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# Mechanisms Regulating the Origins of the Vertebrate Vascular System

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**Abstract** In order to sustain growth, differentiation, and organogenesis, vertebrate embryos must form a functional vascular system early in embryonic development. Intrinsic interest in this process as well as the promise of potential clinical applications has led to significant progress in understanding the mechanisms governing the formation of the vascular system, however the earliest stages of vascular development—the emergence of committed endothelial precursors from the mesoderm—remain unclear. A review of the current literature reveals an unexpected diversity and heterogeneity with respect to where vascular endothelial cells originate in the embryo, when they become committed and the mechanisms governing how endothelial cells acquire their identity. Spatially, a widespread region of the early mesoderm possesses the ability to give rise to vascular endothelial cells; temporally the process is not limited to a small window during embryogenesis, but rather, may continue throughout the lifespan of the organism. On the molecular level, recent findings point to several determinative pathways that regulate, modulate, and extend the scope of the Flk1/VEGF signaling system. An expanding array of novel gene products implicated in endothelial cell type determination appear to act synergistically, with different combinations of factors leading to diverse cellular responses, varying patterns of differentiation, and considerable heterogeneity of endothelial cell types during embryogenesis. J. Cell. Biochem. 93: 46–56, 2004. 

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For reasons of scientific importance as well as clinical relevance, understanding the physiological and molecular mechanisms governing the development of the vascular system has been a subject of longstanding interest [Weinstein, 1999; Baron, 2003; Ema and Rossant, 2003]. Not only is the circulatory system the first functional organ system within the embryo, it is one upon which other developing organs depend for nutrition, oxygen, and even determination and differentiation signals [Cleaver and Melton, 2003]. With the prospect of employing regenerative medicine and gene therapy to terminate the progression of neovascularization in solid tumors as well as to promote the process of revascularization in damaged tissues, there is also considerable clinical interest in the field of vascular endothelial cell development. The convergence of basic research and clinical investigations has resulted in significant progress in understanding the mechanisms regulating virtually every stage of vascular development from cell fate determination to vascular remodeling and the integration of vessels into heterogeneous organ systems [Weinstein, 1999; Baron, 2003; Ema and Rossant, 2003]. This is particularly true for angiogenesis, that is, the means whereby new vessel growth occurs by extension from pre-existing vessels. However, vasculogenesis, the process in which blood vessels coalesce in situ from endothelial progenitor cells, is less well understood. In fact, it is widely agreed that the mechanisms governing the earliest stages of vascular development remain unclear [see, e.g., Baron, 2003; Guo et al., 2003; Moser et al., 2003]. This review will focus specifically on the poorly understood process of the early steps of vasculogenesis, that is, how a population of mesodermal cells progressively becomes

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specified and determined to adopt a vascular endothelial fate. More specifically, it will examine how the current research in the field of vascular endothelial development addresses three fundamental and closely intertwined questions—where vascular endothelial cells originate in the embryo, when they become committed, and the mechanisms governing how they acquire a vascular endothelial identity—and then conclude with an overview of an emerging consensus, unresolved issues, and directions for further research.

## WHERE IN THE VERTEBRATE EMBRYO DO VASCULAR ENDOTHELIAL CELLS ORIGINATE?

While the identification of the anatomical sites within the embryo that support vasculogenesis may appear to be a relatively simple task, this straightforward question has been the subject of considerable debate and shifting scientific views. Early investigators proposed that in amniotes and mammals, only the mesoderm that migrated from the primitive streak into the yolk sac (a structure analogous to the ventral blood islands in amphibians and fish) produced vascular endothelial and hematopoietic stem cell progenitors in situ. However, subsequent experiments demonstrated that such progenitors arose intraembryonically as well, specifically in the para-aortic mesoderm which serves as the precursor to the aorta-gonad-mesonephros (AGM) region [Caprioli et al., 2001]. More recently, additional areas have been added to the list of regions in which vasculogenesis occurs, namely, the allantois and the placenta. Caprioli et al. [2001] demonstrate that the chick allantois expresses the full array of molecular markers that characterize angiogenic tissue in the yolk sac and AGM region including Flk1, Scl-Tal1, GATA-1, and GATA-2. By using grafts of quail allantois into chick embryos, they also show that the resulting vascular network arises from intrinsic progenitors present in the donor tissue. They conclude that the capacity to give rise to angioblasts as well as hematopoietic stem cells appears to be far more extensively dispersed than previously thought [Caprioli et al., 2001]. The placenta has also been invoked as an organ with both angioblast and hematopoietic activity [Alvarez-Silva et al., 2003]; although gene expression studies suggest that these progenitors originate in situ, the authors cannot exclude the possibility that their presence is due to colonization. The highly migratory nature of these progenitor cells renders the question of their ultimate origins difficult to answer definitively, particularly once the circulatory system is functional.

