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REVIEW

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Oxygen, genes, and development: An analysis of the role of hypoxic gene regulation during murine vascular development

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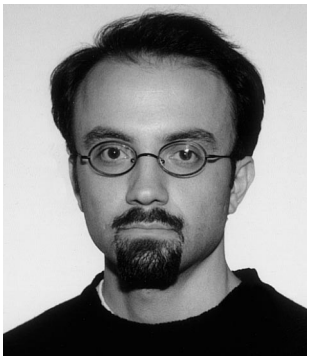
Abstract Development of the mammalian cardiovascular system is a complex process guided by both genetic and environmental components. Significant advances in the genetics of vascular development have been accomplished most recently by the analysis of multiple "knockout" and transgenic mice which exhibit varying degrees of impaired vascularity. This review focuses on the potential of the environment of the developing embryo to affect its development. In particular we analyze the evidence implicating the ability of physiological parameters such as oxygen and glucose concentrations within and surrounding the embryo to affect the expression of genes critical for vascular development. We conclude that the vascularization of a developing mammalian embryo is a plastic process dependent on the dynamic interaction between fundamental genetic and physiological factors.

Key words Hypoxia · Arylhydrocarbon receptor nuclear translator (ARNT/HIF-1β) · Gene expression · Vascular development · Mouse

Abbreviations *aFGF* Acidic fibroblast growth factor · *bFGF* Basic fibroblast growth factor · *Epo* Erythropoietin · *HIF* Hypoxia-inducible factor · *PDGF* Platelet derived growth factor · *TF* Tissue factor · *TGF* Transforming growth factor · *tr* Thrombin receptor · *VEGF* Vascular endothelial growth factor



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Introduction

The formation of a complex multicellular organism involves the carefully orchestrated interplay between a predetermined genetic program and a variety of environmental cues. When speaking of developmental processes, however, there tends to be a bias toward focusing on the actions of the gene. Perhaps the most important reason for this bias is that a mammalian embryo develops within the remarkably well-controlled environment of the womb. Hence there is little variability to affect ontogenic processes. And even when geneticists do speak of environmental factors affecting development they tend to think of secreted proteins diffusing through the embryo to generate morphogenic gradients, or cell surface proteins exerting their inductive influence in a direct cell-to-cell fashion. While these factors are proteins and hence products of genes, other kinds of morphogens have also been described. The most famous of these is perhaps retinoic acid, a natural

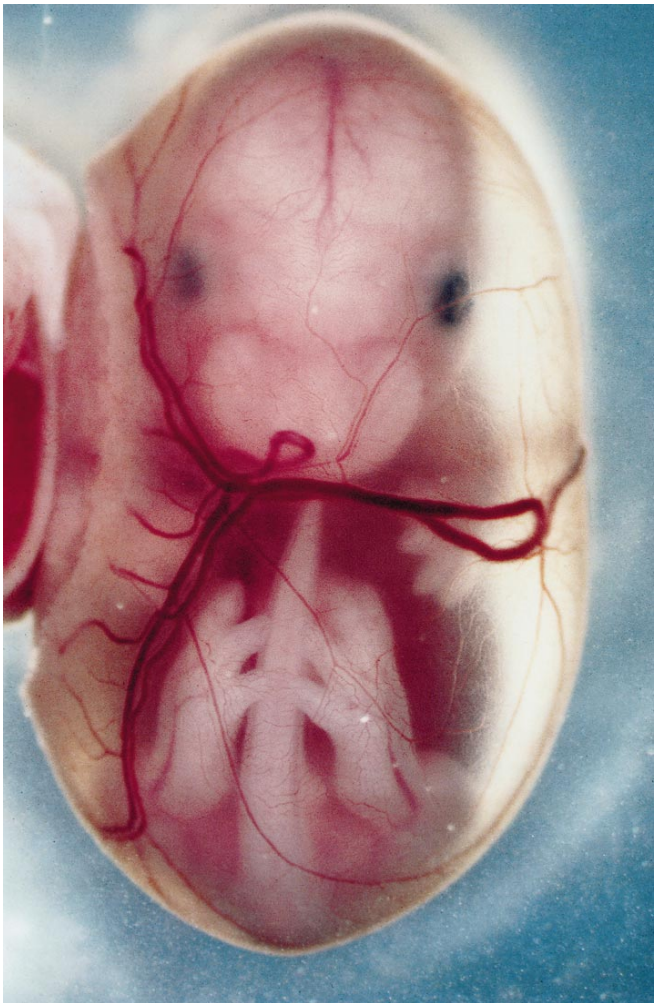

Fig. 1 A day E16.5 mouse embryo within its yolk sac displaying extensive vascularization of the yolk sac and embryo proper. Note the vessels of differing size ranging from large muscular arteries to capillaries forming a distribution network covering the entire surface of the yolk sac

metabolite of vitamin A which plays a critical role in limb development and embryonic patterning [1–3].

