Published in final edited form as:

Stem Cells. 2010 April ; 28(4): 799–809. doi:10.1002/stem.316.

Hypoxia influences the vascular expansion and differentiation of embryonic stem cell cultures through the temporal expression of VEGF- receptors in an ARNT-dependent manner

Yu Han^{a,†}, Shu-Zhen Kuang^{b,†}, Alla Gomer^a, and Diana L. Ramirez-Bergeron^{a,*}

^a Case Cardiovascular Research Institute and University Hospitals Harrington-McLaughlin Heart & Vascular Institute, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

^b Department of Pathology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

Abstract

Adaptive responses to low oxygen (O₂) tension (hypoxia) are mediated by the heterodimeric transcription factor Hypoxia Inducible Factor (HIF). When stabilized by hypoxia, bHLH-PAS α - and β -(HIF-1 β or ARNT) HIF complex regulate the expression of multiple genes including vascular endothelial growth factor (VEGF). In order to address the mechanism(s) through which hypoxia contributes to blood vessel development, we utilized embryonic stem cell (ESC) differentiation cultures that develop into embryoid bodies (EBs) mimicking early embryonic development. Significantly, low O₂ levels promote vascular development and maturation in wild type (WT) ESC cultures measured by an increase in numbers of CD31⁺ endothelial cells (ECs) and sprouting angiogenic EBs but refractory in *Arnt*^{-/-} and *Vegf*^{-/-} ESC cultures. Thus, we propose that hypoxia promotes the production of ECs and contributes to the development and maturation of vessels. Our findings further demonstrate that hypoxia alters the temporal expression of VEGF receptors Flk-1 (VEGFR-2) and the membrane and soluble forms of the antagonistic receptor Flt-1 (VEGFR-1). Moreover, these receptors are distinctly expressed in differentiating *Arnt*^{-/-} and *Vegf*^{-/-} EBs. These results support existing models wherein VEGF signaling is tightly regulated during specific biological events, but also provide important novel evidence that, in response to physiological hypoxia, HIF mediates a distinct stoichiometric pattern of VEGF receptors throughout EB differentiation analogous to the formation of vascular networks during embryogenesis.

Keywords

HIF; ARNT; hypoxia; endothelial cells; vasculogenesis; angiogenesis; VEGF; Flk-1; Flt-1; embryonic stem cells; embryoid bodies

INTRODUCTION

Mammalian embryonic development unfolds under conditions of limiting O₂ levels and poor nutrient delivery, and in order to survive, embryos must adapt to these environmental

*Corresponding Author: 2103 Cornell Rd Rm. 4-532, Cleveland, OH 44106, TEL: 216-368-2036, FAX: 215-368-0556, dlr36@case.edu.

[†]These author's contributed equally to this paper

AUTHOR CONTRIBUTION: Yu Han: Collection and assembly of data, data analysis and interpretation.

Shu-Zhen Kuang: Collection and assembly of data, data analysis and interpretation.

Alla Gomer: Collection of data

Diana L. Ramirez-Bergeron: Conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing

constrains. Ultimately, the early formation of the vascular system is essential to the embryo as it provides factors to promote further growth. Vascular development is believed to originate with the emergence of progenitor cells (hemangioblasts) that further differentiate into angioblasts and form a primitive vascular network through the process of vasculogenesis [1]. In subsequent angiogenic mechanisms, the cells undergo remodeling that includes pruning and sprouting, recruitment of supporting cells, and production of extracellular matrix proteins.

The Hypoxia Inducible Factor (HIF-1) is a transcription factor that is regulated by changes in O₂ tension and is responsible for upregulating multiple genes including those involved in glycolysis, cell proliferation and survival, erythropoiesis, and angiogenesis [reviewed in 2]. HIF-1 is a ubiquitously expressed heterodimeric transcription factor composed of members of the basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) family of proteins. In the presence of O₂, prolyl hydroxylases target the HIF- α (HIF-1 α , HIF-2 α) subunits for rapid proteosomal degradation. In contrast, as oxygen becomes limiting, HIF- α subunits stabilize, heterodimerize with constitutively expressed ARNT (Aryl hydrocarbon Receptor Nuclear Translocator, ARNT, HIF-1 β), bind to and transactivate multiple gene promoters containing hypoxia response elements (HREs). Not surprisingly, HIF-deficient mice die in utero with hematopoietic, vascular, placental, and cardiac defects [reviewed in 3].

As ARNT is the obligate HIF- β binding partner responsible for the regulation of hypoxia-specific gene expression in most cell types, the use of genetic models that target deletion of ARNT serve to test the consequences of HIF transcriptional inactivity in response to hypoxia. In previous analyses, we determined that the expression of ARNT in early differentiating ES cell cultures enhances the expression of Vascular Endothelial Growth Factor (VEGF), numbers of Flk-1⁺ (VEGF-R2, KDR) cells, and the formation of hemangioblast precursor cells in response to hypoxia [4]. Furthermore, using mouse embryonic Paraaortic-Splanchnopleural explant cultures we demonstrated that ARNT is necessary for proper hematopoietic, vasculogenic, and angiogenic intraembryonic processes [5]. However, the precise mechanistic role of HIF's downstream effects in response to hypoxic embryonic environment during the emergence and maturation of functional vessels remains unclear.

The aim of this study was to discern the effects of ARNT and hypoxia in the development and maturation of the vasculature. To overcome inherent difficulties in analyzing the biological and molecular consequences to in vivo changes in O₂ tension during embryogenesis, we employed mouse embryonic stem cells (ESCs). ESCs can be differentiated into embryoid bodies (EBs), resembling early embryogenesis and facilitate the analysis of reduced O₂ levels in the programming of particular developmental pathways. EBs can mimic embryonic vascular development whereby around day 3 of EB differentiation, early vasculogenesis is evident by presence of hemangioblast precursors expressing Brachyury transcription factor and Flk-1 receptor [6,7,8]. The EBs further mature and express endothelial cell (EC) markers [9]. As during embryogenesis, the vessels in EBs undergo vascular remodeling beginning at Day 6. When replated in 3D Collagen Type I gel matrix, angiogenic potential can be characterized and quantified by the outgrowth and sprouting of ECs [10,11].

