Hypoxia affects mesoderm and enhances hemangioblast specification during early development

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Summary

Hypoxia Inducible Factor (HIF), consisting of HIF1α and ARNT (HIF1β) subunits, activates multiple genes in response to oxygen (O_2) deprivation. *Arnt*$^{-/-}$ mice exhibit substantial defects in blood cell and vessel development. We demonstrate that hypoxia accelerates the expression of Brachyury (a mesoderm-specific transcription factor), BMP4 (a mesoderm-promoting growth factor) and FLK1 (a marker of hemangioblasts, the bipotential progenitor of endothelial and hematopoietic cells) in differentiating ES cell cultures. Significantly, proliferation of embryonic hemangioblasts (BL-CFCs) is regulated by hypoxia, as *Arnt*$^{+/+}$ ES cells generate increased numbers of FLK1^{+} cells, and BL-CFCs with accelerated kinetics in response to low O_2. This response is HIF-dependent as *Arnt*$^{-/-}$ ES cells produce fewer FLK1^{+} cells and BL-CFCs, under both normoxic and hypoxic conditions. Interestingly, this defect is rescued when *Arnt*$^{-/-}$ ES cells are co-cultured with *Arnt*$^{+/+}$ ES cells. *Vegf*$^{+/-}$ or *Vegf*$^{-/-}$ ES cells generate proper numbers of FLK1^{+} cells but fewer BL-CFCs, suggesting that additional factors regulated by HIF (other than VEGF) are involved in these early events. Thus, hypoxic responses are important for the establishment of various progenitor cells, including early mesoderm and its differentiation into hemangioblasts. Together these data suggest that ineffective responses to hypoxia in *Arnt*$^{-/-}$ embryos abrogate proper cardiovascular development during early embryogenesis, including the pathways controlling hemangioblast differentiation.

Supplemental data available online

Key words: HIF, ARNT, Hypoxia, Hemangioblast, Mesoderm

Introduction

During embryogenesis, O_2 delivery by diffusion becomes limiting shortly after gastrulation. The cardiovascular system, composed of the heart, vascular network, blood cells and placenta, ensures continued delivery of O_2 and nutrients essential for proper growth and development of the embryo. Molecular responses to O_2 gradients contribute to the proper differentiation and maintenance of the cardiovascular system by directly or indirectly regulating various genes required for these events, including erythropoietin (EPO), transferrin and its receptor, vascular endothelial growth factor (VEGF) and its receptors FLK1 (KDR – Mouse Genome Informatics) and FLT1, platelet derived growth factor-β (PDGFβ), basic fibroblast growth factor (bFGF), and multiple genes encoding glycolytic enzymes (Bianchi et al., 1999; Forsythe et al., 1996; Gerber et al., 1997; Gleadle et al., 1995; Kourembanas et al., 1990; Liu et al., 1995; Lok and Ponka, 1999; Maxwell et al., 1993; Rolfs et al., 1997; Semenza et al., 1994; Tacchini et al., 1999; Wood et al., 1996).

HIF modulates the transcription of these genes and is globally activated during embryonic development in organs that naturally experience an O_2 gradient (Iyer et al., 1998; Mitchell and Yochim, 1968; Rodesch et al., 1992). HIF is a member of the basic helix-loop-helix (bHLH)-PAS family of proteins that regulate many essential processes, including O_2 homeostasis, circadian rhythms, neurogenesis and toxin metabolism (Gu et al., 2000; Wang et al., 1995). Members of the HIF subfamily of bHLH-PAS proteins heterodimerize to form transcriptional complexes that induce gene expression by binding an ~50-bp Hypoxia Response Element (HRE) (Semenza, 1998; Semenza, 1999; Semenza et al., 1991). Under normoxic conditions, the HIFα subunits HIF1α, HIF2α (EPAS) and HIF3α are targeted for proteasome degradation by the von Hippel-Lindau (VHL) protein (Cockman et al., 2000; Maxwell et al., 1999; Ohh et al., 2000). However, under hypoxic conditions, HIFα subunits are stabilized, translocate to the nucleus, and dimerize with the β-subunits ARNT (aryl hydrocarbon receptor nuclear translocator) or ARNT2 (Semenza, 1999). Null mutations in either HIF1α or ARNT lead to midgestational lethality of embryos, with phenotypes that include defects in the vasculature, blood, placenta and heart (Adelman et al., 2000; Adelman et al., 1999; Iyer et al., 1998; Kotch et al., 1999; Maltepe et al., 1997; Ryan et al.,

1998). Thus, improper responses to low O_2 in the embryo can lead to lesions in multiple aspects of cardiovascular development.

Our original characterization of *Arnt*$^{-/-}$ embryos demonstrated a vascular remodeling defect in the extraembryonic yolk sac (Maltepe et al., 1997). Further clonogenic analysis of *Arnt*$^{-/-}$ yolk sacs revealed a defect in the generation of hematopoietic progenitors (Adelman et al., 1999). As the yolk sac endothelial cell defect also extends to embryonic tissues (Keith et al., 2001; Maltepe et al., 1997), we postulated that embryonic lethality could be the result of a defect in an early progenitor of the cardiovascular system. Because embryonic hematopoiesis is spatially and temporally linked to endothelial cell development, *Arnt* may independently be required for the differentiation of both lineages, or alternatively be essential for the production of ‘hemangioblast’ progenitors.

The notion that endothelial and hematopoietic cells are derived from a common hemangioblastic precursor is based on observations that these lineages emerge simultaneously and in proximity to each other during organogenesis (Sabin, 1920): extraembryonically in the yolk sac blood islands, and intraembryonically in the aorta-gonad-mesonephros (AGM) (Haar and Ackerman, 1971; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Endothelial and hematopoietic cells also share expression of multiple genes (reviewed by Choi, 1998). FLK1$^+$ cells are first detected in blood island mesodermal aggregates that contribute to the extraembryonic vasculature of the yolk sacs in 7.0 dpc mouse embryos (Choi, 1998; Choi et al., 1998; Dumont et al., 1995). More recently, an intraembryonic source of potential hemangioblast cells has been identified in the endothelium of the dorsal aorta (de Bruijn et al., 2002; North et al., 2002).

Although attempts to isolate hemangioblasts from embryos have not been successful, a morphologically distinct cell or ‘blast colony-forming cell’ (BL-CFC) has been described in embryonic stem (ES) cell-derived ‘embryoid bodies’ (EBs) (Choi, 1998; Choi et al., 1998; Kennedy et al., 1997). BL-CFCs are likely to represent hemangioblasts, as they exclusively produce both endothelial and blood cells in vitro. Significantly, early embryonic development can be mimicked by in vitro differentiation of ES cells into EBs composed of all three germ layers (Keller et al., 1993; Keller, 1995). The differentiating ES cell masses are an advantageous model system in which mutant ES cells can be synchronized, manipulated and analyzed for their production of various cell lineages. Using this assay system, we have previously demonstrated that hypoxia increases hematopoietic precursor numbers (Adelman et al., 1999). These in vitro assays confirmed that *Arnt*$^{-/-}$ ES cells generate significantly fewer numbers of hematopoietic progenitors, consistent with results obtained from *Arnt*$^{-/-}$ yolk sacs (Adelman et al., 1999). The putative hemangioblast, or BL-CFC, appears transiently within the mesoderm of differentiating EBs. Indeed, mesodermal progenitors that express Brachyury, a T-box transcription factor, differentiate into hemangioblasts, and subsequent hematopoietic and endothelial lineages (Fehling et al., 2003). The EB differentiation system has facilitated the elucidation of cell-intrinsic factors required for the generation of hemangioblasts, including the VEGF receptor FLK1, the bFGF receptor FGFR1, the Ephrin receptor EPHB4, and the transcription factors SCL (TAL1) and RUNX1 (AML1) (Ema et al., 2003; Faloon et al., 2000; Fehling et al., 2003; Lacaud et al., 2002; Robertson et al., 2000; Wang et al., 2004).

Given the observed hematopoietic and endothelial defects in *Arnt*$^{-/-}$ embryos, we investigated the role of hypoxia in the commitment of mesoderm to the hemangioblast lineage. We report here that hypoxia indeed promotes the generation of hemangioblasts from ES cells. In fact, hypoxia induces earlier expression of Brachyury, *Flk1* and *Bmp4* in EBs. Interestingly, both *Arnt* and *Vegf* mutant ES cells are deficient in the generation of BL-CFCs. Moreover, we establish that the hemangioblast defect in *Arnt*$^{-/-}$ cells is not cell-intrinsic, although multiple growth factors (including VEGF and bFGF) are not sufficient to rescue the phenotype. These findings suggest that hypoxia-mediated generation of mesoderm, and of blood and vascular progenitor cells, is crucial for early embryonic development.