While the yolk sac, AGM, and the allantois are now regarded as areas where de novo vasculogenesis routinely occurs, it is equally important to recognize that there are many other areas within the embryo that possess angiogenic potential. The classic and elegant work of Noden and colleagues in avians has unequivocally demonstrated that virtually the entire mesoderm, with the exception of the notochord and prechordal mesoderm, possesses angiogenic potential during embryogenesis and is able to support vasculogenesis [Noden, 1990]. The expanding list of angioblast-producing regions within the embryo is therefore consistent with the wealth of embryological data demonstrating this widespread vasculogenic potential.

Defining the precise origin of vascular endothelial progenitors on the cellular level has been an even more challenging endeavor. As early as 1920, the painstaking work of Florence Sabin not only provided detailed descriptions of the anatomical sites where the vascular endothelium emerged, but also the insightful observation that angioblasts developed in close apposition to erythroid cells. P.D.F. Murray noted that cells removed from the primitive streak of chick embryos and cultured in vitro gave rise to both endothelial cells and blood islands, and subsequently formalized the hypothesis by formulating the name "hemangioblast" for these bipotential precursor cells [reviewed in Bailey and Fleming, 2003].

While the in vivo identification and isolation of hemangioblasts remains challenging due to their transient nature, there are several lines of evidence from a number of different model organisms supporting the existence of the hemangioblast [Weinstein, 1999; Baron, 2003; Ema and Rossant, 2003]. First, vascular endothelial and hematopoietic precursor cells generally express the same suite of genes, and have similar or identical molecular signatures, including the expression of *Flk1*, *Scl-Tal1*, *Tie1*, and *Tie2*. Secondly, manipulation of the expression of these genes affects both the vascular endothelial as well as the hematopoietic lineage with outcomes being similar among different species. For example, homozygous *flk1* mutants in mice

fail to develop both vasculature and blood, while ectopic expression of *Scl-Tal1* results in excessive production of blood and endothelial cells at the expense of somitic and pronephric duct cells in zebrafish embryos [Gering et al., 2003]. Third, single blast colony-forming cells (BL-CFC) have been successfully identified from murine embryoid bodies (EB) that give rise to both lineages [Choi et al., 1998]. Similarly, experiments in chick demonstrate that single VEGF-R2+ (Flk1) cells from the caudal mesoderm of the blastodisc can develop into either hematopoietic or endothelial colonies under in vitro culture conditions. Moreover, this close association of vascular endothelial cells and blood cells has been observed not only in the yolk sac and AGM, but in the allantois as well [Caprioli et al., 2001].

Despite the compelling evidence in favor of a bipotential hemangioblastic progenitor, it is highly unlikely that all vascular endothelial cells arise from a hemangioblast lineage. Employing the chick system, Paradenaud and Dieterlen-Lievre document two distinct cellular origins of angioblasts, one present primarily in the splanchnopleural mesoderm producing hemangioblasts and the other derived from the somites which gives rise to angioblasts [reviewed in Weinstein, 1999; Caprioli et al., 2001; Vokes and Krieg, 2002]. This is also supported by evidence from *Xenopus* fate mapping experiments showing that virtually every blastomere of the 16- and 32-cell stage embryo gives rise to vascular structures, while blood arises from a subset of these blastomeres, suggesting both a hemangioblastic and an alternative pathway for vascular endothelial determination [Mills et al., 1999; Walmsley et al., 2002]. The hemangioblast stage does not appear to be a necessary prerequisite for all endothelial cells given the documented evidence of alternative and heterogeneous determination pathways. This heterogeneity combined with early plasticity is certainly a major factor contributing to the fact that, despite the considerable amount of effort devoted to developing a "molecular ID tag" for the hemangioblast [Chung et al., 2002], recent investigators acknowledge that the specific phenotypes used to identify these cells are still matter of debate [Guo et al., 2003]. In summary, despite the considerable evidence supporting the hypothesis, the existence of "the hemangioblast" remains controversial, and its in vivo isolation remains problematic [Wu et al., 2003].