In this study, we examine the influence of hypoxia on the angiogenic potential of differentiating ESC cultures. *Arnt*^{-/-} ESCs cells provide an important genetic tool to examine the requirements for HIF transcriptional activity in vessel outgrowth and sprouting. In addition, as VEGF is believed to play a critical role in EC proliferation, differentiation, and sprouting, we examined the direct role of hypoxia independent of VEGF utilizing *Vegf*^{-/-} ES cells [12]. Our data indicate that hypoxia promotes angiogenesis in a VEGF independent manner and that HIF transcriptional activity is important for this process. We show distinct requirements for VEGF in that while *Vegf*^{-/-} ES cell cultures are refractory to hematopoietic defects, they display angiogenic deficiencies similar to *Arnt*^{-/-} cultures [13]. Also, our results demonstrate

that hypoxia temporally regulates the expression of Flk-1 and the antagonistic receptor Flt-1 (VEGFR-1) in the developing EB. We conclude that hypoxia promotes vascular development in part by regulating VEGF signaling through multiple mechanisms linked to the temporal alteration in the expression of VEGF receptors.

MATERIALS AND METHODS

ESC culture and differentiation

The generation and maintenance of *Arnt*^{-/-}, *Vegf*^{+/-}, and *Vegf*^{-/-} ES cells have been previously described [14–17]. ESCs were then differentiated into suspension EBs as described [7,18]. Collagen differentiation of EBs has also been described [19].

To examine vascular differentiation, EBs were differentiated in methylcellulose (M3131, SCT) as previously described with a few modifications [6]. Briefly, ES cells were trypsinized, washed, and resuspended in 10% IMDM. Cells ranging from 1000–5000 in number were mixed in 1% methylcellulose containing 15% FBS (SCT), 450 μ M monothioglycerol (MTG), 1% L-glutamine, 1% penicillin-streptomycin and various concentrations of bFGF or VEGF (Pharmingen) in a final volume of 1.5 ml and incubated under either 21% or 3% O₂ for a period of 7–14 days. At Day 6, cultures were supplemented with an additional 1 ml of the methylcellulose mixture.

In order to quantify the angiogenesis, the differentiated EBs were replated in Collagen. Briefly, after dissolving the methylcellulose and washing in PBS at 37° C, EBs were then enumerated and triplicate cultures containing about 50–100 EBs were mixed in 1.25 ml of 1.2 mg/ml of type I Collagen (BD Biosciences, Bedford, MA) containing 15% FBS (Gemini), 450 μ M MTG, 5 ng/ml VEGF, 25 ng/ml bFGF, and 10 μ g/ml insulin in IMDM (Invitrogen). Finally, 1 N NaOH was added to polymerize the cultures in 35-mm bacterial dishes (SCTs). After 4 days, EBs were individually scored for the extent of angiogenic outgrowth and sprouting and data was assessed using a Student *t*-test.

For hematopoietic progenitor cell analysis, ESCs were differentiated as previously described [15]. D9 EBs were disassociated with Collagenase (SCT) and 15,000 were replated in M3434 methylcellulose media (SCT). CFCs were scored after 6 days of culture.

Gene Expression Analysis

RNA was isolated by the TRIzol method (Invitrogen). After treatment with DnaseI (Invitrogen), reverse transcription was performed with Superscript-II reverse transcriptase (Invitrogen) using supplied oligo dT primers. Real-time detection PCR (RTD-PCR) was performed as previously described [20]. Reactions were carried out in a 15 μ l volume with 2x Taqman Master Mix (ROCHE) with using specific ROCHE Universal probes. Primer sequences are listed in the Supplemental Table 1.

Immunostaining

Collagen gels were transferred onto glass slides and dehydrated with adsorbent cards (SCTs), fixed with 4% paraformaldehyde for 30 minutes, washed with PBS, and blocked in 1% BSA, 0.1% Triton X-100 in PBS for 2 hours. Slides were incubated overnight at 4° C with 1:250 anti-CD31 (MEC13.3, Pharmingen) or for 1 hour at room temperature with 1:500 anti-SMA () or 1 μ g/ml of anti-NG-2 in blocking buffer. After three washes, samples were incubated with secondary immunofluorescent conjugated antibodies (Pharmingen, or Invitrogen) and mounted with DAPI containing mounting media (Vector Labs). Alternatively, biotinylated anti-rat IgG antibody (Pharmingen) was used with DAB kit (Vector Labs).

Flow cytometry

EBs were dissociated in 0.25% collagenase to obtain single cell suspensions. Cells were then blocked with CD16/32 for 10 minutes before staining with 1:200 FITC-CD31, 1:50 PE-Flk-1 or 1:100 FITC-mFlt1 (BD Biosciences) in PBS containing 1% BSA on ice. After washing, surface expression was detected by flow cytometry (BD LSR I) and analyzed using FloJo software (Tree Star).

ELISA

Cell lysates or suspension media were used to quantify sFlt-1 as described by the manufacturer (Biosciences).

RESULTS

Hypoxia promotes EC expansion and vascular gene transcription

In order to evaluate the requirements of ARNT for angiogenesis in a system that emulates embryonic vascular development, mouse ESCs differentiation cultures were employed. In the initial characterization of angiogenic events, aggregated ESCs from drop cultures were differentiated with reduced growth factor and serum conditions in suspension into cystic EBs (CEB) or Collagen Type I matrix (Figure 1A and B). Vessel maturation was observed in the majority of the developing WT EBs with the ability to differentiate into vessel- containing CEBs in suspension and into CD31⁺ ECs that emerge, sprout, lumenize, and shown to recruit supporting cells in 3- dimensional Collagen cultures (Figure 1B upper panels). In contrast, under these limiting growth conditions, *Arnt*^{-/-} ES cells failed to form CEBs and sprouting vessels consistent with a vascular deficiency phenotype (Figure 1B, lower panels).

We had previously described that hypoxia promotes the early emergence of Flk-1⁺ cells associated with the early temporal presence of hemangioblast progenitor cells in suspension EB cultures [4]. We first examined by western blot analysis that chronic exposure to physiological levels of 3% O₂ induced the accumulation of HIF-1α in EB cultures (Figure S1). To assess the effects of hypoxia on the differentiation and proliferation of ECs in EB cultures, the surface expression of CD31 (PECAM) EC cell marker was quantified by flow cytometry. CD31^{lo} and CD31^{hi} cells from methylcellulose and suspension EB cultures in the absence of exogenous VEGF were analyzed over 5–9 days of differentiation. Hypoxia promoted the expression of CD31 in both culture conditions, but interestingly, suspension cultures promoted the earlier production and an increase in numbers of CD31⁺ cells (Figure 1C). Specifically, hypoxia induced the early expression of CD31^{lo}, followed by the induction of CD31^{hi} cell numbers in WT EB (Figure 1C). In contrast, while the percent of CD31^{lo} cell numbers increased over time in either *Arnt*^{-/-} EB culture conditions, the numbers of CD31^{hi} cells were greatly reduced (7–12% for methylcellulose and 2% to 12% for suspension) compared to WT control (2% to 25% for methylcellulose and 11– 40% for suspension) cultures (Figure 1C). Moreover, hypoxia either doubled (18% for normoxia and 40% for hypoxia) or tripled (8% for normoxia and 24% for hypoxia) the peak production of CD31^{hi} cells in WT suspension or methylcellulose cultures, respectively. Under these conditions, no significant numbers of Annexin V⁺ apoptotic cells were detected by flow cytometry suggesting that the phenotypes of *Arnt*^{-/-} EBs are not due to decreased cell survival.