Materials and methods

ES cell culture and differentiation

The generation and maintenance of *Arnt*$^{-/-}$ (Adelman et al., 1999), *GFP-Bry* (Fehling et al., 2003), *Vegf*$^{+/-}$ and *Vegf*$^{-/-}$ (Carmeliet et al., 1996) ES cells have been previously described. Undifferentiated trypsinized ES cells were initially plated for 1 hour in cell culture dishes to remove adherent embryonic fibroblasts, followed by two further replatings onto 0.1% gelatinized plates in the presence of 1% leukemia inhibitory factor conditioned media (LIF). ES cells were then differentiated into EBs as described (Choi et al., 1998; Kennedy et al., 1997). Hypoxic cultures were maintained at 3% O_2 for 1.5 to 5 days in Jouan incubators. For ‘rescue’ experiments, combinations of growth factors, including rmVEGF (5-10 ng/ml), bFGF (10-20 ng/ml), EPO (2 U/ml), BMP2 (50 ng/ml), BMP4 (50 ng/ml), TGFβ1 (2 ng/ml), TGFβ3 (1 ng/ml), Angiopoietin 1 [BowAng1-TDF (1 μg/ml)] and Angiopoietin 2 [BowAng2-Fc (1 μg/ml)], were added to differentiating ES cultures. All cytokines were obtained from R&D Systems, except for BowAng1-TDF and BowAng2-Fc, which were a kind gift from of Dr Gavin Thurston (Regeneron Pharmaceuticals).

Methylcellulose colony assays

EBs were disaggregated in 0.25% Trypsin-EDTA (Invitrogen), and further dissociated with a 21-gauge needle. 5×10^4 cells were replated in triplicate in 1% methylcellulose medium (H4100, SCT), supplemented as described (Choi et al., 1998; Kennedy et al., 1997). For mixing conditions, equal numbers of *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ ES cells were co-cultured during EB differentiation. Disaggregated EBs were replated in methylcellulose as above. One set of triplicate cultures was treated with 0.4 mg/ml of G418. X-gal staining for the expression of β-galactosidase was performed on individually picked colonies that were fixed in 5% paraformaldehyde for 10 minutes, washed in PBS, and stained as previously described (Schuh et al., 1999).

Matrigel cultures

Individual colonies were transferred to Matrigel™ (Collaborative Research)-coated 96-well plates and cultured for 10 days, as described (Choi et al., 1998). All cytokines were purchased from R&D Systems, except rhEpo (Amgen) and ECGS (Collaborative Bioresearch). DiI-acetylated low density lipoprotein (DiI-Ac-LDL; Biomedical Technologies) endothelial uptake was performed by adding 10 μg/ml to Matrigel cultures for 4 hours at 37°C. Cultures were fixed with 4% paraformaldehyde, washed, and observed by fluorescence microscopy using a rhodamine filter.

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 oligo dT primers (Promega). PCR reactions were performed as previously described (Schuh et al., 1999), with sequence-specific primers (10 pmol per reaction) published by Roberston or Schuh et al. (Roberston, 2000; Schuh et al., 1999) for β-actin. Radioactive PCR was performed by adding 0.01 μl of α-dCTP to PCR reactions, which were separated on acrylamide gels, dried, and visualized by PhosphoImager analysis.

Real-time detection PCR (RTD-PCR) was performed as previously described (Seagroves et al., 2003). PCR reactions were performed using default cycling parameters of the ABI Prism 7900HT Sequence Detector. Reactions were carried out in a 20 μl reaction, with 2×Taqman Master Mix (ABI) and the following primers:

GLUT-1F, 5′-ATGGCGGCGGTCCTATAAA-3′;
GLUT-1R, 5′-CGCCCTGACGCACGGAAGA-3′;
GLUT1-PROBE, 5′-(6FAM)CAGCTCCGCGCGCGCGCCC(TAMRA)-3′;
VEGF-F, 5′-AGGAGTACCCCGACGAGATAGAG-3′;
VEGF-R, 5′-CTCCAGGGCTTCATCGTTACA-3′;
VEGF-PROBE, 5′-(6FAM)TCAAGCCGTCCTGTGTGCCGC(TAMRA)-3′;
BRY-F, 5′-AAGCGGTGGCGAGAGAAGT-3′;
BRY-R, 5′-CCCTCTCCACCTTCCAGGA-3′;
BRY-PROBE, 5′-(6FAM)AAGGTGGCTGTTGGGTAGGGAGTCAAGA(TAMRA)-3′;
BMP4-F, 5′- GGGCCAAACGTAGTCCCAA-3′;
BMP4-R, 5′-GGCGACGGCAGTTCTTATTC-3′;
BMP4-PROBE, 5′-(6FAM)CATCACCCACAGCGGTCCAGGAA-(TAMRA)-3′;
FLK1-F, 5′-TCATTATCCTCGTCGGCACTG-3′;
FLK1-R, 5′-CCGCTTAACGGTCCGTAGG-3′; and
FLK1-PROBE, 5′-(6FAM)CCATGTTCTTCTGGCTCCTTCTTGTCATTG(TAMRA)-3′.

Each target gene was normalized to 18S RNA (ABI, catalog number 4308329) for each sample using the ΔΔCt method (threshhold values) (Muller et al., 2002). Relative mRNA levels were then compared, at each time point, to wild-type normoxic samples, which were normalized to a value of one, and data was expressed as fold induction of mRNA.

Flow cytometry

After 1.5 to 4 days of differentiation, EBs were disaggregated as previously reported (Faloon et al., 2000). Cells were incubated in 1:100 blocking buffer FcγIII/II receptor (Pharmingen) for 20 minutes at 4°C. After washing in 1% BSA/PBS, cells were incubated with PE-conjugated FLK1 [Avas12α1 (Pharmingen)] at 1:100 in wash buffer for 20 minutes at 4°C, washed and visualized by FACS Vantage (Becton Dickinson). Results were analyzed by Flo-Jo (Tree-Star).

Results

Phenotypes of colonies in BL-CFC assays

Because of the hematopoietic and endothelial defects noted in *Arnt*$^{-/-}$ embryos, we examined the requirement for ARNT in BL-CFC development. First, we assessed the production of BL-CFCs using the established blast assay (Choi et al., 1998; Kennedy et al., 1997). An earlier progenitor, the ‘transitional’-colony forming cell (Trans-CFC), has also been identified in these culture conditions (Faloon et al., 2000; Robertson et al., 2000). Trans-CFCs maintain undifferentiated mesodermal potential in addition to hematopoietic/endothelial characteristics, representing an earlier stage of development than BL-CFCs. Cells disaggregated from primary (1°) EBs replated in methylcellulose generate three distinct colony types: secondary (2°) EBs that represent differentiation of the three germ layers, including mesodermal differentiation (Fig. 1A,A′); transitional colonies (Trans-CFCs) that contain residual undifferentiated mesoderm but undergo some commitment to hematopoietic and/or endothelial lineages (Fig. 1A,D′); and hemangioblast colonies (BL-CFCs), representing progenitors with exclusively hematopoietic and endothelial potential (Fig. 1A,G′) (Faloon et al., 2000; Robertson et al., 2000). The current model proposes that these colonies represent specific stages of early differentiation (Fig. 1A). To confirm the phenotype of these three colonies, individual colonies were replated onto matrigel to promote their differentiation into mesodermal, endothelial and/or hematopoietic cell types. Individual colonies were also assayed for the expression of cell type-specific genes by radioactive RT-PCR.

Replatings of 2° EBs gave rise to adherent cells, including mesodermal cell types (Fig. 1A,A′-C′). *Rex1*, a marker of undifferentiated ES cells, and *Bry*, a mesoderm-specific transcript, were amplified from both *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ 2° EBs (Fig. 1C). By contrast, Trans-CFCs exhibited a more ruffled and dense appearance, as previously described (Faloon et al., 2000; Robertson et al., 2000) (Fig. 1A,D′). Matrigel platings of Trans-CFCs from *Arnt*$^{-/-}$ cultures gave rise to colonies of various types, but primarily mesoderm and non-adherent hematopoietic cells (see below and Fig. 1A,E′-F′). Undifferentiated ES cell (*Rex1*), mesoderm-specific (*Bry*) and endothelial/hematopoietic (*Gata1*, *Scl*) transcripts were detected in individual Trans-CFC colonies from *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ cultures (Fig. 1C). However, the pattern for *Rex1* levels are reduced compared with 2° EBs. These data are consistent with previous reports indicating that Trans-CFCs represent more immature precursors than the BL-CFCs, expressing markers for pre-committed vascular/hematopoietic lineages, while retaining mesodermal characteristics. Of note, replatings from *Arnt*$^{-/-}$ EBs generated mostly Trans-CFCs (see below).