## WHEN DO CELLS BECOME COMMITTED TO A VASCULAR ENDOTHELIAL FATE?

The question of precisely when a given population of mesodermal cells becomes specified and eventually committed to adopt a vascular endothelial fate is closely intertwined with the question of where this specification occurs; the progenitor cells at different locations differentiate with chronologically specific and unique developmental programs. It is generally accepted that in mammalian and amniote embryos, vasculogenesis begins first in the yolk sac, followed by the AGM region, and then the allantois—a pattern similar to that in amphibians and zebrafish with the yolk sac analogous to the ventral blood islands [Weinstein, 1999; Baron, 2003; Ema and Rossant, 2003]. However, the question of "when" has recently assumed a new level of complexity and is now being thoroughly re-examined in light of the growing body of evidence demonstrating the persistence of stem cells with hemangioblastic, and even more primitive mesodermal qualities, well past embryonic stages and even into adulthood [Minasi et al., 2002; Bailey and Fleming, 2003].

Guo et al. [2003] demonstrate that a population of Flk1-expressing cells derived from human fetal bone marrow possesses hemangioblastic characteristics, that is, they differentiate in vitro into both endothelial and hematopoietic cells. Similar reports from murine systems corroborate these data. Bailey et al. [2004] show that adult bone marrow hematopoiteic stem cells positive for c-kit, Sca-1 are able to give rise to endothelial cells that stably integrate into a wide range of vascular structures. Notably, these latter experiments employed the transfer of a single cell to establish unequivocally the clonal origin of the resulting endothelial and hematopoiteic cells in the host. Comparable results were obtained by Cogle et al. [2004] who used a novel mouse xenotransplantation model in which human hematopoietic cells were transplanted into a mouse induced to develop retinal ischemia. The resulting neovascularized vessels efficiently incorporated the donor cells, thus demonstrating the hemangioblastic activity of these cells. Taken together, these studies suggest that angiogenesis, that is, vascular growth by sprouting from existing vessels, may not be the sole mechanism of neovascularization in later embryonic and fetal stages or even in the adult; rather, vasculogenesis may

be commonplace throughout postnatal life as various types of circulating progenitor cells move to sites of injury and re-activate what used to be regarded as a strictly embryonic program of vascular differentiation. However, many questions remain including the level of plasticity and the degree of heterogeneity among the putative "pure" adult hemangioblasts, and even whether these are truly adult hemangioblasts or represent hemogenic endothelium—or if there is a functional difference [Orkin and Zon, 2002].

## HOW DO CELLS ACQUIRE A VASCULAR ENDOTHELIAL FATE?

### Mechanisms of Vascular Endothelial Cell Determination: Tissue Interactions

The major issue dominating the field of vascular development, and one clearly tied to questions of the timing and location of vascular development, is deciphering the mechanisms governing how angioblasts or hemangioblasts initially arise. An important aspect of fully understanding this process is identifying the specific tissue interactions, as well as the corresponding signaling pathways, that lead various cells to adopt a vascular endothelial fate. It is widely accepted that the prime candidate for inducing the vascular endothelium is the endoderm, tissue which is regarded as both necessary and sufficient for induction of endothelial cells [see discussion in Vokes and Krieg, 2002]. This view initially arose because of the close physical association of vasculogenic tissue in the yolk sac, AGM, and allantois with the adjacent endoderm. Additional support for the determinative role of the endoderm derives from the identification of the putative signals secreted from this germ layer, specifically Indian hedgehog (Ihh). In the yolk sac, Indian hedgehog is expressed "at the right time and at the right place," namely, in the visceral endoderm of the yolk sac at E6.5 [Byrd et al., 2002]. Loss of hedgehog signaling results in blood island defects in embryonic stem cell (ES) derived EB, inhibition of hematopoiesis, and vasculogenesis in the neighboring epiblast, and a 50% lethality rate in vivo by midgestation due to vascular defects. Overexpression of the hedgehog pathway leads to hypervascularization of the neural ectoderm [Byrd et al., 2002] and even the respecification of neural tissue to hematopoietic and endothelial fates [Dyer et al., 2001].