To begin to characterize the molecular events that result from hypoxia stimulated vascular development, the expression of various vascular markers was examined by real-time PCR. WT and *Arnt*^{-/-} EBs were differentiated under normoxic and hypoxic (3% O₂) conditions for 7 and 10 days and cDNA was used to amplify transcripts including a known HIF target Adrenomedullin (ADM) whose levels are enhanced in hypoxic WT cultures (Figure 1D). VEGF mRNA levels were comparable between normoxic and sustained hypoxic treatment.

By Day 7 of differentiation, WT cells displayed elevated *Angiopoietin-1* [*Ang-1* (3.0X)], *Angiopoietin-2* [*Ang-2* (10X)], *Flk-1* [*VEGFR-2*, *KDR* (2.5X)], *Tie-2* (4X) and *Tie-1* (5X) transcript levels. In Day 10 EBs, the expression of *EPO* (3.5X), *Tie-2* (1.5X) and *Tie-1* (1.8X) message levels in WT cultures were also induced by hypoxia. The overall transcript levels of angiogenic genes were greatly reduced in *Arnt*^{-/-} EBs. Of interest, independent of ARNT the Tie receptors were induced by hypoxia in D7 EBs although significant differences were indistinguishable in D10 *Arnt*^{-/-} EBs. Taken together, these results show that in an EB differentiation system hypoxia treatment promotes vascular development in an ARNT dependent manner as measured by the expression of CD31 and select blood vessel gene profile.

Hypoxia promotes the emergence of highly angiogenic EBs

To quantitatively assess hypoxia's role in sprouting angiogenesis, an established angiogenesis assay was used to differentiate ESCs into 14 Day EBs in methylcellulose containing 25 and 100 ng/ml of exogenous VEGF and bFGF, respectively [21]. Angiogenic competence was measured by replating the EBs in 3D Collagen Type I gel matrix for 4 days and evaluating the extent of vessel length and sprouting of individual EBs (Figure 2A, upper panel). From these EBs, the outgrowth of sprouting endothelial tubes was further characterized by staining for SMA⁺ or NG2⁺ pericytes (Figure 2A, middle panels). Following this protocol, the requirement of HIF transcriptional activity for angiogenic growth was assessed by contrasting WT and *Arnt*^{-/-} ESCs differentiated in either normoxic (21% O₂) or physiological hypoxic (3% O₂) conditions. The replated EBs were individually categorized into least (Type A) to most (Type E) angiogenic with Types B and C (Low angiogenic) having few emergent ECs while Types D and E (High angiogenic) displaying significant numbers of vessels infiltrating the collagen with profuse sprouting (Figure 2A, lower panels). Since we observed no measureable differences between normoxia and hypoxia in 14 day EBs, the differentiation period was reduced to 10 days revealing a significant increase of angiogenic WTs EB differentiated under hypoxic (59%) compared to normoxic (37%) conditions (Data not shown, Figure 2B). In contrast, *Arnt*^{-/-} ES cultures contain reduced numbers of angiogenic EBs under either condition (Figure 2B). These results show that hypoxia promotes more rapid vascular differentiation.

To further optimize our experimental system, the concentration of exogenous growth factors was lowered to 25 ng/ml bFGF with either 5 (Figure 2C) or 0 (Figure 2D and E) ng/ml of exogenous VEGF. As a result, replated Day 7 WT EBs differentiated with 5 ng/ml displayed elevated numbers of highly angiogenic EBs (Types D and E) induced by hypoxia compared to normoxia conditions (Figure 2C). In the absence of exogenous VEGF, hypoxia significantly induced numbers of highly angiogenic EBs in WT EB cultures that were further expanded by day 10 of differentiation (Figure 2D). Phenotypically, no discernable differences were observed between control and VEGF treated WT EBs differentiated under 3% O₂ (Figure 2E). In contrast, in cultures treated with 5 ng/ml VEGF, *Arnt*^{-/-} EBs failed to generate highly angiogenic EBs or demonstrate significant hypoxic effects (Figure 2C). While in the absence of VEGF *Arnt*^{-/-} Day 7 EBs showed an increase in numbers of low angiogenic EBs in response to hypoxia, the numbers of highly angiogenic EBs were significantly reduced in both Day 7 and 10 cultures (Figure 2D). Thus, these findings demonstrated that chronic hypoxia promotes angiogenic sprouting in an ARNT- dependent manner.

Exogenous VEGF is not sufficient to rescue the angiogenic defects in *Arnt*^{-/-} EBs

It has been debated whether differing degrees of VEGF signaling affect specific aspects of vessel development [22–24]. Since VEGF is significantly regulated by HIF, experiments were undertaken to examine the potential for VEGF to rescue and promote angiogenesis in *Arnt*^{-/-} EB cultures and to test whether hypoxia alone promotes vascular differentiation. Day 7 and Day 10 EBs cultures were supplemented with 25, 5, or 0 ng/ml VEGF. In Day 7 EBs,

hypoxia promoted the differentiation of angiogenic EBs (EB Types C, D, and E) in WT cultures in the absence of exogenous VEGF (Figure 2F). Furthermore, by Day 10 of differentiation, while overall numbers of angiogenic EBs were reduced in WT cultures with no VEGF, hypoxia significantly increase (16%) highly angiogenic EBs compared to control normoxic conditions (6%) (Figure 2F). Titration experiments further revealed that addition of 25 ng/ml of exogenous VEGF was not sufficient to promote further angiogenesis in *Arnt*^{-/-} EB cultures. Surprisingly, the percentage of highly angiogenic EBs in *Arnt*^{-/-} cultures never exceeded 10%. Therefore, since VEGF did not rescue *Arnt*^{-/-} EBs, we conclude that dysregulation of other important vascular growth factors, including Angiopoietins-1 and 2, might contribute to the vascular phenotype as a result of HIF deficiency (Figure 1C).