BL-CFCs appeared as loosely adherent cell clusters in methylcellulose (Fig. 1A,G′), and when replated on matrigel yielded both adherent endothelial cells that formed characteristic tube-like structures and non-adherent hematopoietic cells (Fig. 1A,H′-I′). Subsequent differentiation of BL-CFCs confirmed that the adherent cells were endothelial cells, based on their uptake of fluorescent DiI-Ac-LDL (Fig. 1B). We further confirmed the BL-CFC phenotype by demonstrating the absence of early markers (*Bry*, *Rex1*) and the presence of endothelial/hematopoietic markers (*Gata1*, *Scl*) in both *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ cultures (Fig. 1C). These replating studies reveal that BL-CFC colonies obtained from *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ EBs have the potential to generate both hematopoietic and endothelial cells.

ARNT is required for appropriate generation of hemangioblast colonies

Although both *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ EB cultures are capable of generating functional BL-CFCs in vitro, hematopoietic and endothelial defects observed in *Arnt*$^{-/-}$ embryos may arise from reduced numbers of functional hemangioblasts. To assess the quantitative effect of the *Arnt* mutation on hemangioblast formation, *Arnt*$^{-/-}$ ES cells were analyzed for their capacity to generate appropriate numbers of BL-CFCs. *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ cells were differentiated into EBs for 3 days and assayed for the development of BL-CFCs. Of note, five independent *Arnt*$^{-/-}$

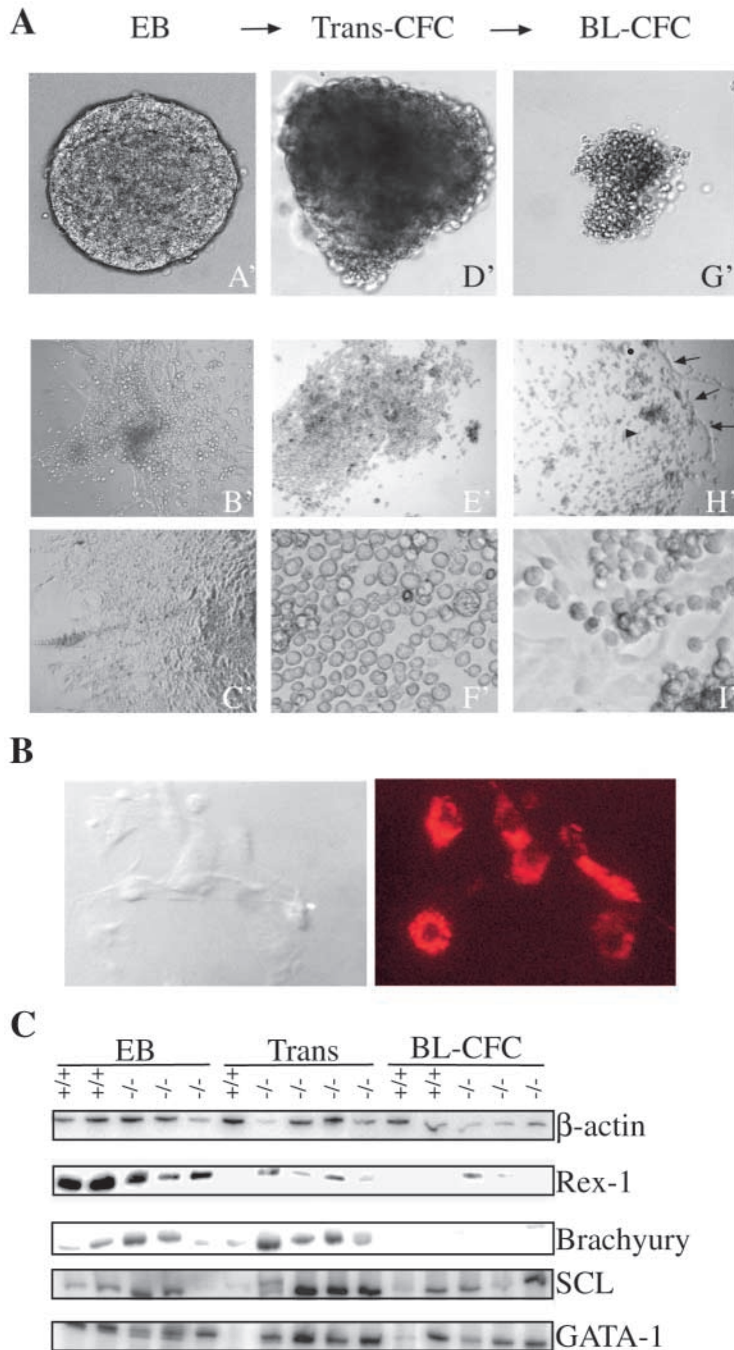


Fig. 1. Characterization of methylcellulose colonies. (A) Micrographs of representative colonies from methylcellulose cultures: 2° EB colony (A′), transitional colony (Trans-CFC; D′) and hemangioblast colony (BL-CFC; G′). Microwells of colonies replated on matrigel: 2° EB with adherent mesodermal cell type (B′,C′); two independent transitional colonies showing mostly hematopoietic cells (E′,F′); BL-CFCs showing both adherent endothelial cells (arrows) and overlaying non-adherent hematopoietic cells (arrowhead; H′,I′). (B) Single hemangioblasts were replated on matrigel for one week and treated with fluorescent DiI-Ac-LDL. Phase contrast (left panel) and fluorescent micrograph (right panel) images are shown. (C) RNA from individual $Arnt^{+/+}$ and $Arnt^{-/-}$ 2° EB, Trans-CFC and BL-CFC colonies were analyzed for the expression of the indicated genes by radioactive RT-PCR: β-actin (control), *Rex1* (ES cells), *Bry* (mesoderm), *Scl* (endothelial/hematopoietic cells) and GATA1 (hematopoietic cells). As BL-CFCs are generally smaller colonies, the signal for β-actin is lower.

clones from two independent $Arnt^{+/-}$ ES cell lines generated significantly fewer BL-CFCs compared with $Arnt^{+/+}$ controls (*P* values ranging from 0.0117 to 0.03; Fig. 2A). By contrast, $Arnt^{-/-}$ cultures generated a higher proportion of 2° EBs and Trans-CFCs in methylcellulose when compared with wild-type cultures (Fig. 2B and data not shown), suggesting proper mesoderm formation but an arrest in subsequent differentiation into the hemangioblasts.

Hypoxia influences the kinetics and numbers of hemangioblast colonies via ARNT

To mimic the hypoxic environment of a developing murine embryo, EBs were differentiated under low O_2 levels. We previously demonstrated that hypoxic conditions stimulate the number of hematopoietic progenitors from day 9 EBs in an ARNT-dependent manner (Adelman et al., 1999). Surprisingly, hypoxic (3% O_2) differentiation of $Arnt^{+/+}$ cells for 3 days resulted in a decreased number of BL-CFCs compared with normoxic (21% O_2) conditions (Fig. 2B). By contrast, BL-CFC and Trans-CFC numbers from $Arnt^{-/-}$ cultures were not statistically different between both conditions (Fig. 2B).

One explanation for the unexpected lack of a hypoxic stimulation of BL-CFCs may be that mesoderm differentiation is influenced by low O_2 conditions, hastening the kinetics of hemangioblast development. Although the peak for BL-CFC generation is at 3.5 days of differentiation, the presence of BL-CFCs is brief during EB differentiation (Kennedy et al., 1997; Robertson et al., 2000). Kinetic analysis of Trans-CFCs and BL-CFCs previously revealed that the highest numbers of transitional colonies appear one day earlier than blast colonies (Robertson et al., 2000). To delineate the kinetics of early progenitor colony formation during EB differentiation, we performed differentiation time courses of 1.5-4 days under normoxic and hypoxic conditions. Sample data from experiments performed for 2 to 3.5 days are represented in Fig. 2B,C. First, the number of Trans-CFCs in $Arnt^{+/+}$ cultures was higher at 2 and 2.5 days of differentiation for normoxic and hypoxic conditions than at day 3. Interestingly, hypoxia increased Trans-CFC numbers on day 2 of differentiation in $Arnt^{+/+}$ cultures (P=0.008, Fig. 2B). Second, under normoxic conditions, wild-type ES cells exhibited an increase in BL-CFC numbers between days 2 and 3.5. Of note, 2-2.5 days of hypoxic differentiation further increased the generation of BL-CFCs compared with normoxia (day 2, P=0.001; day 2.5, P=0.0013; Fig. 2B,C). However, hypoxia decreased BL-CFC numbers by day 3 of EB differentiation (Fig. 2B,C). These results suggest that low O_2 not only influences the number of BL-CFCs formed, but also alters the kinetics of EB differentiation into transitory BL-CFCs (Fig. 2C). Conversely, hypoxia did not greatly influence $Arnt^{-/-}$ Trans- or BL-CFC colony numbers (Fig. 2B). When compared with $Arnt^{+/+}$ cells, the total numbers of Trans-CFCs were higher for $Arnt^{-/-}$ cells under normoxic conditions, and they were unaffected by hypoxia (Fig. 2B, see also Fig. 5A). Therefore, it appears that $Arnt^{-/-}$