There is, however, equally compelling evidence that the endoderm may not be necessary, or even sufficient, to induce vasculogenesis. Byrd et al. [2002] have shown that the earliest stage of differentiation can take place in the absence of endodermal Ihh signaling, and that the primary role of the endodermal signal lies in vascular remodeling. Although $Ihh^{-/-}$ ES lines show a substantial decrease in expression of *flk1* and *Scl-Tal1*, which serve as markers for the hemangioblast, these genes are still expressed; moreover, $Ihh^{-/-}$ yolk sacs can form blood vessels, even though they are undersized and few in number. Vokes and Krieg [2002] provide even more compelling evidence that endoderm may not be required for initial induction. Using an array of techniques to remove the endoderm from the developing *Xenopus* embryo (including manual removal and morpholino knockdown of *VegT*) as well as corroborating experiments in quail embryos, they convincingly demonstrate that endoderm is not required for angioblast specification, but rather, for subsequent morphogenesis and the formation of vascular tubular structure. Given that the endoderm does not induce hemangioblastic tissue and that the ectoderm actually represses endothelial development, these data raise the possibility of a mechanism of determination intrinsic to the mesoderm.

### Mechanisms of Vascular Endothelial Cell Determination: Genetic Interactions

The primary thrust of current research on vascular development is directed toward identifying the genes that mediate critical endothelial cell determination events. As one of the earliest and most specific genetic markers for the endothelial lineage, and as a gene of clear functional importance, *flk1* has received the lion's share of scientific attention. However, recent work has now begun to expand the repertoire of relevant genes, examining not only upstream players but also mediators and modulators of the Flk1 pathway. The emerging theme has been one of combinatorial and synergistic regulation and a growing complexity of the genetic players governing endothelial cell determination (Fig. 1).

### Early Determinants of the Endothelial Lineage

There has been significant progress in the past few years in identifying the cascade of interacting genes regulating the determinative

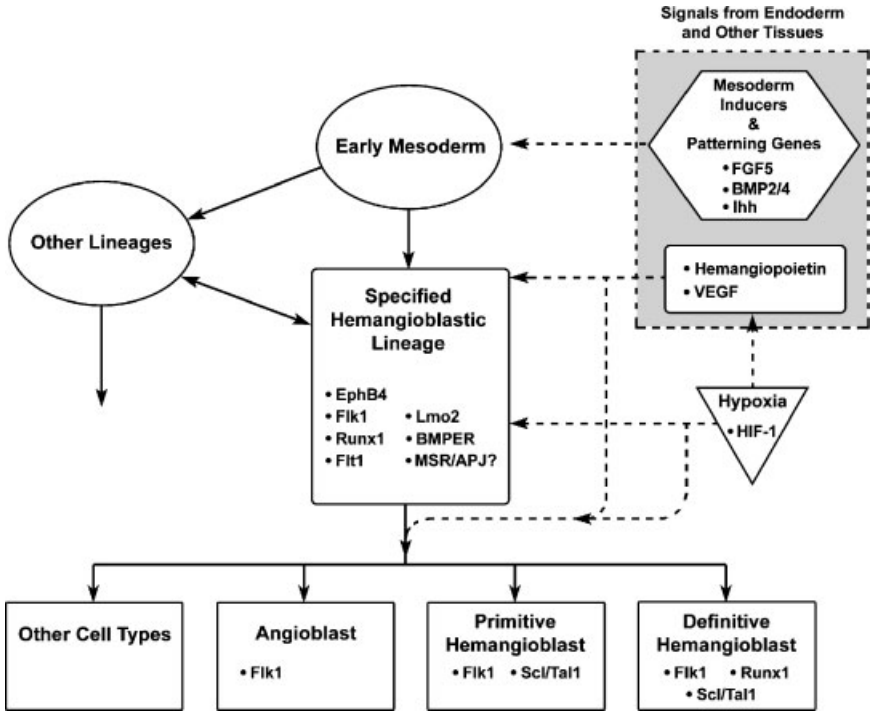


**Fig. 1.** Schematic diagram of vascular endothelial cell development. Solid vertical lines indicate lineage relationships among the tissue and cell types with arrows designating the direction of differentiation. Broken lines denote putative factors mediating the specification of the various cell types. Bullets indicate genes expressed at the various stages of development. However, each of the cell types denoted in the boxes may actually represent a heterogeneous population of cells expressing various subsets of the listed genes. The figure is adapted from Ema and Rossant [2003] and incorporates the findings of the many investigators cited in the current review.

events upstream of Flk1 activation. Not surprisingly, several of the factors associated with general mesoderm induction and patterning are likewise implicated in the early phases of hemangioblast and angioblast development. bFGF, for example, is critical for the proliferation of the hemangioblast lineage [Faloon et al., 2000]. The BMP pathway, although less well studied, is also implicated in early vascular development [Dyer et al., 2001; Moser et al., 2003]. Interference with normal gene expression of either of these pathways in *Xenopus*, mice, or zebrafish perturbs vascular and/or hematopoietic development.