An often overlooked category of developmental cues, however, may be comprised of the very compounds which are required for the most basic of physiological processes, namely oxygen and glucose. Not only does a mammalian embryo develop within an environment exhibiting oxygen concentrations in the hypoxic range, but it also undergoes a large increase in tissue mass prior to vascularization. The ability to survive and grow in such an environment stipulates the presence of an intact hypoxia response machinery. Additionally, the generation of oxygen/nutrient gradients within the embryo as a result of growth, coupled with an oxygen/nutrient level sensing and response machinery, could enable the developing embryo to utilize this gradient in a manner not unlike other morphogen gradients.

Decades of research into the nature of the physiology of the developing embryo and its relationship to its immediate physical environment is being complemented by more recent research into the genetic mechanisms which help coordinate this interaction. This review concentrates on the events leading to the formation of a complete vascular system in the mouse, with only minor reference to cardiac development or hematopoiesis. The cardiovascular system is not only the earliest organ system to become functional in the developing mouse embryo, it is also the first organ system whose proper functioning is critical for the embryo's homeostasis and continued growth. It is responsible not only for the delivery of metabolites to developing tissues but also for the removal from these tissues of the toxic byproducts of metabolism itself. Given that this system plays such a critical physiological role, it is not surprising that many of the genes which are required for proper vascular development are now known to be regulated by physiological factors such as oxygen and glucose concentrations. An analysis of the role of the immediate physical environment in regulating the formation of this organ system can reveal much about the nature of the physiological parameters which govern early mammalian development.

Genes and vessels

Two fundamental events are involved in blood vessel formation. The de novo formation of endothelial cells from primitive mesenchymal precursors and their organization into vascular channels are termed *vasculogenesis*. Structures thought to arise via this process are the dorsal aorta and the early vascular network in the yolk sac [4]. The vascularization of tissues as a result of the sprouting of new vessels from preexisting ones is termed *angiogenesis*. The combination of these processes lays the foundation for the subsequent maturation of vascular networks into ones which contain bona fide venules, veins, arteries and arterioles (Fig. 1).

A number of recent studies utilizing targeted mutagenesis has uncovered numerous genes that are required for the proper formation of blood vessels within a developing mouse embryo. While many of the results obtained by these experiments have substantiated the known roles of particular agents in blood vessel formation, other results have led to the incorporation of mechanisms previously thought to lie outside of this realm. We have learned that the process of vascularization requires the products of numerous genes acting in concert, and that this process can be disrupted at multiple points, with varying consequences. It has also become clear that an important modulator of many of these genes involves a dynamic interaction between the developing embryo and its environment.

Growth factors and their receptors

Factors able to induce endothelial cell division, migration, and survival, known as angiogenic agents, are obvious candidates for genes required for the proper vascularization of a developing organism. One of the best character-

ized is vascular endothelial growth factor (VEGF) [5]. Although now the focus of intense research due to its potent angiogenic properties, VEGF was initially characterized as a protein produced by tumors which exhibits profound permeability inducing effects on the vessels which supplied them [6]. Thus it was previously termed “vascular permeability factor”. It was not until several years later, however, that the mitogenic actions of this protein on endothelial cells were appreciated [7]. The observation that tumors can become vascularized when implanted into avascular sites such as the cornea led to the initial search for angiogenic agents. The biochemical isolation of the first angiogenic factor was accomplished by Shing et al. in 1983 [8] utilizing heparin-Sepharose chromatography techniques to purify an endothelial cell mitogen from a rat chondrosarcoma homogenate. An additional related factor was rapidly isolated and the two were identified as acidic (a) and basic (b) fibroblast growth factors [9, 10]. FGFs exhibit high-affinity heparin binding and both lack signal sequences to mediate their secretion from cells. This latter point is interesting in that factors able to recruit vessels must act at a distance. FGFs can, however, be liberated from cells in the event of cell damage or by exocytosis and can be sequestered in the extracellular matrix until their release via the actions of various heparinases [11]. Both VEGF and the FGFs exert their angiogenic effects via multiple receptor tyrosine kinases. VEGF can bind to three receptors, flt-1, flk-1/KDR, and flt-4 (VEGF receptors 1–3) which are expressed primarily on endothelial cells [5, 12, 13]. FGFs (1–9) bind to FGF receptors 1–4 which exhibit a much broader expression profile [14].