Hypoxia promotes angiogenic outgrowth and sprouting in collagen

To test whether hypoxia could affect the extent of outgrowth and sprouting of vessels, replated EBs were further exposed to two differing O₂ tensions (Figure 3A). Exposure of *Arnt*^{-/-} EBs to hypoxia during their replating into Collagen did not promote further sprouting (Figure 3B). In contrast, hypoxia promoted angiogenic outgrowth from differentiated WT EBs in the Collagen matrix, the sprouting phase of the experiment (Figure 3B). Furthermore, hypoxic stimulation during differentiation and vascular growth stages resulted in an enriched percentage of highly angiogenic WT EBs. These results suggest that 1) hypoxia stimulates vascular differentiation of EBs by enhancing their angiogenic potential, and 2) hypoxia promotes angiogenesis by increasing the numbers of EBs eliciting vessel outgrowth and sprouting.

Hypoxia promotes EB vascular differentiation independent of VEGF

To assess a requirement for VEGF in hypoxia induced EB vascular differentiation, soluble Fc-Flt1 was used in EB methylcellulose cultures to compete with endogenous VEGF receptors thereby inhibiting VEGF activity [25]. The addition of 1 µg/ml Fc-Flt1 (solid blue line) during normoxic differentiation of WT EBs substantially reduced the outgrowth of angiogenic EBs relative to cultures treated with 0.25 µg/ml (stippled blue line) of the inhibitor (Figure 4A). The numbers of angiogenic EBs were also reduced in hypoxic cultures treated with 1 µg/ml (solid red line) compared to 0.25 µg/ml (stippled red line) of Fc-Flt1. Yet, hypoxia protected the EBs from the antagonizing effects of Fc-Flt1 as the percentage of angiogenic EBs (Types C, and/or D in particular) remained significantly higher compared to normoxic conditions (Figure 4A).

SU5416, a potent and selective inhibitor of Flk-1 receptor tyrosine kinase activity, was also employed to downregulate VEGF signaling [25]. While there was no consequence to the numbers of low angiogenic EBs by hypoxia as a result of SU5416 treatment, a statistically significant reduction of highly angiogenic EBs from Day 10 WT hypoxic cultures was observed (Figure 4B and C). In contrast, no vessel growth or sprouting was observed with the exclusive addition of SU5416 to the Collagen matrix (Data not shown). Taken together, extrinsic and intrinsic inhibition of VEGF reduced but did not inhibit vascular development in EB cultures in response to hypoxia. These observations further suggest that while intracellular VEGF signaling is required for EC outgrowth and sprouting of replated EBs, enhanced angiogenic differentiation by hypoxia is not exclusively mediated through VEGF.

Vegf^{-/-} ES cells generate hematopoietic progenitor colonies but are angiogenic deficient

We previously determined that while Flk-1⁺ ES cells numbers present in Day 3 *Vegf*^{-/-} EB cultures can be induced by hypoxia, the numbers of hemangioblast progenitor colonies were limited compared to WT cultures [4]. Since VEGF-A plays a critical role during embryonic development, we tested *Vegf*^{-/-} mESCs in differentiation conditions that promote either hematopoietic or vascular differentiation [14]. Using clonogenic hematopoietic assays, ES cells were differentiated into 9 Day EBs, dissociated into single cell suspension by collagenase

treatment, and replated in methylcellulose with a cocktail of growth factors for 6 days. The hematopoietic potential from these EBs were evaluated by categorizing and quantifying hematopoietic progenitor colony forming units (CFU's). Under these conditions, *Vegf*^{+/-} and *Vegf*^{-/-} EBs phenocopied WT control cultures, effectively generating all types of hematopoietic progenitor colonies (Figure 4D). This contrasts with *Arnt*^{-/-} ES cells which have been reported to ineffectively generating multiple hematopoietic colony types [13].

Vascular development was first assessed by quantifying CD31^{lo} and CD31^{hi} cell numbers derived from methylcellulose and suspension EB cultures (Figure 1C). Hypoxia promoted the temporal upregulation of CD31^{hi} expression in *Vegf*^{-/-} EBs, albeit numbers were distinctly reduced compared to WT cultures. Next, the angiogenic capacity of *Vegf*^{-/-} EBs was analyzed by replating in 3D collagen gels. In the absence of exogenous VEGF, *Vegf*^{-/-} and *Arnt*^{-/-} ES cultures inadequately generated sprouting EBs, even in response to hypoxic stimulus (Figure 4E). Collectively, these results suggested that EBs have specific requirements for VEGF during the differentiation of progenitor, blood, and vascular cells.

Hypoxia influences the temporal expression of VEGF receptors

Distinct VEGF receptors are functionally associated with VEGF-A in ECs whereby Flk-1 and Flt-1 are considered positive and negative regulators of VEGF signaling, respectively [26]. Using suspension cultures, we previously showed that Flk-1 levels are transiently induced in early differentiating Day 2.5–3 WT EBs (during the emergence of early progenitor cells which we also measured as hemangioblast colonies, BL-CFCs) in response to hypoxia and that *Arnt*^{-/-} EB cultures are Flk-1⁺ cell deficient [4]. In the present study, we examined the levels of VEGF receptors from angiogenic EB cultures during later time points when they are considered to be actively angiogenic [27] (Figure 5A). Suspension EB cultures were also employed since they permit analyses of soluble molecules from conditioned media. Single cell suspensions obtained from collagenase treated EBs were used to evaluate the surface expression of Flk-1 and mFlt-1 (Figure 5B). In more mature suspension WT EBs, Flk-1 expression ranged from 2% at D5 to 7% by D9 of differentiation and with a maximum 6% percent of double positive (DP) Flk-1⁺mFlt-1⁺ cells. In contrast, single positive (SP) mFlt-1⁺ cell numbers were transiently induced by hypoxia in WT EBs with a peak level (57%) achieved by D7 of differentiation and tapering in D9 EBs. The numbers of SP Flk-1⁺ cells in *Arnt*^{-/-} EB cultures peaked with 18% and 45% at Day 5 of differentiation for normoxia and hypoxia, respectively. A high percentage of Flk1⁺ cells (SP and DP combined) were detected in *Arnt*^{-/-} cultures (ranging from 46% to 17%) relative to WT cultures (< 13%). In contrast, a delay in the emergence of SP or DP mFlt-1⁺ cells was observed, and levels remained below those seen for hypoxic WT EBs. (Figure 5B, upper panels).