In terms of cell intrinsic factors mediating the various signals, Runx1, Flt1, and EphB4 all appear to play an important and relatively early role in mediating initial commitment to the hemangioblast lineage. In addition to its recognized role in definitive hematopoietic cell fate, Lacaud et al. [2004] demonstrated that *runx1* heterozygosity leads to an acceleration of mesodermal commitment and specification to the BL-CFCs and to the hematopoietic lineages in EBs, with the *runx1*$^{-/-}$ mice producing hematopoietic stem cells a full day earlier than wild type mice both in the yolk sac and in the AGM region. This progression is accompanied by premature *flk1* expression as well.

Somewhat surprisingly, Flt1, which had previously been implicated only in the later stages of angiogenic morphogenesis, has been shown to play a much earlier role in hemangioblast and endothelial cell fate determination, perhaps even acting prior to Flk1. In an elegant series of experiments, Fong et al. [1999] demonstrate that it is the increase in the number of endothelial progenitors that causes the *flt*$^{-/-}$ mutant phenotype of vascular disorganization and that when the mutant cells are appropriately "diluted," vascular endothelial cells undergo normal morphogenesis. These experiments support the view that Flt1 is required to control the development of hemangioblast number by potentially binding untrapped VEGF.

While the family of ephrins and their receptors was recognized to play an important role in later vascular development, specifically the distinction between venous and arterial fates, Wang et al. [2004] convincingly demonstrate

that EphB4 alters the rate and magnitude at which ES cells acquire genotypic and phenotypic characteristics of mesodermal tissues. They show that in EB derived from EphB4 deficient ES cells, the number of BL-CFCs and Flk1-expressing cells is significantly reduced and is accompanied by a preservation of the more primitive phenotypes. EphB4 signaling appears to modulate the response to mesoderm induction signals as a competence factor or perhaps by modifying sensitivity to various signals. The authors suggest the EphB4 serves as a means of "creating boundaries" of different cell types, specifically segregating or restricting the more primitive, undifferentiated cells to areas of EphB4 activity. Their hypothesis is supported by data from zebrafish in which perturbed ephrin expression profoundly disrupts muscle cell differentiation and MyoD expression.

While the factors described above potentially affect the broader spectrum of mesodermal cell populations, Wu et al. [2003] took a more direct approach to define those factors specifically affecting the transcriptional processes regulating the earliest steps in vasculogenesis by examining the regulation of the murine *flk1* gene as a means to understand transcriptional events upstream of *flk1* expression. Using a variety of molecular and biochemical approaches, Wu et al. [2003] identified HoxB5 as the transcription factor binding the *cis*-acting element in first intron of *flk1* which is responsible for endothelial-specific expression of *flk1*. Not only does HoxB5 colocalize with *flk1* expression in vivo in the yolk sac and lateral plate mesoderm, but its overexpression leads to an appreciably increased number of *flk1*-expressing angioblasts in differentiating EB and an increased number of PECAM-positive blood vessels. The authors conclude that HoxB5 is both necessary and sufficient to activate the cell autonomous program regulating the differentiation of angioblasts from mesodermal precursors. Taken together, these data indicate an extensive network of pathways acting upstream of Flk1.

### The Role of the Flk1/VEGF Pathway

As the high affinity receptor for VEGF, Flk1 has undoubtedly been the focus of intensive study as both a marker and mediator of (hem)angioblast commitment. This is largely due to the dramatic phenotypes resulting from gene inactivation studies; $flk^{-/-}$ mice die in utero by E8.5 to E9.5 due to vascular and hematopoietic defects while $VEGF^{+/-}$ heterozygotes display profound vascular patterning defects. However, a number of recent papers have put forth evidence indicating that, functionally, the Flk1/VEGF pathway is not primarily involved in vascular endothelial cell commitment, but rather in the expansion, migration, and survival of this lineage. Schuh et al. [1999] have shown that early endothelial progenitors are produced in absence of Flk1, with normal numbers of endothelial cells present on E7.5, but a severely reduced number detectable on E8.5. The authors conclude that Flk1 is not essential for initial hemangioblast commitment, but is required for the migration of endothelial progenitors into the appropriate microenvironment required for their survival. Cleaver and Krieg obtained similar results in *Xenopus laevis;* they showed that the diffusible form of VEGF expressed by the hypochord serves as a chemoattractant for angioblasts, which migrate from lateral plate mesoderm to the midline region of the embryo [reviewed in Vokes and Krieg, 2002].