Targeted disruption of the murine VEGF gene revealed a strict dose-dependent regulation of embryonic vessel formation by this agent. Embryos heterozygous for an inactivated VEGF allele exhibit early embryonic lethality due to compromised intra- and extraembryonic vessel formation and impaired cardiac development apparent at embryonic day E8.5 with no viable embryos surviving to term [15, 16]. This phenotype is more pronounced in the homozygotes. These embryos form endothelial cells; however, their organization into vessels is impaired, as indicated by the rudimentary formation of the dorsal aortae and yolk sac vasculature. Blood island formation and cardiac development are also affected. This contrasts with disruption of VEGF receptor 2, or flk-1. These mice exhibit an almost complete block to both hematopoiesis and vasculogenesis [17]. No blood islands or vessels can be detected in the yolk sacs of these animals, potentially due to an inability of precursor cells to properly migrate and populate the yolk sac [18]. In addition, the yolk sacs of *Flk-1*$^{-/-}$ embryos contain almost no hematopoietic progenitor cells as assayed by methylcellulose culture. Flk-1 is the earliest VEGF receptor expressed, appearing at day E7.0 [19], consistent with its requirement for the development of endothelial cell precursors. While VEGF is also expressed at this stage, the increased severity of the flk-1 mutation raises the possibility of undefined additional ligand(s) for this receptor. Deficiency of VEGF receptor 1, the flt-1 receptor tyrosine kinase, leads to a less severe phenotype with blood island and vessel formation present, although disorganized. The blood vessels of *Flt-1*$^{-/-}$ embryos are enlarged and form an irregular plexus in the yolk sac leading to midgestational embryonic lethality [20]. Although present, the dorsal aortae, as well as the yolk sac blood islands, contain differentiated endothelial cells not only on the periphery but also within them. These observations led the authors to conclude that the flt-1 signaling pathway plays a role in regulating endothelial cell-cell or cell-matrix interactions during vessel formation. Although potent angiogenic agents, the roles of aFGF, bFGF, and their receptors during vascular development are uncertain. Individual knockout of these genes does not lead to disrupted vascular development, perhaps due to compensation by other family members [14].

Another family of endothelial specific receptor tyrosine kinases is comprised of TIE-1 and TIE-2 (or TEK) [21–23]. These receptors are detected in all embryonic vasculature beginning at day E8.0 and are also present in some hematopoietic lineages. Their expression patterns during development are identical, and they exhibit a high degree of sequence and structural similarity [24]. It is thus interesting that the individual knockouts of these genes produced markedly different phenotypes. Disruption of the *Tie-1* gene results in the formation of normaly appearing vascular plexuses comprised of endothelial cells that exhibit decreased integrity and survival [25]. Leakiness of the vessels results in large scale edema and impaired lung function. Most *Tie-1*$^{-/-}$ embryos die between day E13.5 and 14.5 of gestation due to vascular compromise; those that survive to term die immediately after birth due to respiratory difficulties. In contrast, all *Tie-2* deficient embryos die by embryonic day 10.5 due to the formation of an irregular vascular network unable to supply the circulatory demands of the developing embryo [25]. Although vasculogenesis does not seem to be affected, angiogenesis clearly is abnormal in *Tie-2*$^{-/-}$ animals. In addition, cardiac development is also impaired.

The recent cloning of a specific ligand for TIE-2, termed angiopoietin-1 [26], and its targeted disruption [27] have further elucidated the role that these receptors play in blood vessel formation. Angiopoietin-1, unlike angiogenesis factors, does not induce endothelial cell division and thus is not a classic growth factor. It is expressed primarily by the mesenchymal cells surrounding developing vessels and in the myocardial layer of the heart and is critical to cardiac development and vascular network formation. The most severe phenotype of angiopoietin-1 null mice is abnormal heart trabeculae formation. In addition, these animals form a much less complex vascular network than their wild-type littermates. This is most striking in the yolk sacs of mutant embryos where the initially homogeneous capillary network is unable to remodel itself into one which contains both large and small vessels. A closely related factor, angiopoietin-2, has recently been cloned and shown to antagonize the actions of angiopoietin-1 on the TIE-2 receptor [28]. The presence or absence of VEGF may be able to modify the impact of these interactions on the endothelial cell. In the presence of VEGF an-

giopoietin-2 is thought to maintain endothelial cells in a plastic state, enabling them to grow in the direction of the VEGF gradient. Since angiopoietin-1 activity is thought to induce the maturation of blood vessels, the inhibition of this activity at the leading edge of vessel migration is desirable. In the absence of VEGF, i.e., in tissues which do not need to be vascularized, angiopoietin-2 expression may contribute to blood vessel regression [28]. Neither angiopoietin-1 nor -2 affects TIE-1, which remains an orphan receptor.

Two other growth factor deficiencies also result in vascular defects. Some 50% of transforming growth factor β1 (TGFβ1$^{-/-}$) and 25% of TGFβ1$^{+/-}$ mice die embryonically due to impaired yolk sac vascularization at day E9.5 [29]. All platelet-derived growth factor β (PDGFβ) deficient embryos have vessels which lack proper pericytes [30] and die at late gestation due to hemorrhage. The pericyte is an ill-defined cell type derived from primitive mesenchymal cells which encircles microvessels and exhibits contractile properties [31]. The recruitment of the pericyte to the developing vessel is thought to be mediated in large part by PDGFβ, as evidenced by the lack of such support cells in its absence. TGFβ secreted by the pericyte as a result of contact with the endothelial cell induces endothelial cell quiescence and an alteration of integrin expression and promotes the deposition of extracellular matrix by the maturing pericyte [31]. Thus, an absence of proper TGFβ expression results in a disruption of the endothelial cell pericyte interaction, resulting in compromised vessel integrity and impaired vascular development.