Hypoxia's effect on the expression of VEGF receptors from EBs differentiated in methylcellulose in the absence of exogenous VEGF was also evaluated. In a time dependent manner, the expression of either DP Flk-1⁺mFlt-1⁺ cells or SP mFlt-1⁺ cells in WT cultures was measured. Consistently, hypoxia treatment increased the expression of VEGF receptors under these differentiation conditions. The percent number of cells for normoxia and hypoxia in WT cultures were respectively: Day 5, SP Flk-1⁺ 3% and 17%; Day 7, total mFlt-1⁺ 19.6% and 46%; and Day 9 total number of cells expressing either Flk-1 and/or Flt-1 were 42% and 50% (Figure 5B, lower panels). In contrast, a distinct expression pattern for VEGF receptors in *Arnt*^{-/-} EB cultures was observed. First, the relative levels of SP Flk-1⁺ cells were significantly higher in *Arnt*^{-/-} than WT EB cultures in both normoxic and hypoxic Day 5 cultures (42% and 26%, respectively) and maintained in Day 7 cultures (22% and 23%, respectively). Second, mFlt-1⁺ cell numbers were overall reduced (3–20%) and no significant differences between normoxia and hypoxia were observed. In contrast, SP or DP Flk-1⁺ cell numbers were distinctly different between both culture conditions.

In order to examine the presence of sFlt-1, conditioned media from EB suspension cultures were tested by ELISA. While sFlt-1 levels were induced by hypoxia in a time-dependent manner in WT cultures, there were no significant differences in sFlt-1 protein levels between normoxic and hypoxic *Arnt*^{-/-} EBs at Day 5 or 7, though concentrations were higher than supernatants from control WT normoxia cultures (Figure 5D). sFlt-1 levels from protein cell lysates obtained from D5–9 EBs differentiated in methylcellulose were also analyzed. A similar trend of hypoxic induction of sFlt-1 levels in WT EBs was observed. While the concentrations of sFlt-1 in *Arnt*^{-/-} EB cultures were increased over control WT normoxia cultures, the levels of cellular and supernatant sFlt-1 were inversely proportional in these mutant cultures. Surprisingly, more sFlt-1 was retained within the cells of *Arnt*^{-/-} EBs when compared to WT cultures. Thus, it appears that while hypoxia enhances the expression of sFlt-1 in an *Arnt* - independent manner, *Arnt* may be responsible for the formation of a sFlt-1 gradient by affecting the expression of mFlt-1 and retention/release of sFlt1.

To examine the contribution of VEGF to hypoxia-regulated expression of VEGF receptors, *Vegf*^{-/-} ES cells were differentiated in the absence of exogenous VEGF. While Flk-1⁺ cell numbers were relatively high at Day 5, overall numbers were progressively lower in hypoxia treated *Vegf*^{-/-} cultures (Figure 6A). Moreover, mFlt-1 cell numbers increased in a time dependent manner. Overall, the expression profile of VEGF receptors in *Vegf*^{-/-} EBs appeared intermediate of *Arnt*^{-/-} and WT cultures. sFlt-1 levels either from cell lysates or conditioned media were increased in hypoxia treated *Vegf*^{-/-} EB cultures (Figure 6B). Together, these results argue that HIF activity in response to physiological hypoxia contributes to the stringent control of the VEGF signaling pathway by influencing the temporal expression of VEGF receptors independent of VEGF.

DISCUSSION

We had previously described that hypoxia alters the kinetics and promotes the emergence of hemangioblast progenitor cells in early differentiating EBs [4]. Our present analyses demonstrate how HIF, in response to hypoxic cues, participates in the differentiation of the vasculature and angiogenic expansion using an ESC differentiation model that permits the quantification of vascular competence.

While VEGF is a critical transcriptional target of HIF, VEGF itself plays an important autocrine role in ECs during vascular development and survival [28]. Specific effects for VEGF have been extensively documented including its ability to induce EC and reduce hematopoietic differentiation [29–31]. While VEGF is required for the formation of blood vessels, its necessity for vessel maintenance differs between embryogenesis and adult homeostasis [14, 22,28]. We previously showed that early *Vegf*^{-/-} EBs expressed significant levels of Flk-1 inducible by hypoxia, though ineffectively generate hemangioblast colonies [4]. We now confirm reports that *Vegf*^{-/-} derived EBs fail to organize vascular networks and that while *Vegf*^{-/-} EBs have altered levels of VEGF receptors, our analyses demonstrate that hypoxia additionally affects their expression [32,33]. The results from our present study corroborate the concept that there are unique requirements for intrinsic VEGF, as *Vegf*^{-/-} EBs successfully generate hematopoietic progenitor cell colonies but are poorly angiogenic.

While competition (Fc-Flt1) or inhibition (SU5416) of Flk-1 during the differentiation of WT ES cultures affected angiogenesis, hypoxic treatment was nonetheless able to promote vascular differentiation. One plausible explanation is that the experimental parameters do not permit the adequate inhibitory effects. On the other hand, we observed that EBs treated with 1 µg/ml of Fc-Flt1 significantly inhibited vascular growth from normoxic cultures and that the addition of SU5416 inhibitor exclusively during the replating of EBs completely blocked outgrowth of vessels in WT cultures in both culture conditions. These results corroborate our previous

interpretation that reduced PECAM⁺ vessels observed in *Arnt*^{-/-} embryos may be due to limited numbers of endothelial and hematopoietic precursors and that hypoxic induction of VEGF is required for further development and maturation [5].

VEGF receptors are known to regulate EC proliferation, migration, and branching. HIF has been demonstrated to induce the transcription of Flk-1 and Flt-1 [34–36]. Moreover, exposure of in vitro human EC cultures to chronic hypoxia resulted in the downregulation of Flk-1 thus attenuating VEGF signaling activity [37]. By analyzing the expression of these receptors, our present study demonstrates that hypoxia can influence the levels of Flk-1, membrane- and soluble- Flt1. Indeed it appears that in a physiological hypoxic environment HIF initially upregulates the expression of Flk-1 in Day 2–3 WT EBs, and then in a reciprocal manner, downregulates its expression while “secreted” and “cellular” sFlt-1 levels increase. A recent ESC study describing hypoxia’s affect on the expression of sFlt-1 emphasized that the balance between Flk-1 and sFlt-1 directs the differentiation of hemangioblasts into ECs and hematopoietic progenitor cells, respectively [38]. In *Vegf*^{-/-} or *Arnt*^{-/-} genetic ESCs systems, we determined that hypoxia alters the expression of these receptors independent of VEGF or HIF, respectively, although in a pattern clearly distinct from WT cells. Moreover the overall stoichiometry of VEGF receptors is significantly delayed in *Arnt*^{-/-} cultures relative to WT cultures indicative of inadequate vascular differentiation.