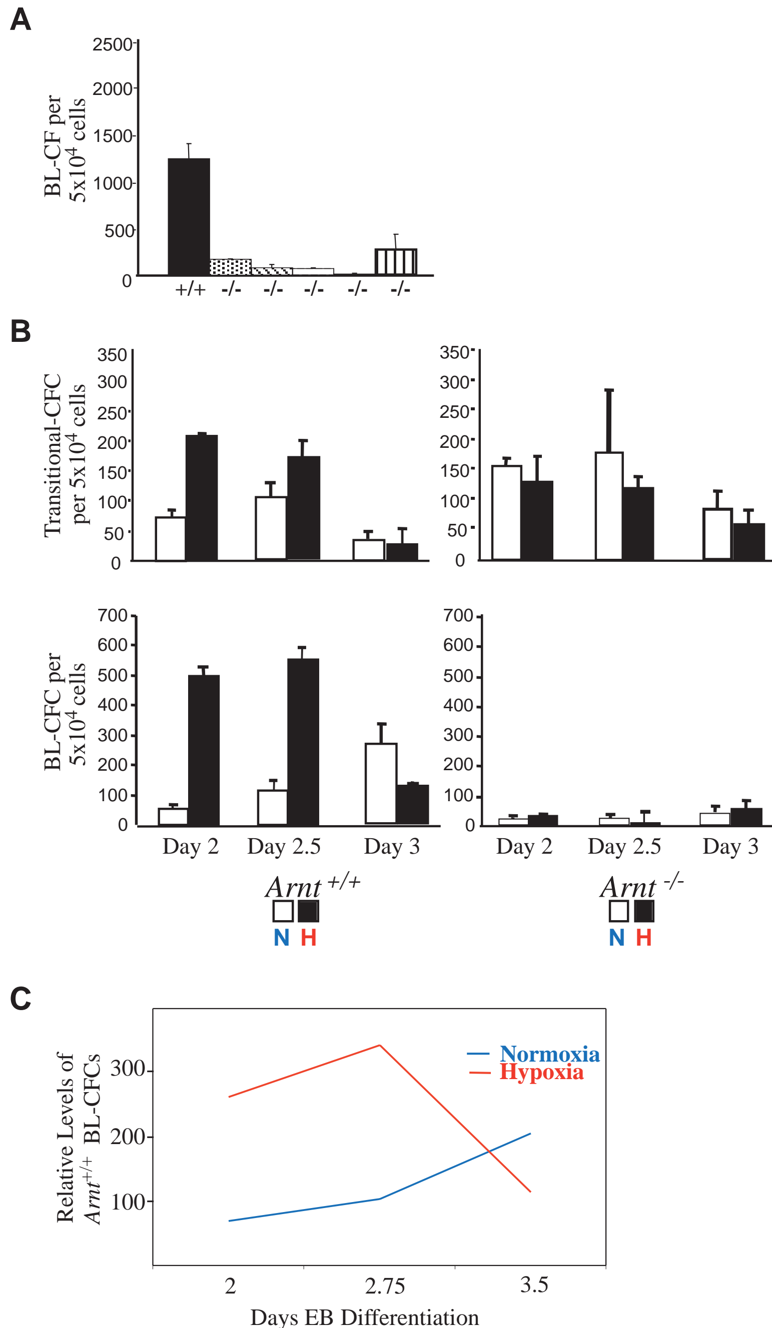


Fig. 2. ARNT is required for the appropriate development of hemangioblasts, and hypoxia accelerates the kinetics of their appearance. ES cell lines were differentiated into EBs and replated in a BL-CFC methylcellulose assay. (A) Five independent *Arnt*$^{-/-}$ clones (patterned bars) were differentiated for 2.75 days at 21% O_2, replated and compared with the wild-type control (black bar). (B) *Arnt*$^{+/+}$ (left panels) and *Arnt*$^{-/-}$ (right panels) ES lines were differentiated into EBs under normoxic (white bars, N; 21% O_2) or hypoxic (black bars, H; 3% O_2) conditions, for the indicated times, and replated for hemangioblast analysis. The number of transitional colonies is stimulated by hypoxia in *Arnt*$^{+/+}$ cultures (days 2 and 2.5), but mutants are not affected (upper panels). Hypoxia also accelerates the kinetics and increases the number of BL-CFCs for wild-type cells but not for *Arnt*$^{-/-}$ clones (lower panels). Error bars represent the standard error of the mean (s.e.m.) for triplicate assays. (C) Kinetics of *Arnt*$^{+/+}$ BL-CFC formation from 2 to 3.5 days, comparing normoxic (blue) and hypoxic (red) conditions.

cells undergo a developmental arrest and are blocked at the transitional stage. In all assays performed, an increase in BL-CFCs was consistently observed in hypoxic *Arnt*$^{+/+}$ cultures, although the peak time of induction varied by 12 hours between experiments. The kinetic experiments suggest that ‘physiological’ hypoxia encountered during embryogenesis contributes to the proper and timely development of hematopoietic/endothelial progenitors, and is dependent upon ARNT.

Hypoxia influences the expression of FLK1, Brachyury and BMP4

Faloon et al. previously showed that BL-CFCs develop from a small number of cells expressing FLK1, a receptor for VEGF and a putative hemangioblast marker (Faloon et al., 2000). Interestingly, FLK1$^+$ cells can also give rise to other vascular components, including smooth muscle cells (Yamashita et al., 2000). The number of FLK1$^+$ cells in developing EBs is not significant until day 2.75 of differentiation, when BL-CFCs peak in the experiments of Faloon et al. (Faloon et al., 2000). To determine whether low O_2 levels influence the formation of FLK1$^+$ cells, EBs were differentiated under normoxic (21% O_2) or hypoxic (3% O_2) conditions and analyzed for FLK1 expression by flow cytometry. Of note, FLK1 surface expression was significantly induced by hypoxia on multiple days of *Arnt*$^{+/+}$ EB differentiation, although the intensity diminished by day 4 (Fig. 3A, Fig. 5C). By contrast, two independent *Arnt*$^{-/-}$ clones exhibited decreased FLK1$^+$ cell numbers under both conditions (Fig. 3A, Fig. 5C). These results indicate that FLK1$^+$ cell numbers increase under hypoxia in accord with increased numbers of BL-CFCs, and suggest that the BL-CFC deficit in *Arnt*$^{-/-}$ EBs is secondary to a decrease in FLK1$^+$ cells, proposed to be required for hemangioblast development (Robertson et al., 2000; Schuh et al., 1999).

To more accurately define the role of hypoxia in early development, ES cells with GFP targeted to the Brachyury locus, an early primitive streak mesoderm marker, were employed (Fehling et al., 2003). In this system, it has been established that GFP$^+$FLK1$^-$ cells represent a pre-hemangioblast mesoderm stage that can further differentiate into GFP$^+$FLK1$^+$ hemangioblasts, demonstrating that Brachyury (BRY$^+$; T – Mouse Genome Informatics) mesodermal progenitors can give rise to BL-CFCs. The *GFP-Bry* ES cells were cultured under normoxia (21% O_2) and hypoxia (3% O_2), and assayed for GFP expression between 1.5 to 5 days of EB differentiation by flow cytometry. Similar kinetics of GFP expression were observed in both culture conditions. However, a twofold induction of GFP$^+$ cells was detected at days 2.0 and 2.5 of differentiation in normoxic (0.2-2.0%) versus hypoxic (0.6-3.8%) conditions, demonstrating that hypoxia influences GFP$^+$ cell numbers as the mesoderm emerges. At days 3.0 (42.3% versus 44.0%) and 3.5 (63.0% versus 70.5%) of

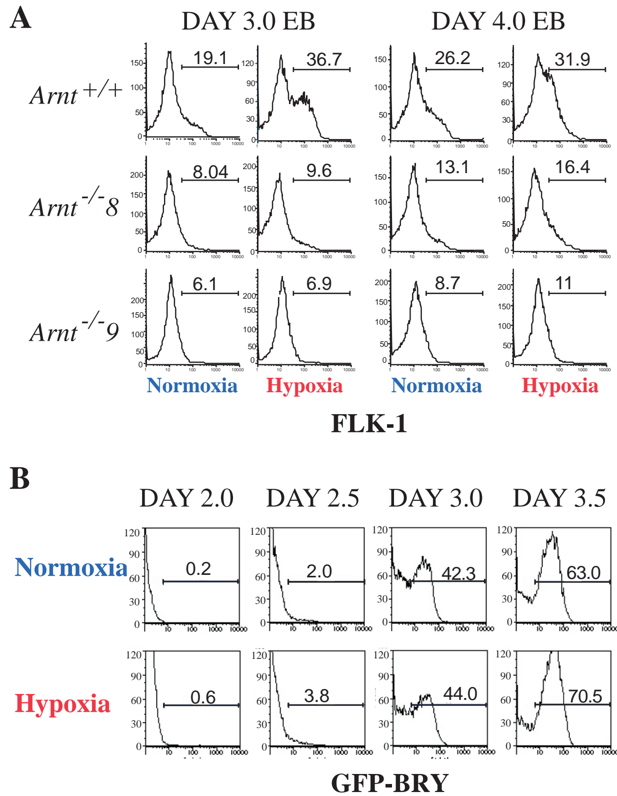


Fig. 3. Hypoxia influences the kinetics of mesoderm differentiation. Flow cytometry was performed on dissociated cells obtained from EBs harvested at the indicated time points. (A) The number of FLK1+ cells detected in differentiated *Arnt*+/+ EBs is increased by hypoxic conditions. In direct contrast, the number of FLK1+ cells in two independent *Arnt*–/– cell lines is reduced in normoxia and is not enhanced by 3% O_2. (B) Hypoxia stimulates the production of GFP+ cells in GFP-BRY ES cells as early as day 2 of differentiation.

differentiation, hypoxia reproducibly yielded higher BRY expression (Fig. 3B). In all experiments performed, low O_2 conditions enhanced BRY expression, whereby the percent of GFP+ cells was higher for hypoxia (e.g. 19.0%, 20.3%) than normoxia (e.g. 10.3%, 13.4%) (data not shown). However, numbers of GPF+ cells where not as substantially different as the levels of FLK1+ cells in hypoxic treatment.