The view of the Flk1/VEGF pathway mediating angioblast migration is also consistent with the role of Fps/Fes, a potential downstream signal transduction mediator of the VEGF signal. By using chimeras, Haigh et al. [2004] show that activated Fps/Fes protein in *flk1* deficient ES cells rescue the Flk1 contribution to the developing vascular endothelium, specifically by restoring the ability of $flk1^{-/-}$ progenitors to migrate from the primitive streak to the yolk sac.

While the data presented above argue for a predominately later role of the Flk1 pathway in regulating migration, it is likely that the Flk1/VEGF system plays multiple roles at various stages during vascular endothelial determination. Not only is this view supported by experiments perturbing *flk1* or *VEGF* expression [Weinstein, 1999; Baron, 2003; Ema and Rossant, 2003], but also by the evidence showing that activated Fps/Fes increased the number of hemangioblast colonies in vitro and the number of progenitors in vivo, as well as being linked with formation of hemangiomas. While some of the conflicting data and interpretations regarding the role of the Flk1 pathway in cell fate commitment may be due to species differences, the multitasking role of Flk1 can also be explained by recent results showing that it works synergistically with a host of other factors

which may serve to rescue or partially compensate for Flk1's function in its absence. In addition to regulating migration and expansion of the hemangioblast lineage, the Flk1/VEGF pathway may promote the initial commitment of the hemangioblast lineage, but in its absence the role is assumed by other factors such as Scl-Tal1 or Lmo2.

### Potential Modulators of the Flk1 Pathway

Although Scl-Tal1 was originally considered to function primarily in hematopoiesis and significantly downstream of Flk1 because it is not expressed in *flk1* mutants, several lines of evidence suggest that Scl-Tal1 may have an earlier role in hemangioblast cell fate determination. First, expression of *scl-tal1* in mouse and zebrafish is similar to that of *flk1*, and *scl-tal1* homozygous mutants fail to differentiate in vitro to form blast colonies. Secondly, ectopic expression of *scl-tal1* results in excessive production of blood and endothelial cells from the lateral mesoderm at the expense of other mesodermal derivatives such as somitic and pronephric tissue in zebrafish embryos. Finally, Ema et al. [2003] showed that expression of *scl-tal1* under the control of the *flk1* promoter was sufficient to effect partial rescue of the *flk1* deficiency in vivo and result in successful blast colony formation in vitro. Taken together, these data support the view that Scl-Tal1 not only has an earlier role in cell fate specification, but that Scl-Tal1 may act in a combinatorial fashion with the Flk1 pathway.

Additional evidence supporting synergistic genetic interactions comes from work on the *lmo2* gene [Gering et al., 2003]. Previously shown to have a role in hematopoiesis and in angiogenic remodeling, Gering and colleagues demonstrate that Lmo2 works cooperatively with Scl-Tal1. Ectopic expression of coinjected *lmo2* and *scl-tal1* mRNA induces blood and endothelial lineage markers along the entire anterior–posterior axis of the embryo, although the erythroid lineage is only expressed in the pronephos which, interestingly, corresponds to the expression of the *gata-1* gene in this region. These results suggest that in the absence of a specific hematopoiteic inducer, Scl/Tal1 induces hemangioblasts to differentiate into endothelial cells, with vascular tissue serving as a default state.

A particularly intriguing gene that also interacts, albeit indirectly, with the Flk1 pathway, is *BMPER*, an ortholog of *Drosophila crossveinless* [Moser et al., 2003]. *BMPER* mRNA colocalizes with *flk1* and parallels its spatial and temporal expression. Serving as a BMP inhibitor, BMPER effectively represses BMP-dependent differentiation of endothelial cells. This is consistent with reports that BMP2 and BMP4 are required for the genesis of hematopoiteic cells and that deletion of proteins involved in the BMP signaling pathway frequently leads to significant vascular defects. The authors hypothesize that Flk1-expressing cells may express *BMPER* to inhibit recruitment of additional Flk1 positive cells in order to prevent vascular overgrowth. Alternatively, BMPER may promote the differentiation of other cell lineages by determining which cells respond to local BMPs and which cells do not.