The abundance of mFlt-1 and sFlt-1 can vary physiologically suggesting their importance during various vascular processes [24,26,27,39–41]. In recent observations in an angiogenic model of tumorigenesis, the inhibition of HIF degradation in *PHD2*^{+/Tie2Cre} mice promoted the ‘normalization’ of ECs by upregulating sFlt1 and Ve-Cadherin, which re-specify ECs to become quiescent [42]. sFlt-1 isoform modulates a VEGF – concentration gradient by affecting ligand availability correlating with patterns of Flk-1 activation in EBs [43]. In a previous analysis, the amount of Flt-1 mRNA from EB derived Flk-1⁺ cells was upregulated when cells were further differentiated and may explain the greater requirement for VEGF during the late phase of EC differentiation [23]. Indeed, we observed that in WT EB vascular cultures Flk-1⁺ cell numbers decreased relative to earlier differentiation phase of hemangioblast expansion while the inhibiting Flt-1 receptor increases in a time-dependent manner. Specifically, hypoxia expanded the numbers of mFlt-1⁺ or DP cells, and as vascular remodeling proceeded, sFlt-1 levels were also enhanced.

Thus, as a result of physiological hypoxia, HIF influenced the critical stoichiometric pattern of VEGF dose responsiveness during specific vascular events (Figure 7). A local gradient of sFlt-1 may function to direct the guidance of emerging and sprouting vessels consistent with the *Arnt*^{-/-} mouse embryonic phenotype featuring congested vessel growth and reduced sprouting [5,17,44]. ECs isolated from human hemangiomas (hemECs) are associated with increased GLUT-1 and VEGF, both important HIF transcriptional targets. Strikingly, the abundance of Flt-1 is significantly reduced in hemECs [45]. Since we observed opposing expression patterns for VEGF receptors during vascular differentiation of EBs, we suspect that hemECs that arise exclusively in neonates may develop as a consequence of ineffective responses to physiological hypoxia [46]. While we propose that a physiological hypoxic environment during embryogenesis directs HIF to regulate the temporal expression of VEGF receptors, a major challenge is to clarify how these events are orchestrated during the various morphogenic events in vessel generation that include proliferation, migration, sprouting, remodeling and survival of vascular cells contributing to our understanding of vascular pathologies.

CONCLUSION

The use of ESC differentiation cultures permitted us to demonstrate that hypoxia is able to promote the emergence of CD31⁺ ECs, hasten vascular development, and promote angiogenic outgrowth and sprouting. However in the absence of ARNT, and thereby ineffective HIF transcriptional activity, vascular development is delayed and significantly reduced in *Arnt*^{-/-} EBs. In our present model we consider that the transcriptional activity of HIF, in response to hypoxia, is important in effectively directing vascular development. In particular, in this study we report a novel mechanism of HIF's ability to moderate the temporal expression of VEGF receptors known impart either positive or negative effects on VEGF signaling, thus affecting the stoichiometric pattern of VEGF dose responsiveness critical to the formation of vascular networks. Further studies on the mechanisms of hypoxia-induced angiogenesis may define novel elements that control vascular development as well as illuminate new therapeutic approaches against pathological angiogenesis. Moreover, these experiments impart an important experimental tactic to promote the expansion of ECs for further experimental analyses and ultimately regenerative cell therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Andras Nagy for the *Vegf*^{+/-} and *Vegf*^{-/-} ES cells. We would also like to thank Aaron Proweller for critically reading this manuscript. This work was supported by the National Institutes of Health (HL-073153 D.R.B).

References

1. Risau W. Mechanisms of angiogenesis. *Nature* 1997;386:671–674. [PubMed: 9109485]
2. Semenza GL. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. *Physiology (Bethesda)* 2009;24:97–106. [PubMed: 19364912]
3. Simon MC, Liu L, Barnhart BC, et al. Hypoxia-induced signaling in the cardiovascular system. *Annu Rev Physiol* 2008;70:51–71. [PubMed: 17850210]
4. Ramirez-Bergeron DL, Runge A, Dahl KD, et al. Hypoxia affects mesoderm and enhances hemangioblast specification during early development. *Development* 2004;131:4623–4634. [PubMed: 15342485]
5. Ramirez-Bergeron DL, Runge A, Adelman DM, et al. HIF-dependent hematopoietic factors regulate the development of the embryonic vasculature. *Dev Cell* 2006;11:81–92. [PubMed: 16824955]
6. Vittel D, Prandini MH, Berthier R, et al. Embryonic stem cells differentiate in vitro to endothelial cells through successive maturation steps. *Blood* 1996;88:3424–3431. [PubMed: 8896407]
7. Choi K, Kennedy M, Kazarov A, et al. A common precursor for hematopoietic and endothelial cells. *Development* 1998;125:725–732. [PubMed: 9435292]
8. Chung YS, Zhang WJ, Arentson E, et al. Lineage analysis of the hemangioblast as defined by FLK1 and SCL expression. *Development* 2002;129:5511–5520. [PubMed: 12403720]
9. Vittel D, Buchou T, Schweitzer A, et al. Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc Natl Acad Sci U S A* 1997;94:6273–6278. [PubMed: 9177207]
10. Li X, Claesson-Welsh L. Embryonic stem cell models in vascular biology. *J Thromb Haemost* 2009;7 (Suppl 1):53–56. [PubMed: 19630768]
11. Jakobsson L, Kreuger J, Claesson-Welsh L. Building blood vessels--stem cell models in vascular biology. *J Cell Biol* 2007;177:751–755. [PubMed: 17535968]
12. Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. *Nature* 2005;438:937–945. [PubMed: 16355211]