Coagulation factors

As mentioned above, VEGF was originally identified as a vascular permeability inducing factor secreted by many tumor types. The induction of microvascular permeability leads to the leakage of plasma components, resulting in the formation of an extravascular clot. In fact, transplantation of guinea pig carcinomas into syngeneic hosts is accompanied by the regular deposition of cross-linked fibrin [32]. This fibrin deposition precedes the influx of granulation tissue, a mixture of invading capillaries and fibroblasts, in a manner analogous to that seen during normal wound healing [33]. That fibrin itself may be an important mediator of angiogenesis had previously been demonstrated by the observation that blood vessels and fibroblasts invade fibrin gels deposited into the subcutaneous space of experimental animals [34]. Thus it appeared that the fibrin deposited around tumors as a result of their permeability-inducing and procoagulant properties might play an important role in their subsequent vascularization. These earlier studies may be helpful in analyzing the recently reported phenotypes obtained by the mutagenesis of three important components of the coagulation cascade – tissue factor (TF), factor V and the cloned thrombin receptor (tr), each of which results in embryonic lethality.

Mouse embryos rendered deficient for TF via homologous recombination exhibit an early embryonic lethality with almost all TF$^{-/-}$ embryos dead by day E10.5 [35]. The primary cause of death in these embryos is the formation of aberrant vasculature predominantly within the embryonic yolk sac. These vessels exhibit decreased integrity due to the lack of cell types which characteristically provide support for them: pericytes and smooth muscle cells. The authors concluded that the inability to recruit such cells results in the inability of the vessels to withstand increasing pressures, leading to their eventual dissolution. TF is chemotactic for vascular smooth muscle cells [36]; however, this activity seems to be independent of its procoagulant properties. TF levels are elevated within the vasculature and tumor cells of malignant breast cancers but not in benign fibrocystic disease [37], suggesting that TF expression is correlated with a malignant phenotype. And while tumor cells engineered to overexpress TF are highly angiogenic, this activity is independent of coagulation and may be due to increased expression of VEGF in these tumors [38]. Other studies suggest an important role for the cytoplasmic domain of TF in signal transduction independent of the coagulation inducing extracellular domain [39]. Thus, although TF is crucial for the development of embryonic vasculature, it is not clear that it mediates this effect through activation of the coagulation cascade. Furthermore, mice lacking platelets [40] or fibrinogen [41] develop to term without obvious defects in their vasculature. Fibrinogen-deficient mice, however, display impaired penetration of organizing cells into hematomas which routinely develop in them, consistent with the view that fibrin provides a critical matrix for the movement of cells.

Factor V deficient animals also exhibit vascular defects with 50% of the embryos dead by day E9-10 due to impaired yolk sac vascularization [42], suggesting a link between coagulation and vessel formation. The other factor V$^{-/-}$ embryos die immediately after birth due to massive hemorrhage. The 50% mortality in factor V$^{-/-}$ mice is also observed at day E9-10 of tr-deficient mice. The remaining tr$^{-/-}$ animals, however, develop to term and exhibit normal hemostasis [43]. While platelets from tr$^{-/-}$ liveborns are still able to respond to thrombin, suggesting the presence of a second thrombin receptor in these cells, tr$^{-/-}$ fibroblasts fail to show thrombin-induced phosphoinositide hydrolysis, calcium mobilization, or mitogenic responses. The inability of thrombin to stimulate and recruit mesenchymal cells in tr$^{-/-}$ animals may help explain the embryonic lethality. This is also suggested by the inability of G protein subunit α_{13} deficient mice to produce proper yolk sac vasculature [44]. Fibroblasts from these mice do not migrate in response to thrombin, the receptor for which couples to Gα_{13}. Thus factor V mediated thrombin generation and subsequent signaling through the thrombin receptor may be essential for the recruitment of mesenchymal cells to developing vessels.

The role of coagulation in blood vessel development still remains uncertain. The leakiness of growing vessels leads to the extravasation of serum components, triggering clot formation. Extracellular clots could act as provisional matrices that provide support for growing vessels until the generation of proper scaffolding composed of pericytes,

smooth muscle cells, and extracellular matrix components deposited by them [45]. Such support would be especially important in the yolk sac where there is little else to maintain vessel integrity. This hypothesis is rendered less likely by the observation that fibrinogen- or platelet-deficient mice are able to develop proper vasculature. The disruption of vascular development when members of the coagulation cascade are genetically inactivated is most likely due to an inability to recruit support cells to developing vessels and not simply to a lack of structural support provided by the clot itself. The obvious hemostatic benefits of blood clots are known to be augmented by the chemotactic properties of the various components of the coagulation cascade to recruit macrophages, fibroblasts, and smooth muscle cells to help regenerate the original tissue and vasculature. These chemotactic properties also seem to play a crucial role in the maturation of developing vascular networks into ones which contain proper support structures required for their maintenance and continued growth. Thus a process active in the adult only in response to pathology may also play an important role during normal development.

