, Tunnacliffe A, Favier R, Bernstein A. Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. *Immunity*. 2000;13:167–177.

16. Vandenbunder B, Wernert N, Stehelin D. [Does oncogene c-ets 1 participate in the regulation of tumor angiogenesis?]. *Bull Cancer*. 1993;80: 38–49.

17. Minegishi N, Ohta J, Yamagiwa H, Suzuki N, Kawauchi S, Zhou Y, Takahashi S, Hayashi N, Engel JD, Yamamoto M. The mouse GATA-2 gene is expressed in the para-aortic splanchnopleura and aorta-gonads and mesonephros region. *Blood*. 1999;93:4196–4207.

18. Burbach KM, Poland A, Bradfield CA. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proc Natl Acad Sci U S A*. 1992;89:8185–8189.

19. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci U S A*. 1995;92:5510–5514.

20. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*. 1996;16: 4604–4613.

21. Maltepe E, Schmidt JV, Baunoch D, Bradfield CA, Simon MC. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature*. 1997;386:403–407.

22. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*. 1996;380:435–439.

23. Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlaenderen I, Demunck H, Kasper M, Breier G, Evrard P, Muller M, Risau W, Edgington T, Collen D. Role of tissue factor in embryonic blood vessel development. *Nature*. 1996;383:73–75.

24. Kotch LE, Iyer NV, Laughner E, Semenza GL. Defective vascularization of HIF-1α-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. *Dev Biol*. 1999;209:254–267.

25. Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev*. 1997;11:72–82.

26. Tian H, Hammer RE, Matsumoto AM, Russell DW, McKnight SL. The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. *Genes Dev*. 1998;12:3320–3324.

27. Peng J, Zhang L, Drysdale L, Fong GH. The transcription factor EPAS-1/hypoxia-inducible factor 2α plays an important role in vascular remodeling. *Proc Natl Acad Sci U S A*. 2000;97:8386–8391.

28. Arany Z, Huang LE, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, Bunn HF, Livingston DM. An essential role for p300/CBP in the cellular response to hypoxia. *Proc Natl Acad Sci U S A*. 1996;93:12969–12973.

29. Oike Y, Takakura N, Hata A, Kaname T, Akizuki M, Yamaguchi Y, Yasue H, Araki K, Yamamura K, Suda T. Mice homozygous for a truncated form of CREB-binding protein exhibit defects in hematopoiesis and vasculo-angiogenesis. *Blood*. 1999;93:2771–2779.

30. Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ, Maxwell PH. Hypoxia inducible factor-α binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem*. 2000;275:25733–25741.

31. Mazure NM, Chen EY, Laderoute KR, Giaccia AJ. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidyl-inositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood*. 1997;90:3322–3331.

32. Richard DE, Berra E, Gothie E, Roux D, Pouyssegur J. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1α (HIF-1α) and enhance the transcriptional activity of HIF-1. *J Biol Chem*. 1999;274:32631–32637.

33. Schreiber M, Wang Z, Jochum W, Fetka I, Elliott C, Wagner EF. Placental vascularisation requires the AP-1 component fra1. *Development*. 2000;127:4937–4948.

34. Kuo CT, Veselits ML, Barton KP, Lu MM, Clendenin C, Leiden JM. The LKLF transcription factor is required for normal tunica media formation and blood vessel stabilization during murine embryogenesis. *Genes Dev*. 1997;11:2996–3006.

35. Steingrimsson E, Tessarollo L, Reid SW, Jenkins NA, Copeland NG. The bHLH-Zip transcription factor Tfeb is essential for placental vascularization. *Development*. 1998;125:4607–4616.

36. Boudreau N, Andrews C, Srebrow A, Ravanpay A, Cheresh DA. Induction of the angiogenic phenotype by Hox D3. *J Cell Biol*. 1997; 139:257–264.

37. Liao W, Ho C, Yan YL, Postlethwait J, Stainier DY. Hhex and scl function in parallel to regulate early endothelial and blood differentiation in zebrafish. *Development*. 2000;127:4303–4313.

38. Kennedy M, Firpo M, Choi K, Wall C, Robertson S, Kabrun N, Keller G. A common precursor for primitive erythropoiesis and definitive haematopoiesis. *Nature*. 1997;386:488–493.

39. Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. *Development*. 1998; 125:725–732.

40. Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development*. 1998;125:1747–1757.

41. Xiong JW, Leahy A, Lee HH, Stuhlmann H. Vezf1: A Zn finger transcription factor restricted to endothelial cells and their precursors. *Dev Biol*. 1999;206:123–141.

42. Henderson AM, Wang SJ, Taylor AC, Aitkenhead M, Hughes CC. The basic helix-loop-helix transcription factor HESR1 regulates endothelial cell tube formation. *J Biol Chem*. 2001;276:6169–6176.

43. Zhong TP, Rosenberg M, Mohideen MA, Weinstein B, Fishman MC. Gridlock, an HLH gene required for assembly of the aorta in zebrafish. *Science*. 2000;287:1820–1824.

44. Myers C, Charboneau A, Boudreau N. Homeobox B3 promotes capillary morphogenesis and angiogenesis. *J Cell Biol*. 2000;148:343–351.

45. Xin X, Yang S, Kowalski J, Gerritsen ME. Peroxisome proliferator-activated receptor γ ligands are potent inhibitors of angiogenesis in vitro and in vivo. *J Biol Chem*. 1999;274:9116–9121.