13. Adelman DM, Maltepe E, Simon MC. Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. *Genes Dev* 1999;13:2478–2483. [PubMed: 10521392]
14. Carmeliet P, Ferreira V, Breier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;380:435–439. [PubMed: 8602241]
15. Adelman DM, Simon MC. Hypoxic gene regulation in differentiating ES cells. *Methods Mol Biol* 2002;185:55–62. [PubMed: 11769012]
16. Carmeliet P, Ferreira V, Breier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996;380:435–439. [PubMed: 8602241]
17. Maltepe E, Schmidt JV, Baunoch D, et al. Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature* 1997;386:403–407. [PubMed: 9121557]
18. Kennedy M, Firpo M, Choi K, et al. A common precursor for primitive erythropoiesis and definitive haematopoiesis. *Nature* 1997;386:488–493. [PubMed: 9087406]
19. Hermant B, Desroches-Castan A, Dubessay ML, et al. Development of a one-step embryonic stem cell-based assay for the screening of sprouting angiogenesis. *BMC Biotechnol* 2007;7:20. [PubMed: 17437635]
20. Seagroves TN, Hadsell D, McManaman J, et al. HIF1alpha is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. *Development* 2003;130:1713–1724. [PubMed: 12620994]
21. Feraud O, Cao Y, Vittet D. Embryonic stem cell-derived embryoid bodies development in collagen gels recapitulates sprouting angiogenesis. *Lab Invest* 2001;81:1669–1681. [PubMed: 11742037]
22. Argraves WS, Larue AC, Fleming PA, et al. VEGF signaling is required for the assembly but not the maintenance of embryonic blood vessels. *Dev Dyn* 2002;225:298–304. [PubMed: 12412012]
23. Hirashima M, Ogawa M, Nishikawa S, et al. A chemically defined culture of VEGFR2+ cells derived from embryonic stem cells reveals the role of VEGFR1 in tuning the threshold for VEGF in developing endothelial cells. *Blood* 2003;101:2261–2267. [PubMed: 12406893]
24. Wirzenius M, Tammela T, Uutela M, et al. Distinct vascular endothelial growth factor signals for lymphatic vessel enlargement and sprouting. *J Exp Med* 2007;204:1431–1440. [PubMed: 17535974]
25. Roberts DM, Kearney JB, Johnson JH, et al. The vascular endothelial growth factor (VEGF) receptor Flt-1 (VEGFR-1) modulates Flk-1 (VEGFR-2) signaling during blood vessel formation. *Am J Pathol* 2004;164:1531–1535. [PubMed: 15111299]
26. Shibuya M. Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *J Biochem Mol Biol* 2006;39:469–478. [PubMed: 17002866]
27. Li X, Edholm D, Lanner F, et al. Lentiviral rescue of vascular endothelial growth factor receptor-2 expression in flk1^{-/-} embryonic stem cells shows early priming of endothelial precursors. *Stem Cells* 2007;25:2987–2995. [PubMed: 17702981]
28. Lee S, Chen TT, Barber CL, et al. Autocrine VEGF signaling is required for vascular homeostasis. *Cell* 2007;130:691–703. [PubMed: 17719546]
29. Eichmann A, Corbel C, Nataf V, et al. Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. *Proc Natl Acad Sci U S A* 1997;94:5141–5146. [PubMed: 9144204]
30. Koibuchi N, Taniyama Y, Nagao K, et al. The effect of VEGF on blood vessels and blood cells during *Xenopus* development. *Biochem Biophys Res Commun* 2006;344:339–345. [PubMed: 16630570]
31. Pearson S, Sroczynska P, Lacaud G, et al. The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. *Development* 2008;135:1525–1535. [PubMed: 18339678]
32. Bautch VL, Redick SD, Scalia A, et al. Characterization of the vasculogenic block in the absence of vascular endothelial growth factor-A. *Blood* 2000;95:1979–1987. [PubMed: 10706864]
33. Ng YS, Ramsauer M, Loureiro RM, et al. Identification of genes involved in VEGF-mediated vascular morphogenesis using embryonic stem cell-derived cystic embryoid bodies. *Lab Invest* 2004;84:1209–1218. [PubMed: 15220937]
34. Kappel A, Ronicke V, Damert A, et al. Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) promoter/enhancer sequences sufficient for angioblast and endothelial cell-specific transcription in transgenic mice. *Blood* 1999;93:4284–4292. [PubMed: 10361126]

35. Elvert G, Kappel A, Heidenreich R, et al. Cooperative interaction of hypoxia-inducible factor- 2alpha (HIF-2alpha) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). *J Biol Chem* 2003;278:7520–7530. [PubMed: 12464608]
36. Dutta D, Ray S, Vivian JL, et al. Activation of the VEGFR1 chromatin domain: an angiogenic signal-ETS1/HIF-2alpha regulatory axis. *J Biol Chem* 2008;283:25404–25413. [PubMed: 18625704]
37. Olszewska-Pazdrak B, Hein TW, Olszewska P, et al. Chronic hypoxia attenuates VEGF signaling and angiogenic responses by downregulation of KDR in human endothelial cells. *Am J Physiol Cell Physiol* 2009;296:C1162–1170. [PubMed: 19244479]
38. Purpura KA, George SH, Dang SM, et al. Soluble Flt-1 regulates Flk-1 activation to control hematopoietic and endothelial development in an oxygen-responsive manner. *Stem Cells* 2008;26:2832–2842. [PubMed: 18772315]
39. Graubert MD, Ortega MA, Kessel B, et al. Vascular repair after menstruation involves regulation of vascular endothelial growth factor-receptor phosphorylation by sFLT-1. *Am J Pathol* 2001;158:1399–1410. [PubMed: 11290558]
40. Zhang Z, Neiva KG, Lingen MW, et al. VEGF-dependent tumor angiogenesis requires inverse and reciprocal regulation of VEGFR1 and VEGFR2. *Cell Death Differ.* 2009
41. Dumont DJ, Fong GH, Puri MC, et al. Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev Dyn* 1995;203:80–92. [PubMed: 7647376]
42. Mazzone M, Dettori D, Leite de Oliveira R, et al. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell* 2009;136:839–851. [PubMed: 19217150]
43. Kappas NC, Zeng G, Chappell JC, et al. The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching. *J Cell Biol* 2008;181:847–858. [PubMed: 18504303]
44. Chappell JC, Taylor SM, Ferrara N, et al. Local guidance of emerging vessel sprouts requires soluble Flt-1. *Dev Cell* 2009;17:377–386. [PubMed: 19758562]
45. Jinnin M, Medici D, Park L, et al. Suppressed NFAT-dependent VEGFR1 expression and constitutive VEGFR2 signaling in infantile hemangioma. *Nat Med* 2008;14:1236–1246. [PubMed: 18931684]
46. North S, Moenner M, Bikfalvi A. Recent developments in the regulation of the angiogenic switch by cellular stress factors in tumors. *Cancer Lett* 2005;218:1–14. [PubMed: 15639335]

Figure 1.