Oxygen and genes

The current excitement over angiogenesis and angiogenic agents stems largely from the role that these agents play in the vascularization of growing tumors. Due to the limitations of diffusion as a means of oxygen and nutrient transport, a tumor cannot grow beyond a diameter of approximately 2 mm without becoming vascularized [46]. Therefore a tumor must be perfused by a network of capillaries distributed approximately every three cell layers to supply its metabolic needs [47]. It has been postulated that reduced O_2 tension within tumors stimulates release of angiogenic agents, leading to subsequent tumor vascularization and rescue from hypoxia. In fact, rapidly growing tumors frequently have necrotic cores surrounded by a layer of cells adjacent to infiltrating capillaries which express high levels of VEGF stimulated by the hypoxic nature of this core [48]. The better vascularized a tumor is, the better it is able to grow, and the worse the prognosis it carries for the patient [49, 50].

The ability of O_2 tension within a growing tumor to regulate vascularization has parallels with the production of erythropoietin (Epo) by the kidney in response to anemia and the secretion of angiogenic agents by macrophages during wound healing [51]. In fact, the molecular mechanisms responsible for hypoxic responses are active in all mammalian cell types studied thus far [50, 52]. Cells and tissues faced with a hypoxic challenge must respond by: (a) switching from aerobic to anaerobic metabolism, (b) increasing glucose transport into the cell to compensate for the reduced ATP generating capacity of glycolysis (the Pasteur effect), (c) promoting improved vascularization via the liberation of various angiogenic agents, and (d) increasing the O_2 carrying capacity of the blood which supplies them. Each of these processes is mediated in part by the products of genes which are upregulated in response to low oxygen tension. For example, the glycolytic enzymes phosphoglycerate kinase 1, pyruvate kinase m, phosphofructokinase l, lactate dehydrogenase, aldolase A, glyceraldehyde 3-phosphate dehydrogenase and enolase 1 [53–57], the glucose transporters GLUT-1 and GLUT-3 [58–62], the direct and indirect acting angiogenic agents VEGF [48, 63–69], bFGF [70, 71] and PDGFβ [72], and the glycoprotein hormone responsible for increasing red blood cell mass, Epo (see [73], and references therein) are all encoded by such genes. This transcriptional response is mediated in large part by the actions of a hypoxia-inducible protein complex termed hypoxia-inducible factor (HIF) 1. HIF-1 activity is initiated upon heterodimerization of the two bHLH-PAS proteins which comprise it, namely ARNT and HIF-1α, leading to the formation of a functional transcription factor complex [74]. ARNT is the arylhydrocarbon receptor nuclear translocator responsible for also mediating responses to environmental pollutants such as dioxin via heterodimerization with the arylhydrocarbon receptor [75]. HIF-1α is a novel member of the rapidly growing bHLH-PAS family of transcription factors. Binding sites for this complex have been localized to the promoters or enhancers of most of the previously mentioned hypoxia responsive genes [76].

HIF-1 was discovered as a result of the search for factors that regulate Epo expression. It was quickly discovered, however, that HIF-1 activity can be found in nearly every cell type studied, making it a prime candidate for a master regulator of the transcriptional response to hypoxia. In addition to its aforementioned actions, HIF-1 has also been postulated to regulate the enzymes heme oxygenase-1 [77] and inducible nitric oxide synthase [78]. The products of these enzymes, carbon monoxide and nitric oxide, play important roles in modulating vascular tone. Endothelin-1, a potent vasoconstricting agent secreted by endothelial cells [79], is also stimulated by hypoxia [80]. Peripheral vessels dilate in response to low oxygen whereas the vessels comprising the pulmonary vasculature constrict to shunt blood away from poorly ventilated regions. Differential distribution and activities of hypoxia responsive machinery in the smooth muscle cells of the pulmonary versus systemic vasculature could be one mechanism responsible for this action. Another mechanism whereby mammals adapt to hypoxia is hyperventilation. Catecholaminergic brainstem areas responsible for controlling ventilatory and cardiovascular responses to hypoxia accumulate mRNA and protein for tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, in response to long-term hypoxia [81, 82]. Hypoxia also induces dopamine production by carotid body type I cells which regulate the rapid cardiovascular response to reduced O_2 tension [83]. Tyrosine hydroxylase mRNA is induced by hypoxia in the PC12 pheochromocytoma cell line [84], potentially via the HIF-1 site located in its promoter [85].