46. Hirschi KK, Rohovsky SA, D'Amore PA. PDGF, TGF-β, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. *J Cell Biol*. 1998;141:805–814.

47. Hirschi KK, Rohovsky SA, Beck LH, Smith SR, D'Amore PA. Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. *Circ Res*. 1999;84:298–305.

48. Lin Q, Lu J, Yanagisawa H, Webb R, Lyons GE, Richardson JA, Olson EN. Requirement of the MADS-box transcription factor MEF2C for vascular development. *Development*. 1998;125:4565–4574.

49. Yang X, Castilla LH, Xu X, Li C, Gotay J, Weinstein M, Liu PP, Deng CX. Angiogenesis defects and mesenchymal apoptosis in mice lacking SMAD5. *Development*. 1999;126:1571–1580.

50. Adams RH, Diella F, Hennig S, Helmbacher F, Deutsch U, Klein R. The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell*. 2001;104: 57–69.

51. Yamagishi H, Olson EN, Srivastava D. The basic helix-loop-helix transcription factor, dHAND, is required for vascular development. *J Clin Invest*. 2000;105:261–270.

52. Shivdasani RA, Mayer EL, Orkin SH. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature*. 1995; 373:432–434.

53. Oettgen P, Akbarali Y, Boltax J, Best J, Kunsch C, Libermann TA. Characterization of NERF, a novel transcription factor related to the Ets factor ELF-1. *Mol Cell Biol*. 1996;16:5091–5106.

54. Wang LC, Kuo F, Fujiwara Y, Gilliland DG, Golub TR, Orkin SH. Yolk sac angiogenic defect and intra-embryonic apoptosis in mice lacking the Ets-related factor TEL. *EMBO J*. 1997;16:4374–4383.

55. Larson RC, Lavenir I, Larson TA, Baer R, Warren AJ, Wadman I, Nottage K, Rabbitts TH. Protein dimerization between Lmo2 (Rbtn2) and Tal1 alters thymocyte development and potentiates T cell tumorigenesis in transgenic mice. *EMBO J*. 1996;15:1021–1027.

56. Wadman IA, Osada H, Grutz GG, Agulnick AD, Westphal H, Forster A, Rabbitts TH. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *EMBO J*. 1997;16: 3145–3157.

57. Visvader JE, Mao X, Fujiwara Y, Hahm K, Orkin SH. The LIM-domain binding protein Ldb1, and its partner LMO2 act as negative regulators of erythroid differentiation. *Proc Natl Acad Sci U S A*. 1997;94: 13707–13712.

58. Yamada Y, Pannell R, Forster A, Rabbitts TH. The oncogenic LIM-only transcription factor Lmo2 regulates angiogenesis but not vasculogenesis in mice. *Proc Natl Acad Sci U S A*. 2000;97:320–324.

59. Crawford AW, Pino JD, Beckerle MC. Biochemical and molecular characterization of the chicken cysteine-rich protein, a developmentally regulated LIM-domain protein that is associated with the actin cytoskeleton. *J Cell Biol*. 1994;124:117–127.

60. Arber S, Hunter JJ, Ross J Jr, Hongo M, Sansig G, Borg J, Perriard JC, Chien KR, Caroni P. MLP-deficient mice exhibit a disruption of cardiac cytoarchitectural organization, dilated cardiomyopathy, and heart failure. *Cell*. 1997;88:393–403.

61. Jain MK, Kashiki S, Hsieh CM, Layne MD, Yet SF, Sibinga NE, Chin MT, Feinberg MW, Woo I, Maas RL, Haber E, Lee ME. Embryonic expression suggests an important role for CRP2/SmLIM in the developing cardiovascular system. *Circ Res*. 1998;83:980–985.

62. Norton JD. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci*. 2000;113:3897–3905.

63. Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes RO, Zhuang Y, Manova K, Benezra R. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature*. 1999;401:670–677.

64. Porcher C, Liao EC, Fujiwara Y, Zon LI, Orkin SH. Specification of hematopoietic and vascular development by the bHLH transcription factor SCL without direct DNA binding. *Development*. 1999;126: 4603–4615.

65. Takakura N, Watanabe T, Suenobu S, Yamada Y, Noda T, Ito Y, Satake M, Suda T. A role for hematopoietic stem cells in promoting angiogenesis. *Cell*. 2000;102:199–209.

66. Pereira FA, Qiu Y, Zhou G, Tsai MJ, Tsai SY. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev*. 1999;13:1037–1049.

67. Sata M, Perlman H, Muruve DA, Silver M, Ikebe M, Libermann TA, Oettgen P, Walsh K. Fas ligand gene transfer to the vessel wall inhibits neointima formation and overrides the adenovirus-mediated T cell response. *Proc Natl Acad Sci U S A*. 1998;95:1213–1217.

68. Bryant M, Drew GM, Houston P, Hissey P, Campbell CJ, Braddock M. Tissue repair with a therapeutic transcription factor. *Hum Gene Ther*. 2000;11:2143–2158.

69. Vincent KA, Shyu KG, Luo Y, Magner M, Tio RA, Jiang C, Goldberg MA, Akita GY, Gregory RJ, Isner JM. Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1α/VP16 hybrid transcription factor. *Circulation*. 2000;102:2255–2261.