To complement the kinetic analysis of FLK1+ cell and hemangioblast development, semi-quantitative RT-PCR was performed on EB cultures at day 0 to 3.5 of differentiation (Fig. 4A). *Flk1* transcripts were amplified in hypoxic *Arnt*+/+ cultures one day earlier than normoxic cultures, based on RT-PCR analysis (Fig. 4A). Similar results were obtained for *Bry* and *Bmp4*, a growth factor required for extraembryonic mesoderm formation (Winnier et al., 1995). Interestingly, *Arnt*–/– EB cultures expressed low levels of *Flk1*, *Bry* and *Bmp4* under normoxic and hypoxic conditions. By contrast, *Rex1* and *Bmp2* expression was not affected by O_2 or by the presence of ARNT (Fig. 4A).

To more accurately measure changes in gene expression, quantitative real-time detection (RTD)-PCR was performed on RNA obtained from differentiating EBs. As a control for hypoxic gene induction, the HIF target gene *Glut1* (Hu et al., 2003; Maltepe et al., 1997) was assayed and shown to be induced 3.5-fold at days 2 and 3 of differentiation (Fig. 4B). These levels are comparable to experiments using undifferentiated ES cells (Hu et al., 2003). *Vegf*, a HIF target hypoxically induced in ES and day 9 EBs (Adelman et al., 1999; Hu et al., 2003; Maltepe et al., 1997), was also stimulated in day 2 and 3 hypoxic wild-type cultures (13- and

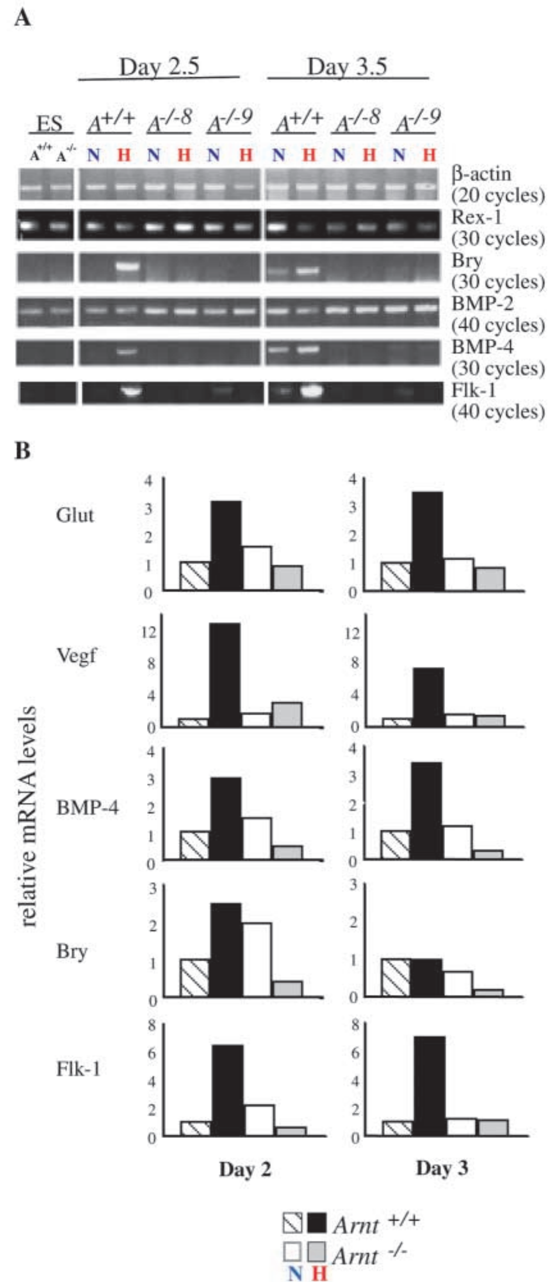


Fig. 4. Hypoxia influences the expression of genes associated with mesoderm differentiation. RT-PCR assays performed on RNA isolated from normoxic or hypoxic undifferentiated (ES) and EB cultures. (A) RT-PCR analyses of *Arnt*+/+ cells and two independent *Arnt*–/– lines differentiated under normoxic (N, 21%) or hypoxic (H, 3%) conditions. β-actin serves as a loading control. (B) Relative levels of gene expression from *Arnt*+/+ and *Arnt*–/– cultures under normoxic (N, 21%) or hypoxic (H, 3%) conditions assayed by real-time detection (RTD)-PCR. Transcripts are normalized to 18S RNA.

8-fold, respectively). Although they are not known to be direct HIF targets, hypoxic stimulation of *Bmp4*, *Bry* and *Flk1* transcripts at day 2 of differentiation in *Arnt*$^{+/+}$ cultures was confirmed by RTD-PCR (3-, 2.5- and 6.5-fold, respectively). Interestingly, although *Bmp4* and *Flk1* transcript levels were still elevated in *Arnt*$^{+/+}$ day 3 hypoxic EBs, *Bry* expression was reduced to normoxic levels. In direct contrast, hypoxia failed to induce *Glut1*, *Vegf*, *Bmp4*, *Bry* or *Flk1* in *Arnt*$^{-/-}$ cultures (Fig. 4B). Thus, hypoxia stimulates the expression of genes involved in mesoderm and hemangioblast development in EB cultures, in a HIF-α/ARNT-dependent manner.

Exogenous growth factors fail to rescue the Arnt$^{-/-}$ FLK1 and hemangioblast defects

VEGF addition to methylcellulose cultures promotes the growth of BL-CFCs (Kennedy et al., 1997; Robertson et al., 2000). If omitted, the resulting colonies retain a more transitional phenotype. Presently, the data demonstrate hypoxic upregulation of *Vegf* mRNA in day 2 and 3 EBs (Fig. 4B). Because HIF is an important transcriptional regulator of *Vegf*, hemangioblast production by *Vegf* mutant ES cells was examined. Interestingly, *Vegf*$^{-/-}$ EBs were deficient in generating Trans-CFCs and BL-CFCs when compared with wild-type cells (Fig. 5A, normoxic conditions), suggesting that VEGF is crucial for their production. Moreover, VEGF levels are important: *Vegf*$^{+/-}$ EBs were also deficient in generating BL-CFCs, although they produced increased numbers of Trans-CFCs, as noted for *Arnt*$^{-/-}$ EBs. In contrast to *Arnt*$^{-/-}$ EBs, hypoxia increased BL-CFC numbers in *Vegf*$^{+/-}$ and *Vegf*$^{-/-}$ EB cultures (Fig. 5A). Owing to the transient nature of BL-CFCs, fewer BL-CFCs were generated by hypoxic *Arnt*$^{+/+}$ EBs on 3 day (see Fig. 2B and Fig. 5A).

As *Vegf* mutant cells were deficient in proper hemangioblast production, and exogenous VEGF rescued the hematopoietic progenitor defect in day 9 *Arnt*$^{-/-}$ EB cultures (Adelman et al., 1999), VEGF was added to differentiating EBs in an attempt to 'rescue' the hemangioblast defect. In *Arnt*$^{+/+}$ cultures, 5-10 ng/ml of VEGF significantly increased the number of BL-CFCs as early as 1.5 days of differentiation (Fig. 5B). Not surprisingly, *Vegf* mutant cultures generated equivalent numbers of BL-CFCs to wild-type cultures upon the addition of exogenous VEGF (data not shown). However, VEGF did not significantly increase BL-CFCs in *Arnt*$^{-/-}$ cultures (Fig. 5B). Interestingly, a new colony type was generated in VEGF-treated *Arnt*$^{-/-}$ cultures that may represent a degenerate progenitor colony with endothelial characteristics, as this colony retains early markers (*Bry*, *Rex1*) based on RT-PCR analysis (see Fig. S1 at http://dev.biologists.org/cgi/content/full/131/18/4623/DC1).