One of the most important outstanding questions involves the nature of the sensing machinery responsible for converting oxygen levels into a message intelligible to the transcriptional apparatus. It is beyond the scope of this review to analyze the experimental evidence arguing for or

against the various proposed models of oxygen sensing (for a detailed analysis see [50]). However, it is important here to emphasize that the transcriptional response to hypoxia is triggered at oxygen concentrations beginning at about 5%, with an exponential increase as O_2 levels drop further [86]. Thus the cell responds to hypoxia well in advance of the point at which oxygen concentrations become limiting for mitochondrial electron transport. In fact, it is under such oxygen concentrations that most cells find themselves, as the tissues which they populate are generally found to be exposed to environments exhibiting oxygen concentrations between 3% and 5% [87–93]. These studies suggest that a range of O_2 concentrations well below atmospheric (20%) constitute a so-called "physiological" hypoxia which represents the appropriate environment for mammalian biological processes. This holds true not only for a fully formed organism but for a developing one as well, as the environment of the developing embryo (i.e., the oviduct and the uterine horn of mammals) resides within the hypoxic range [94–98]. Thus, HIF-1 may be active during development as well as during normal homeostasis of most tissues. Prior to the formation of a complete cardiovascular system, the developing embryo faces hypoxic conditions more severe than those of most vascularized organs. In addition, the increase in embryo mass results in the generation of gradients of oxygen and nutrient levels which may act as other morphogen gradients when coupled with an oxygen/nutrient-sensitive signaling and transcriptional machinery.

Oxygen and vascular development

The effect of oxygen tension on developmental processes has been studied largely by investigators attempting to improve the culture conditions for growing mammalian embryos in vitro. Historically these have involved the optimization of culture conditions for generating blastocysts from one-cell stage embryos. In addition, culture systems have been developed to study the metabolism of later stage mammalian embryos ex vivo. Long-standing research utilizing these methodologies has provided us with a greater understanding of the relationship between the environment of the developing embryo and its metabolism and growth.

Hyperoxia is known to be toxic due to the generation of highly reactive oxygen radicals which can covalently interact with lipids and amino acid side chains leading to membrane and protein damage. Atmospheric oxygen, i.e., approx. 20%, is defined as normoxia; however, outside of the respiratory epithelium and the outer layer of the skin very few tissues are ever exposed to this level of oxygen. Additionally, this level of oxygen has been shown to be detrimental to the development of embryos from many mammalian species. The optimum oxygen concentration for the proper development of mammalian embryos in vitro seems to be 5%, not 20%. This has been shown to be the case for rabbit [99], mouse [100–103], sheep, and cow embryos [104, 105]. A level of 20% oxygen consistently decreases the percentage of single-cell embryos developing to the blastocyst stage, and blastocysts cultured under 20% O_2 contain fewer numbers of cells. Even exposure to 20% O_2 for 1 h can be detrimental to development. These values correlate well with the previously mentioned values obtained for oxygen concentrations within the reproductive tract of mammals and lend further credence to the idea of "physiological" hypoxia.

Growth within such an environment imposes certain constraints on the metabolism of the embryo. Following compaction of the morula and prior to the formation of a vascular system able to transport oxygen and nutrients from the maternal circulation, rat embryos rely nearly exclusively on anaerobic glycolysis to meet their metabolic needs [106–108]. Upon formation of a complete chorioallantoic circulation and the subsequent availability of maternal oxygen at embryonic day 11, the rat embryo decreases its reliance on glycolysis in favor of oxidative phosphorylation. Additionally, increases in enzyme activity concerned with terminal oxidation and phosphorylation occur at this time, along with a corresponding morphological maturation of the mitochondria, as evidenced by increased numbers of their cristae [109]. This switch to aerobic metabolism is correlated with a marked increase in growth rate which requires a much more efficient means of energy production. The ability to coordinate the development of an oxygen delivery system capable of supplying this emerging metabolic demand most likely involves both genetic and environmental components. Many of the previously described genes involved in vascular development have been shown to be responsive either directly or indirectly to hypoxia. These include VEGF, flk-1, flt-1 [110–114], PDGFβ, TF [115], bFGF, and possibly TIE-2 [116]. In addition, VEGF, GLUT-1, and many of the hypoxia-responsive genes are also stimulated by low glucose concentrations [117, 118]. Glucose levels within the uterine horn have been measured around 1 mM [119], well below the amounts used in standard in vitro culture media, and thus potentially acting in accord with low oxygen levels as a stimulus for the expression of these genes.

A critical test of the developmental importance of transcriptional responses to environmental stresses would involve inactivation of the transcriptional machinery mediating this response. As mentioned previously, ARNT forms one-half of the HIF-1 complex. Arnt-deficient hepatoma cell lines are impaired in their ability to induce hypoxic gene expression [120]. In vitro culture of embryonic stem cells lacking both copies of the ARNT gene indicates that not only is this transcription factor required to activate hypoxia responsive genes, it also mediates the transcriptional response to hypoglycemia [115]. In addition, $Arnt^{-/-}$ embryos exhibit an early embryonic lethality with no viable embryos detected past day E10.5 due to the impaired formation of intra- and extraembryonic vasculature. While vasculogenesis seems unaffected, as indicated by proper formation of the dorsal aortae and blood islands lined by endothelial cells in the yolk sac, angiogenesis is impaired. The yolk sac vascularization defect of $ARNT^{-/-}$ embryos is similar to VEGF, TIE-2, and TF knockout mice. $ARNT^{-/-}$ embryos exhibit decreased VEGF expression and the

primitive vessels that form lack proper supporting pericytes/smooth muscle cells as observed in $TF^{-/-}$ mice. While TF expression has been shown to be responsive to low oxygen tension [115], this may be an indirect result of increased VEGF expression [121]. $Arnt^{-/-}$ embryos also exhibit decreased vascularity of growing solid structures such as the branchial arch. Thus, the inability to respond to a condition normally associated with pathologic stress, namely hypoxia, leads to embryonic lethality in the mouse, primarily due to impaired vascular development.