Loss of *Arnt* disrupts proper vascular differentiation in ES differentiation cultures. (A,B) *Arnt*^{-/-} ESCs stained for CD31 demonstrate a failure to generate vascular cystic embryoid bodies in 20 day EBs grown in suspension (A) or sprouting EBs differentiated in Type I collagen (B) compared to WT controls. (C) Flow cytometry was used to assess CD31 levels from cells obtained by dissociating EBs from WT, *Arnt*^{-/-}, or *Vegf*^{-/-} ESCs differentiated in methylcellulose or suspension under normoxia or hypoxia (3% O₂). (D) Transcript levels of angiogenic genes from cDNA isolated from WT or *Arnt*^{-/-} Day 7 and 10 EBs were determined by real-time PCR, normalized against 18S, and expressed as mean±SEM relative to the mean of D7 WT normoxia sample.

Figure 2.

The presence of ARNT in response to hypoxia influences angiogenic growth in differentiating ESC cultures independent of exogenous VEGF. (A) Experimental model used to differentiate EBs and quantify their angiogenic potential. Representative fields of vessels sprouts immunostained to reflect vessels containing CD31⁺ EC and α -SMA⁺ vascular smooth muscle cells. At least 50 individual EBs replated in 3D Type I Collagen are classified as Low or High angiogenic EBs. Experiments were performed in triplicate and data represented as percent mean values $\pm$ SEM, * p < .05, ** p < .005, *** p < .001. (B–E) Quantitative analysis of angiogenesis from WT or *Arnt*^{−/−} ES cells differentiated under normoxia or hypoxia (3% O₂) and replated in Type I collagen. (B) Percent of high angiogenic EBs of cultures differentiated for 10 Days in the presence of 25 ng/ml VEGF and 100 ng/ml bFGF (C) Results from similar EB cultures differentiated for 7 days with growth factors reduced to 5 ng/ml VEGF and 25 ng/ml bFGF. (D, E) Results from EB differentiated for 7 or 10 days in the absence of exogenous VEGF. Representative micrographs of 10 day WT cultures grown under normoxia (a,b,c,d) or hypoxia (e,f,g,h) with (a,b,e,f) or without (c,d,g,h) exogenous VEGF and replated in Type I collagen to assess angiogenic potential. (F) WT or *Arnt*^{−/−} ES cells differentiated in methylcellulose with 25, 5, or 0 ng/ml VEGF for 7 or 10 days and then replated in Type I collagen and individual EBs were scored for displaying low or high angiogenic outgrowth.

Figure 3.

Hypoxia promotes vascular differentiation and angiogenic sprouting in an ARNT-dependent manner. (A) Experimental model outlining the normoxic or hypoxic (3% O₂) conditions used for the differentiation of EBs in methylcellulose (in the absence of exogenous VEGF) and during vascular outgrowth in Type I collagen. (B) Individual EBs were scored for angiogenic outgrowth for each condition. Data represent mean±SEM from triplicate experiments, ***p* < .01, ****p* < .001.

Figure 4.

Hypoxia promotes vascular differentiation independent of VEGF. (A,B,C) Inhibiting VEGF is not sufficient to block hypoxia's angiogenic effects. Inhibitors were added during the differentiation of EBs for 7 or 10 days in methylcellulose EBs exposed to normoxic or hypoxic (3% O₂) conditions and at least 50 EBs per treatment were then replated in Type I Collagen and EBs were individually scored for angiogenic outgrowth. (A) Fc-Flt1 was used at 1 and 0.25 µg/ml, and results are represented as percent of each EB angiogenic type from total numbers of EBs per treatment. (B,C) WT or *Arnt*^{-/-} EBs were differentiated under normoxic or hypoxic conditions with and without with 50 µM SU5416 and replated in Type I collagen. (B) Micrographs of representative cultures demonstrate that WT EBs preserve some angiogenic EBs. (C) Quantification of Low and High angiogenic EBs in response to SU5416 treatment. Data represent means from triplicate samples ± SEM. (D,E) *Vegf*^{-/-} ESCs contain all hematopoietic colony forming potential but are angiogenic deficient. (D) Hematopoietic progenitor activity of day 9 *Vegf*^{-/-}, *Vegf*^{+/+}, and WT EB-derived cells. 1500 cells were replated in triplicate and individual colonies were scored 6–7 days after replating. Error bars represent SEM. (E) Quantitative analysis of angiogenesis from WT, *Arnt*^{-/-}, or *Vegf*^{-/-} ES cells differentiated for 7 days under normoxia or hypoxia (3% O₂) in the absence of exogenous VEGF. Over 50 EBs replated in Type I collagen in triplicate and individual colonies were scored for angiogenic sprouting.

Figure 5.

Hypoxia regulates the temporal expression of VEGF receptors. (A) EBs were differentiated in suspension or methylcellulose cultures in the absence of exogenous VEGF. Cultures were exposed to normoxia or hypoxia (3% O₂) and analyzed for the surface expression of Flk-1 or mFlt-1 by flow cytometry (B,C) and secreted Flt-1 by ELISA (D). (C) In WT cultures, hypoxia (solid red squares) alters the expression of each receptor compared to normoxic conditions (solid blue squares) and are further dysregulated in *Arnt*^{-/-} EB cultures. (D) Although distinct from each other, the concentration of sFlt-1 from protein cell lysates isolated from methylcellulose EB cultures and conditioned media from suspension EB cultures is increased by hypoxic treatment in both WT and *Arnt*^{-/-} EB cultures.

Figure 6.

(A) *Vegf*^{-/-} ES were differentiated in suspension or methylcellulose in the absence of exogenous VEGF. Cultures were exposed to normoxia or hypoxia (3% O₂) and dissociated cells were analyzed for the surface expression of Flk-1 or mFlt-1 by flow cytometry. (B) Concentrations of secreted Flt-1 from supernatant or protein lysates of *Vegf*^{-/-} EB cultures were analyzed by ELISA.

Physiological Hypoxia:

Figure 7.

Model illustrating that hypoxia influences the expression of VEGF receptors either promoting (Flk-1) or inhibiting (sFlt-1) vessel sprouting, thereby controlling the formation of vascular networks. Angiogenic defects in absence of *Arnt* or *Vegf* may result in part from the altered expression of these receptors.