Although FLK1 is necessary for proper BL-CFC generation (Schuh et al., 1999), its expression may require independent conditions from those required for BL-CFC production. In contrast to *Arnt*$^{-/-}$ EBs, *Vegf*$^{+/-}$ and *Vegf*$^{-/-}$ cultures generated significant numbers of FLK1^{+} cells (Fig. 5C). These results suggest an independent requirement for VEGF in the production of BL-CFCs, distinct from the production of FLK1^{+} progenitors. VEGF was not sufficient to fully rescue the *Arnt*$^{-/-}$ FLK1^{+} cell or the BL-CFC defects (data not shown, Fig. 5B). Therefore, bFGF was also added, based on its ability to induce FLK1 surface expression in differentiating EBs (Faloon et al., 2000). The addition of these growth factors did not significantly increase FLK1^{+} cell numbers in either *Arnt*$^{+/+}$ or *Arnt*$^{-/-}$ EB cultures, but did enhance FLK1 expression in *Vegf*$^{+/-}$ and *Vegf*$^{-/-}$ cultures (Fig. 5C). Although 2.75 days of hypoxia effectively stimulated the production of FLK1^{+} cells in *Arnt*$^{+/+}$ cultures (Fig. 5C), FLK1 expression at 2.75 days in both normoxic and hypoxic conditions was reduced when compared with levels detected at later times (Fig. 3A).

FLK1 expression has been used as a hemangioblast marker as the presence of FLK1 is required for proper BL-CFC generation; furthermore, FLK1^{-} cells produce fewer BL-CFCs (Chung et al., 2002; Faloon et al., 2000; Schuh et al., 1999). Because flow cytometry is a more rapid and convenient assay than BL-CFC production, we employed FLK1 expression to screen for potential rescue of the *Arnt*$^{-/-}$ mesoderm defect using a battery of growth factors. Factors including bFGF, VEGF, TGFβ1, TGFβ3, BMP2, BMP4, ANG1, ANG2 and EPO were added, in multiple combinations, to *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ EB cultures, and FLK1 expression was analyzed by flow cytometry (Table 1). Various growth factor combinations containing BMPs, TGFβs, VEGF and/or bFGF resulted in mild stimulation of FLK1^{+} cell numbers in 3.5-day *Arnt*$^{+/+}$ cultures, whereas hypoxia resulted in a marked FLK1 increase (Table 1, Experiment 1). One additional day of normoxic differentiation further increased FLK1^{+} cells in *Arnt*$^{+/+}$ EBs (4.5 days). However, FLK1^{+} cell numbers decreased on day 4.5 under hypoxia, or in the presence of various growth factors (Table 1). By contrast, no treatment yielded a significant effect on FLK1^{+} cell numbers in two independent *Arnt*$^{-/-}$ cell lines (Table 1, Experiment 1). Experiments using EPO, ANG1 and/or ANG2 in combination with other growth factors also had no effect on the FLK1^{+} cell population in *Arnt*$^{+/+}$ or *Arnt*$^{-/-}$ cultures (Table 1, Experiments 2 and 3). Therefore, *Arnt*$^{-/-}$ EBs appear to be deficient in HIF target(s) that remain unidentified at this time.

Arnt$^{-/-}$ hemangioblast production is a cell extrinsic defect

To determine whether the *Arnt*$^{-/-}$ BL-CFC defect is cell intrinsic, mixing experiments between *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ cell clones were implemented, as shown in Fig. 6A. We took advantage of the presence of the neomycin resistance (*neo*^{r}) gene introduced into the targeted *Arnt*$^{-/-}$ ES cells also tagged with a gene encoding β-galactosidase (*lacZ*). In these assays, *lacZ*$^{+}$ *neo*^{r} *Arnt*$^{-/-}$ cells were mixed with *lacZ*$^{-}$*neo*^{s} (neomycin sensitive) *Arnt*$^{+/+}$ ES cells during EB differentiation. Here, individual EBs consist of a combination of *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ cells. After 3 days, cells dissociated from the EBs were plated into methylcellulose in the presence or absence of G418, which selects for cells with the *neo*^{r} gene. As expected, colonies from *Arnt*$^{+/+}$-only cultures did not grow in the presence of G418, whereas *Arnt*$^{-/-}$-only cultures yielded colonies of mostly transitional phenotypes, as observed in previous experiments (Table 2).

To assess the numbers and genotypes of surviving colonies in mixed-EB replatings, colony numbers obtained from G418-treated and untreated cultures were compared. Assuming that cultures treated with antibiotic would suppress growth of all *Arnt*$^{+/+}$ cells, we would expect half of the number of hemangioblasts to perish. It has been previously determined that cultures containing low cell numbers develop poor blast colonies (Kennedy et al., 1997). Thus, twice the number of cells (1×10^{5} cells) were plated in the methylcellulose cultures