Vascular Development: An Overview

An emerging view of vascular development can be summarized for the yolk sac as follows (Fig. 2). Primitive endothelial and hematopoietic progenitors are generated from mesodermal precursors and migrate to populate the yolk sac at embryonic day E7.5. This process requires the activity of VEGF receptor 2/flk-1, although not VEGF itself. Foci of hematopoietic activity called blood islands emerge within the mesodermal layer of the yolk sac with each such island lined by dividing endothelial cells. The increasing blood mass within these structures is distributed across the surface of the yolk sac by the concurrent growth and sprouting of endothelial cells, leading to the fusion of blood islands and the formation of a primitive vascular network consisting of immature capillaries. This process, which is complete by day E8.5, is collectively referred to as vasculogenesis.

At this point the process of remodeling begins. This primitive plexus that is unable to provide any effective means of O_2 distribution is transformed into a proper vascular network consisting of both arterial and venular components. The growing vessels require structural support in the form of pericytes and smooth muscle cells which are recruited to the endothelial cells by growth factors such as PDGFβ. The activity of the TIE-2 receptor tyrosine kinase is required for this remodeling, being stimulated by the competing activities of angiopoietin-1 and angiopoietin-2. Throughout the yolk sac VEGF is expressed and promotes the growth and survival of the endothelial cells and, along with angiopoietin-2, maintains the endothelial cells in a plastic state. The vasculature is also quite leaky at this point, allowing the extravasation of components of the coagulation cascade such as TF and thrombin, acting to further recruit support cells. Interaction between the recruited pericyte and the endothelial cell leads to the liberation of TGFβ and the subsequent maturation of the pericyte into a smooth muscle cell and the deposition of extracellular matrix components. The entire embryo at this point is situated in a hypoxic atmosphere, thus activating the HIF-1 complex throughout, and thus either directly or indirectly affecting the expression of most of the aforementioned factors. The inability to respond to low O_2 tensions due to an inactivation of the HIF-1 complex leads to an inability to remodel the early vascular plexus similar to that observed in VEGF, TF, and TIE-2 deficient mice.

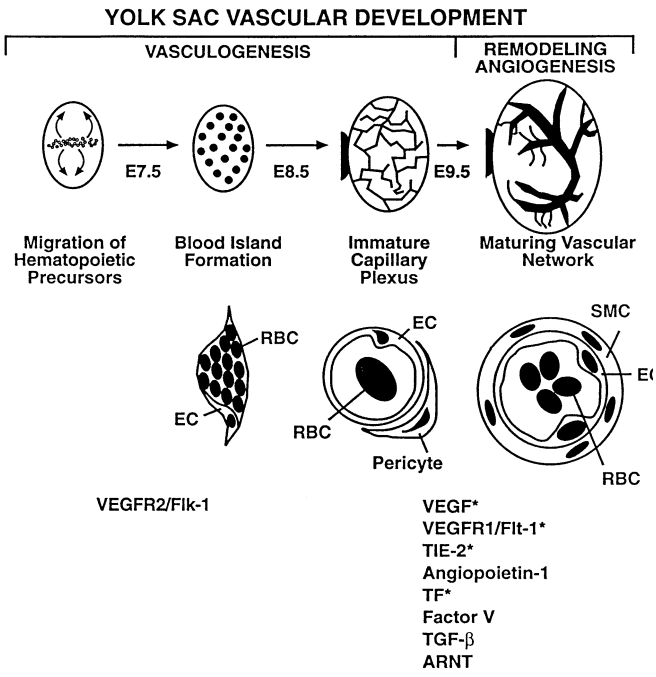

Fig. 2 Formation of the initial capillary plexus involves the differentiation and migration of endothelial/hematopoietic precursors to form blood islands throughout the yolk sac. Indicated here are a cluster of primitive red blood cells along with a representative endothelial cell. Continued growth and sprouting of endothelial cells enables the increasing blood mass within blood islands to be distributed throughout this structure. Eventual fusion of blood islands results in the formation of an irregular vascular plexus composed exclusively of capillaries supported by occasional pericytes. These initial vasculogenic events are followed by the remodeling of this vascular network to form a proper distribution system containing both arterial and venular components. The process of angiogenesis which involves the sprouting of new vessels can only occur after the production of early vascular structures via vasculogenesis. While only one gene is thus far known to be required for vasculogenesis (VEGF receptor 1/Flk-1), multiple genes are required for the subsequent maturation of the vascular tree. Many of these are also known to be regulated by physiological factors such as oxygen and glucose concentrations (*asterisk*). Each gene is listed in the time-line where its expression is critical for continued development