### Mechanisms of Vascular Endothelial Cell Determination: Environmental Interactions

While the hard-wired genetic interactions described above are of undeniable importance, local environmental cues may also exercise a significant influence in the determination of vascular endothelial cell fate. For example, hypoxia has been shown to be a significant stimulus for new blood vessel formation during both vasculogenesis and angiogenesis in a number of model systems. Low oxygen conditions are prevalent over the course of embryonic development, as well as in some pathological circumstances (i.e., tumor growth), and it has been suggested that variation in hypoxic microenvironments may be utilized in the same manner as other morphogen gradients.

The master oxygen regulator on the cellular level is the HIF-1 complex, a heterodimeric transcription factor composed of an $\alpha$ and $\beta$ (also known as ARNT) subunit. The critical role of the HIF-1 complex in vascular development is demonstrated by HIF-1$\alpha^{-/-}$ mutant mice which exhibit early embryonic lethality with impaired formation of intra- and extra-embryonic vasculature [Kotch et al., 1999]. Under hypoxic conditions, HIF-1$\alpha$ protein is stabilized and translocates to the nucleus where it dimerizes and binds to a response element present in the regulatory regions of sensitive genes to promote their transcription. The activation of HIF-1 affects a battery of genes involved in vascular development, including *VEGF*, *flk1*, *flt1*, *Scl-Tal1*, and *tie2*, via direct transcriptional regulation or indirectly through the action of

VEGF. The efforts of many investigators have helped to elucidate the complex system of interactions that exists between the components of this regulatory network; however, a coherent picture of these pathways has yet to be realized.

This situation is further complicated by other major signaling pathways that have also been implicated in the vascular response to hypoxia via interactions with the HIF pathway or in a HIF-independent manner. The TGF-β superfamily's involvement in angiogenesis is well documented by gene ablation experiments in mice which result in phenotypes similar to those observed in HIF-1$\alpha^{-/-}$ mutants. Hypoxia is often accompanied by an increase in TGF-β levels, and recent work demonstrates that TGF-β synergistically cooperates with hypoxia to stimulate VEGF and endoglin expression through physical interactions of Smad3/4, Sp1, and HIF-1 [Sanchez-Elsner et al., 2002]. Another family of signaling proteins, the AMP-activated protein kinases (AMPK) have also been shown to be involved the hypoxic response by sensing the depletion of ATP levels. Nagata et al. [2003] report that AMPK signaling via the Akt pathway plays a role in endothelial cell differentiation and response to VEGF during hypoxia in vivo. Interestingly, this interaction was not observed under normoxic conditions, suggesting a possible signaling mechanism for hypoxia-specific angiogenesis. Mitogen-activated protein kinases (MAPK), key parts of many regulatory systems through which cells integrate a variety of stimuli, are also involved in hypoxic signaling, although the specific kinases and transcription factors involved, as well as the target genes affected, show considerable heterogeneity and differ according to the cell type under investigation. The precise relationships among hypoxia, the signaling pathways stimulated by hypoxia, and the hard-wired genetic programs regulating vascular endothelial cell fate determination remain unclear.

## CONCLUSIONS AND FUTURE DIRECTIONS

The analysis of the literature presented above reveals the significant degree of progress in understanding the molecular, cellular, and physiological mechanisms governing the origins of the vasculature. A recurring theme is the unanticipated extent of diversity—in terms of the location, the timing, and the pathways mediating vascular endothelial development. Not only have several different regions been shown to produce vascular endothelial cells de novo, but perhaps more importantly, the areas that possess angiogenic potential appear to be very widely distributed in the early mesoderm of the vertebrate embryo. This process could involve a hemangioblast progenitor in some regions or alternative pathways in other areas. The spatial diversity of vasculogenesis is mirrored by the corresponding temporal diversity of vasculogenesis; the process is not restricted to a narrow window during early embryonic development, but may occur throughout the lifespan of the organism.

In terms of the genetic interactions and pathways, heterogeneity and complexity have become hallmarks of endothelial cell development (Fig. 1). While the early stages of angiogenesis have become linked to the more general question of mesodermal induction and patterning, recent findings point to a co-ordinated pathway of interactions that regulate, modulate, and extend the Flk1/VEGF signaling system. These modulators act synergistically with different combinations of factors mediating diverse cellular responses and varying patterns of differentiation that sense local microenvironmental cues.