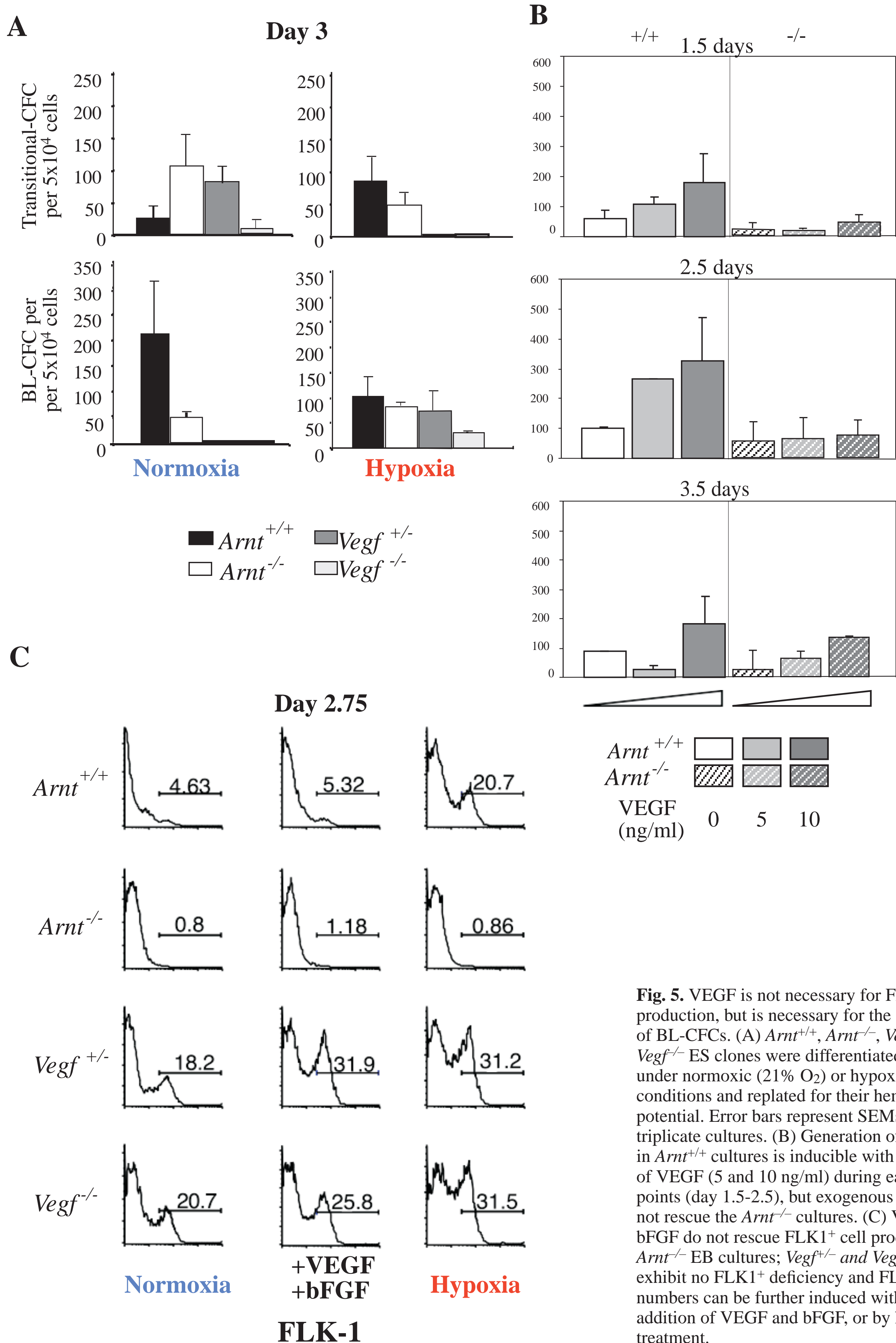


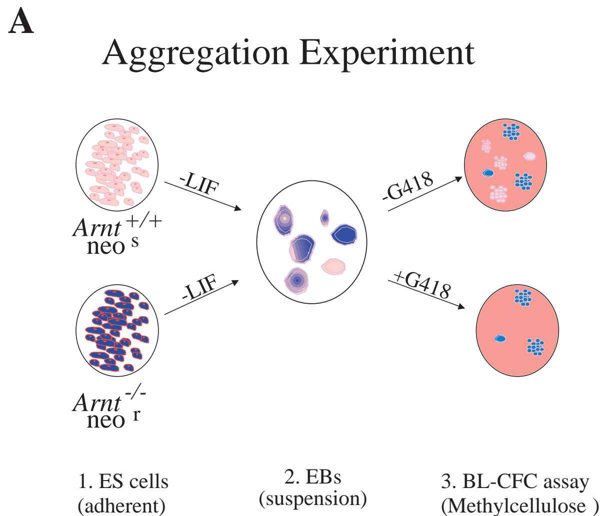
Fig. 5. VEGF is not necessary for FLK1$^+$ cell production, but is necessary for the generation of BL-CFCs. (A) *Arnt*$^{+/+}$, *Arnt*$^{-/-}$, *Vegf*$^{+/-}$ *and Vegf*$^{-/-}$ ES clones were differentiated for 3 days under normoxic (21% O_2) or hypoxic (3% O_2) conditions and replated for their hemangioblast potential. Error bars represent SEMs of triplicate cultures. (B) Generation of BL-CFCs in *Arnt*$^{+/+}$ cultures is inducible with the addition of VEGF (5 and 10 ng/ml) during early time points (day 1.5-2.5), but exogenous VEGF does not rescue the *Arnt*$^{-/-}$ cultures. (C) VEGF and bFGF do not rescue FLK1$^+$ cell production in *Arnt*$^{-/-}$ EB cultures; *Vegf*$^{+/-}$ *and Vegf*$^{-/-}$ cells exhibit no FLK1$^+$ deficiency and FLK1$^+$ cell numbers can be further induced with the addition of VEGF and bFGF, or by hypoxia treatment.

Table 1. Growth factor influence on FLK1 surface expression

	Experiment 1						Experiment 2		Experiment 3	
	Day 3.5			Day 4.5			Day 3.5		Day 3.5	
Cultures	*Arnt*$^{+/+}$	*Arnt*$^{-/-8}$	*Arnt*$^{-/-9}$	*Arnt*$^{+/+}$	*Arnt*$^{-/-8}$	*Arnt*$^{-/-9}$	*Arnt*$^{+/+}$	*Arnt*$^{-/-8}$	*Arnt*$^{+/+}$	*Arnt*$^{-/-8}$
Normoxia	7.1	2.6	0.94	17.6	1.34	1.15	6.7	5.3	4.4	3.26
Hypoxia	33.6	3.8	1.61	12.7	1.1	2.76	21	10.3	17.6	6.5
BMP2,4	12.6	1.8	0.55	7.73	2.08	0.99				
BMP2,4, VEGF, bFGF	8.1	2.47	0.47	8.08	3.07	0.66				
TGFβ1,3	11.6	2.34	0.76							
TGFβ1,3, VEGF, bFGF	12.2	2.1	0.92	4.8	1.57	1.12				
BMP2,4, TGFβ1,3, VEGF, bFGF	10.7	2.03	0.62	4.65	1.68	0.6				
VEGF, bFGF	11.3	4.67	0.81							
ANG1,2							3.0	3.0		
ANG1							6.5	7.6		
ANG2							4.1	4.8		
BMP2,4, ANG1, TGFβ1,3, VEGF, bFGF							3.0	4.8		
BMP2,4, ANG2, TGFβ1,3, VEGF, bFGF							3.1	4.7		
EPO									2.13	2.0

Differentiating *Arnt*$^{+/+}$, *Arnt*$^{-/-8}$ and *Arnt*$^{-/-9}$ EBs were treated under normoxic (21% O_2) and hypoxic (3% O_2) conditions, and with various combinations of growth factors, and analyzed for the number of FLK1$^+$ cells (shown as a percentage) by flow cytometry.

containing cells with mixed genotype. Importantly, surviving colonies from G418-treated, mixed cultures resulted in a robust number of BL-CFCs (Table 2). Untreated and treated cultures yielded 580 (±45.2) and 264 (±50.8) BL-CFCs, respectively (Table 2). Furthermore, to account for any potential plating variability, the percentages of blast colonies obtained from individual cultures were compared. BL-CFCs represent 81% of the total cell population in treated cultures and 82% in untreated cultures. These results suggest that both *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ cells from mixed EBs can contribute equally to the number of BL-CFCs. To confirm that the BL-CFCs were truly of *Arnt*$^{-/-}$ origin, individual colonies were picked and stained for β-gal activity. Importantly, all colonies picked from mixed plates that underwent selection were positive for β-gal (Fig. 6B). Furthermore, co-culture experiments in which *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ cells were separated by 3 μm pore transwells were assayed. To our surprise, we were unable to induce the surface expression of FLK1 in *Arnt*$^{-/-}$ cultures (data not shown). The findings from these analyses indicate that the *Arnt*$^{-/-}$ FLK1$^+$ cells of BL-CFC defects are not cell-intrinsic, but appear to require cell-cell contact or nondiffusable molecules provided by wild-type cells that induce FLK1 expression and the formation of BL-CFCs. The identity of this molecule(s) remains unknown.



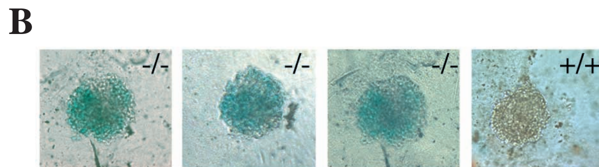


Fig. 6. The BL-CFC defect in *Arnt*$^{-/-}$ cells is cell extrinsic. (A) *Arnt*$^{+/+}$ *neo*^{s} *lacZ*$^-$ and *Arnt*$^{-/-}$ *neo*^{r} *lacZ*$^+$ ES cells co-cultured during EB differentiation, dissociated and replated in methylcellulose with or without G418. (B) Under G418 selection, all surviving BL-CFCs stained blue indicating β-galactosidase activity from *Arnt*$^{-/-}$ *neo*^{r} *lacZ*$^+$ cells. Wild-type clones only survived in the absence of G418.

Discussion

Growing evidence indicates that physiological hypoxia regulates both embryonic angiogenesis and hematopoiesis (Ramirez-Bergeron and Simon, 2001). The intimately coordinated development of endothelial and hematopoietic lineages suggests that hypoxia may control the formation

Table 2. *Arnt*$^{-/-}$ rescue

Cultures	G418†	Trans-CFC	BL-CFC	(%) BL/ (BL+Trans)
Arnt$^{+/+}$	+	0	0	0
Arnt$^{+/+}$	–	76.0 (±40.4)	253 (±30.8)	76
Arnt$^{-/-}$	+	257 (±95.8)	182 (±48.0)	41
Arnt$^{+/+}$:*Arnt*$^{-/-}$*	–	134 (±10.6)	580(±45.2)	81
Arnt$^{+/+}$:*Arnt*$^{-/-}$*	+	58 (±4.8)	264 (±50.8)	82

5×10^4 cells were replated in methylcellulose.
*1×10^5 cells were replated in these cases.
†Selection with 0.4 mg/ml of G418.
Colonies, transitional (Trans-CFC) and hemangioblast (BL-CFC), were counted from triplicate plates and expressed either as an average (±s.d.) or as percentages.

and/or function of the ‘hemangioblast’, a bipotential stem cell precursor of both lineages. Hypoxic regulation of hemangioblast differentiation depends on the function of HIF, which can directly or indirectly activate the expression of many genes crucial for hemangioblast development, including *Flk1*, *Vegf* and *bFgf* (Corpechot et al., 2002; Forsythe et al., 1996; Liu et al., 1995; Wood et al., 1996). In this paper, we present data indicating that hypoxia enhances the number, and accelerates the kinetics, of hemangioblasts (BL-CFCs) generated from differentiating ES cells, and that this regulation is HIF-dependent. In addition, hypoxia regulates early mesodermal events by accelerating *Bry* expression in EB cultures. Moreover, ARNT (HIF1β)-deficient ES cells generate fewer BL-CFCs and display a concomitant increase in Trans-CFCs, which suggests a block in *Arnt*$^{-/-}$ hemangioblast differentiation. We provide data implicating VEGF (an important vascular and hematopoietic cytokine and a direct HIF transcriptional target) in the proper induction of hemangioblast cells by using *Vegf* mutant ES clones. Although exogenous growth factors, including VEGF and bFGF, are not sufficient to fully rescue the *Arnt*$^{-/-}$ mutation, subsequent experiments determined that the BL-CFC defect is not cell-intrinsic, suggesting that additional targets of HIF are required for the early events of mesoderm differentiation into endothelial/hematopoietic precursors.