Upon formation of the uteroplacental circulation and the subsequent interdigitation of maternal and fetal vessels in the placenta, maternal oxygen becomes available to the embryo. The hypoxic environment of the early conceptus is thought to play an important role in the development of the placenta. The ability of cytotrophoblast cells to invade the uterine vessels has been shown to be dependent on oxygen tension, as organ culture of anchoring villi obtained from early gestation placentas indicates that culture under 20% O_2 promotes the differentiation and inhibits the proliferation of these cells, whereas culture under 2% O_2 has the opposite effect [122]. These results suggest that the cytotrophoblast cells, stimulated by the hypoxic nature of the placenta, continue to proliferate and move through the placenta until invading uterine arteries where, encountered by the higher O_2 concentrations of arterial blood, they cease dividing and differentiate to establish the uteroplacental circulation. The availability of maternal oxygen to regions

of the developing embryo results in the decreased expression of angiogenic agents such as VEGF and bFGF, resulting in the regression of unnecessary vessels in these areas. The ability of differences in the distribution of O_2 to serve as a prime stimulus in this remodeling process has been demonstrated for the developing retinal vasculature [123–125]. Additionally, hypoxia-induced expression of VEGF in the developing kidney has been shown to be instrumental in its vascularization and growth, as rat metanephric organ cultures maintained in 3% O_2 exhibit enhanced growth and greater numbers of tubules and blood vessels when compared with similar explants cultured under 20% O_2 [126]. The beneficial effects of hypoxic culture are abrogated by addition of anti-VEGF antibodies. Finally, the ability of cultured endothelial cells to form capillary networks in response to prolonged exposure to hypoxia but not when cultured under 20% O_2 [127], along with the ability of a recently described homolog of HIF-1α expressed in endothelial cells, EPAS1, to activate the TIE-2 promoter in response to hypoxia [116], provide additional evidence for the importance of oxygen sensing mechanisms in vascular development.

Growing solid structures within the embryo may be vascularized via angiogenic processes similar to those observed during tumor angiogenesis. Increasing tissue mass results in the exposure of cells located inside the mass to lower O_2 concentrations than those located peripherally. As mentioned above, this is a prime stimulus for the liberation of VEGF by growing tumors. Vessels also supply nutrients to growing tissues. Diminished glucose levels have been shown to induce VEGF, along with GLUT-1, expression in the C6 glioma cell line and multicellular spheroids generated from them [117, 128]. These investigators also showed that differences in the availability of oxygen and glucose in regions of growing tumors results in the differential expression of these genes, suggesting that gradients of oxygen and nutrient levels within growing tumors or tissues can act as morphogens to enhance their vascularization. We have shown that the transcriptional response to hypoglycemia is also mediated by the ARNT transcription factor, suggesting that the oxygen and glucose sensing and response pathways converge to activate a common set of genes mediated by this protein [118].

Vascular development can now be viewed as a process which relies on both genetic and physiological components. The development of cell types required for the formation of blood vessels and the generation of signaling molecules and their receptors allowing for their interaction occurs via fundamental genetic processes. The formation of a complex vascular network able to supply the metabolic needs of a rapidly growing embryo, however, must be a plastic process guided by changing physiological parameters. It is in part via the activity of an oxygen/nutrient sensing and response machinery that the vasculature is able to accomplish this feat.

Oxygen, genes, and development: bridging physiology and genetics

Study of the vascularization of developing mammalian embryos has yielded a wealth of information in a short period of time. One of the most exciting outcomes of these studies has been a bridging of the gap between physiology and genetics. A fundamental function of blood vessels is to provide nutrients and oxygen to metabolically active tissues. This is true for both normal tissues and growing tumors. The efficiency with which blood vessels accomplish this is worthy of note. The vasculature is able to supply the entirety of an organism with required metabolites by occupying no more than 5% of its volume. By application of a fractal-like repeated branching pattern, blood vessels are able to cover the greatest volume with the least expenditure of energy and greatest conservation of space. In fact, a recent mathematical model which describes allometric scaling laws across species with remarkable precision utilizes as its foundation a distribution network consisting of just such a series of branching tubes that regularly decrease in diameter [129]. This model thus describes not only the vasculature of higher organisms but also the system of tracheal tubes which deliver oxygen to the tissues of fruit flies. It is thus interesting to note that disruption of the homolog of the hypoxia inducible factor gene in *Drosophila*, known as *trachealess*, leads to the disruption of this network, akin to the observed vascular disruption in ARNT-deficient mouse embryos [130]. Additionally, *Drosophila* SL2 cells have been shown to contain a hypoxia-inducible protein complex capable of binding the hypoxia response element contained within the 3' enhancer of the human EPO gene [131]. Thus, the universal dependence on oxygen as the terminal electron acceptor in the mitochondria of organisms which supply their energetic needs via oxidative phosphorylation has resulted not only in the utilization of similar oxygen distribution systems, but also in the conservation of oxygen sensing and hypoxia response mechanisms.

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