Recent investigations, however, suggest a general consensus that may incorporate some disparate results. This view argues for widespread vasculogenic potential throughout the early mesoderm of the embryo—a capacity that may be induced quite early in the mesoderm of some organisms or may be autonomous or intrinsic to the mesoderm in other species. This vasculogenic potential is progressively restricted to the developing vasculature through a series of cell or tissue interactions that repress vascular differentiation or promote the differentiation of other cell types, perhaps through an intrinsic patterning mechanism such as the Delta-Notch pathway in *Drosophila* as suggested by Vokes and Krieg [2002]. However, a small number of progenitor cells may retain their angioblast character long past the embryonic period.

The view is supported by a diverse array of evidence from different model organisms. A wealth of embryological studies, particularly in the chick system, confirms the broad spatial angiogenic potential [reviewed in Weinstein, 1999; Caprioli et al., 2001; Vokes and Krieg, 2002]. Molecular analyses demonstrating the

surprisingly widespread expression of the primary marker for angioblastic potential, namely the *flk1* gene, corroborate the embryological results. Caprioli et al. [2001] maintain that all mesodermal cells in the allantois express *flk1* and many also express *scl-tal1*, *gata-1*, and *gata-2*. Many other studies employing embryoid body differentiation as the model system cite that a significant percentage of differentiating cells (between 40 and 50%) express *flk1* [Kabrun et al., 1997; Moser et al., 2003]. Others demonstrate very extensive *flk1* expression prior to and during the establishment of the yolk sac blood islands. In chick embryos, *flk1* is expressed throughout the entire mesoderm of embryonic day 1 embryos [Flamme et al., 1995]. It is therefore possible that the expression of Flk1 signifies, and perhaps along with other genes regulates, the widespread vasculogenic competence. This view is also consistent with reports suggesting that angioblasts represent the "default" state within the hemangioblast lineage [Gering et al., 2003].

The progressive restriction of vascular endothelial potential is supported by embryological transplant studies showing a reduced angiogenic potential at later stages in development. These observations are corroborated on the molecular level with consistent reports of *flk1* expression gradually being down-regulated both in vivo (in chick and mice) and during embryoid body differentiation where the number of cells declines to a few percent during the course of differentiation. Other genes such as *msr/apj* (mesenchyme-associated serpentine receptor), which has been extensively utilized as a vascular endothelial marker in amphibians, also display a similar pattern of restriction [Mills et al., 1999; Vokes and Krieg, 2002].

The ability of Flt1 and BMPER to repress the development of vascular endothelial phenotypes now offer a molecular mechanism for this gradual restriction of vasculogenic potential. However, while most cells differentiate into mesodermal subtypes and lose this potential, certain cells may retain this potential and represent a population of long term progenitors. These progenitors likely represent a very diverse population of cells depending upon their own state of maturation and specific anatomical location.

Despite the new directions and an emerging consensus in the field, many unresolved issues remain. Does the early mesoderm that expresses Flk1 and other vascular markers have broad potential or plasticity for a variety of mesodermal cell types, or is this widespread potential restricted to (hem)angioblast fates? How heterogeneous are the vascular endothelial progenitors and what are their molecular signatures? And how diverse are the genetic and environmental pathways regulating vasculogenesis both in the embryo and later in development? Does a subset of "differentiated" vascular endothelial cells retain true developmental plasticity or is this observed plasticity of adult cells restricted to a small number of stem cells?

Addressing these and other questions will require a detailed and perhaps single cell analysis of co-localization of the increasing number of markers delineating the various mesodermal cell types in both the vascular and non-vascular lineages. This expression analysis should be accompanied by an in vivo examination of the fates of these "phenotyped" cells in normal development and under a variety of genetically and embryologically perturbed conditions in an effort integrate gene expression with fate, function, and potential plasticity. Double mutants and the use of fluorescently tagged genes as employed by Fehling et al. [2003] combined with novel imaging approaches should be productive in this respect. This analysis should also include the "non-canonical" vascular genes, such as *msr/apj*. Analysis of the various microenvironments—those attributable to genetic hard-wiring as well as those attributable to environmental conditions such as hypoxia and other biophysical conditions—will be essential to identify in order to disentangle the various factors contributing to the determination and differentiation of vascular endothelial progenitor cells. Genomic and proteomic analyses will reveal a more complete array of interacting players. Finally, careful comparative molecular anatomy and functional genomics across species—attempting to perform analogous experiments where possible—will link gene expression and function with the wealth of available embryological information. Discerning the conserved and the divergent elements of the vascular endothelial determination will undoubtedly facilitate the discovery of the range of mechanisms that govern this dynamic process throughout the life of the organism.

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