In this report, we show that hypoxia influences the kinetics of early EB differentiation. First, the expression of the mesodermal T-box gene *Bry* was induced one day earlier under hypoxic conditions, as was *Bmp4*, a gene encoding a growth factor involved in the ventralization of early mesoderm and in blood formation (Czyz and Wobus, 2001; Hogan, 1996; Jones et al., 1996; Winnier et al., 1995). However, although BRY-GFP$^+$ cell numbers are increased, the induction is not as considerable as FLK1 expression. This could be interpreted in two ways. First, flow cytometry is an underrepresentation of true BRY$^+$ cell numbers. Second, hypoxia increases the amount of *Bry* transcript per cell but not the overall number of BRY$^+$ cells. Significantly, Fehling et al. reported that BMP4 expression is limited to the BRY$^+$FLK1$^+$ population of cells in EB cultures (Fehling et al., 2003), supporting its role as a growth factor involved in mesodermal progenitors that give rise to BL-CFCs. We determined that *Bmp4* transcripts are deficient in *Arnt*$^{-/-}$ EBs, suggesting an indirect role of HIFα/ARNT in mesoderm and hemangioblast development. In the developing mouse embryo, mesoderm emerges from the primitive streak at 6.5-7.0 dpc. Of note, we have demonstrated that *Arnt*$^{-/-}$*Arnt2*$^{-/-}$ embryos die prior to 7.0 dpc, possibly because of inappropriate mesoderm differentiation (Keith et al., 2001). Hypoxia also increased the expression of the *Flk1* transcript, the number of FLK1$^+$ cells and the number of BL-CFCs generated. Moreover, hypoxia influenced the kinetics of BL-CFCs by accelerating their interval of appearance, peaking about one day earlier in hypoxic cultures compared with normoxic ones. Thus, it appears that low O_2 triggers an accelerated and increased commitment of mesoderm to hemangioblast progenitors. These hypoxic responses are likely to promote the O_2-delivering capacity of the embryo in accord with increased metabolic demand.

Previous analysis revealed that the frequency of BL-CFCs is lower than the percentage of FLK1$^+$ cells. We demonstrate that the kinetics of BL-CFC production is shorter than of FLK1 expression, as FLK1$^+$ cells are maintained beyond the narrow window of time during which BL-CFCs can be generated. Thus, FLK1$^+$ cells are a heterogeneous population, only a fraction of which represents a true hemangioblast progenitor. FLK1 surface expression is maintained in more mature cell types, such as endothelial cells. Nevertheless, FLK1 has been used as an experimental readout for mesoderm commitment to hemangioblasts because its expression appears to be necessary for BL-CFC formation (Faloon et al., 2000). Thus, FLK1 surface expression is a useful tool in screening cultures for BL-CFC potential, demonstrating that an assortment of growth factors are not sufficient to rescue *Arnt*$^{-/-}$ ES cells.

The defect in *Arnt*$^{-/-}$ BL-CFC formation appears to be at the level of mesoderm commitment to hemangioblast cell fate and/or subsequent hemangioblast differentiation, as *Arnt*$^{-/-}$ cells produce a preponderance of transitional colonies at the apparent expense of hemangioblasts. A similar phenotype was described for both cells and mice with a null mutation in the gene encoding the bHLH transcription factor SCL. In vivo, SCL is essential for hematopoiesis and vascular remodeling (Robb et al., 1995; Shivdasani et al., 1995; Visvader et al., 1998); in vitro, *Scl*$^{-/-}$ EBs fail to generate BL-CFCs but produce transitional colonies (Faloon et al., 2000; Robertson et al., 2000). Thus, both *Arnt* and *Scl* appear to regulate hemangioblast development from early mesoderm precursors. Although these results suggest that the hemangioblast defect should lead to a complete absence of endothelial development, the fact that *Scl*$^{-/-}$ and *Arnt*$^{-/-}$ embryos are able to establish a primary vascular system may be due to the existence of independent sources of endothelial precursors, as have been shown in the avian system (Pardanaud and Dieterlen-Lievre, 1999). Unfortunately, we are unable to explore this possibility further because it is difficult to assess somitic mesoderm in ES cell cultures.

FLK1 is a cell-surface receptor for VEGF, and hemangioblast numbers are elevated in response to VEGF in methylcellulose assays. We have demonstrated that VEGF expression is stimulated under hypoxic conditions in wild-type EBs, and the number of BL-CFCs increases with the addition of VEGF to differentiating *Arnt*$^{+/+}$ EBs. Although *Vegf*$^{+/-}$ and *Vegf*$^{-/-}$ EBs produce a significant number of FLK1$^+$ cells, they are still defective in generating appropriate BL-CFCs. Thus, it appears that appropriate levels of VEGF are required during the differentiation of EBs into hemangioblasts but not FLK1$^+$ cells.

Although our data support a role for VEGF in the production of hemangioblast colonies, other factors are essential. Faloon et al. suggest that bFGF mediates hemangioblast proliferation while VEGF regulates blast migration (Faloon et al., 2000). Moreover, they were able to generate wild-type levels of FLK1$^+$ cell numbers from *Scl*$^{-/-}$ ES cells, and could further induce their numbers with the addition of bFGF during EB differentiation. However, they failed to determine whether the FLK1$^+$ cells generated with the addition of bFGF were rescued for their ability to generate BL-CFC colonies. Indeed, we stimulated FLK1$^+$ cell numbers with the addition of VEGF and/or bFGF in *Vegf* mutant clones, but not in two independent *Arnt*$^{-/-}$ clones (Fig. 5C). Again, *Arnt*$^{-/-}$ clones may be blocked at transitional stages, which are refractory to growth factor signaling, as we have discovered that multiple combinations of a large number of growth factors were insufficient to rescue

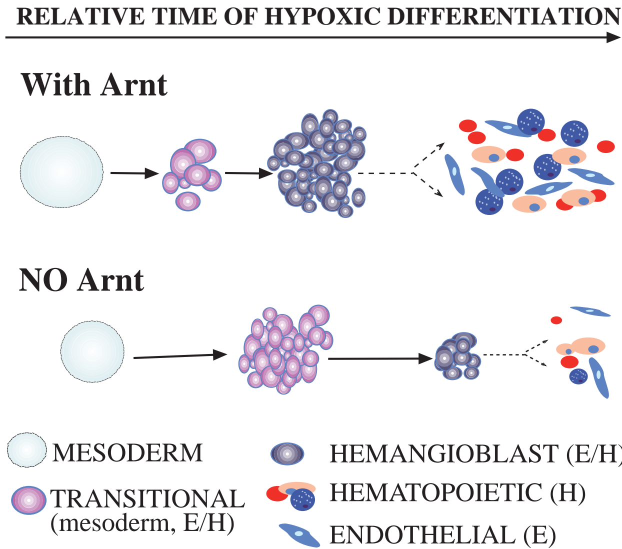


Fig. 7. Model of ARNT activity during early embryonic development. HIF responses are required for the proper differentiation, survival or proliferation of mesoderm and hemangioblasts. In the absence of ARNT, mesoderm differentiation is delayed. Furthermore, ARNT deficiency results in reduced mesoderm differentiation and increased transitional cell numbers, suggesting a block in commitment to the hemangioblast phenotype. Fewer hemangioblasts lead to a reduction in the numbers of hematopoietic and endothelial precursors.

FLK1 expression. Importantly, in a definitive co-culture experiment, we demonstrated that the *Arnt*$^{-/-}$ BL-CFC defect is rescued in the presence of wild-type cells during EB differentiation, but FLK1^{+} cell numbers failed to be stimulated when the *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ cells were separated by 3 μm transwells. These results suggest that signaling via cell surface or poorly diffusible molecules provided by the *Arnt*$^{+/+}$ cells in mixed EBs can induce proper differentiation of *Arnt*$^{-/-}$ ES cells into FLK1^{+} cells. Further experiments will focus on the identification of such a factor(s).

In conclusion, we demonstrated that ARNT, as a subunit of HIF, is important for the generation of the common precursors of cells (blood vessels and blood cells) that supply O_2 and nutrients to a growing embryo. Our model suggests that in response to low O_2, HIF first stimulates *Bry*, a mesoderm gene, and BMP4, a mesodermal promoting factor. These in vitro assays suggest that the hypoxic environment supports further mesoderm maturation, such as the emergence of transitional colonies and their subsequent differentiation into appropriate numbers of hemangioblasts (see Fig. 7). Lack of ARNT and an improper hypoxic response results in a block in differentiation, whereby *Arnt*$^{-/-}$ cultures are arrested and accumulate at the transitional stage. Delayed differentiation and decreased numbers of hemangioblast progenitors are likely to contribute to the vascular and hematopoietic defects noted in the *Arnt*$^{-/-}$ and *Hif1*$\alpha^{-/-}$ embryos. Therefore, development of the blood and vascular systems is regulated at very early stages by appropriate responses to O_2 availability.

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