Multilineage embryonic hematopoiesis requires hypoxic ARNT activity

David M. Adelman,[1,5] Emin Maltepe,[2] and M. Celeste Simon[2–5]

[1]Department of Pathology, [2]Committee on Cancer Biology, [3]Departments of Medicine and Molecular Genetics and Cell Biology, and the Howard Hughes Medical Institute (HHMI), University of Chicago, Chicago, Illinois 60637 USA

Although most cells undergo growth arrest during hypoxia, endothelial cells and placental cytotrophoblasts proliferate in response to low O_2. We demonstrate that proliferation of embryonic multilineage hematopoietic progenitors is also regulated by a hypoxia-mediated signaling pathway. This pathway requires HIF-1 (HIF-1α/ARNT heterodimers) because *Arnt*$^{-/-}$ embryoid bodies fail to exhibit hypoxia-mediated progenitor proliferation. Furthermore, *Arnt*$^{-/-}$ embryos exhibit decreased numbers of yolk sac hematopoietic progenitors. This defect is cell extrinsic, is accompanied by a decrease in ARNT-dependent VEGF expression, and is rescued by exogenous VEGF. Therefore, "physiologic hypoxia" encountered by embryos is essential for the proliferation or survival of hematopoietic precursors during development.

Received July 9, 1999; revised version accepted August 17, 1999.

Low O_2 tension (hypoxia) is a condition commonly associated with pathology. Mammals adapt to hypoxia on an organismal level by increasing expression of erythropoietin (EPO) resulting in increased red blood cell production (Semenza et al. 1991), and vascular endothelial growth factor (VEGF) to promote increased vascularization of affected tissues (Shweiki et al. 1992; Forsythe et al. 1996). On a cellular level, mammals adapt to decreased O_2 by increasing expression of glycolytic enzymes (Bunn and Poyton 1996; Semenza et al. 1996; Wenger and Gassmann 1997) and glucose transporters (Bashan et al. 1992) for increased anaerobic respiration.

Whereas the growth and division of many cell types is suppressed until O_2 tensions return to normoxic levels (20% O_2) (Graeber et al. 1996; Carmeliet et al. 1998), certain cell types must grow and proliferate in response to decreased O_2. These include placental cytotrophoblasts (Genbacev et al. 1997), which form the maternal–fetal interface in the womb (Rodesch et al. 1992; Fischer and Bavister 1993), and vascular endothelial cells, which proliferate to form new capillaries in hypoxic tissues (Phillips et al. 1995). A critical component of the hypoxic response machinery is the bHLH–PAS transcription factor complex hypoxia-inducible factor 1 (HIF-1). HIF-1 activates the expression of genes involved in a broad spectrum of adaptive responses to oxygen deprivation ranging from basic metabolism to angiogenesis and erythropoiesis (Bunn and Poyton 1996; Wenger and Gassmann 1997; Maltepe and Simon 1998), and may impact cell-cycle regulation by interacting with and stabilizing the tumor supressor protein p53 (An et al. 1998). HIFs are obligate heterodimers comprised of the bHLH–PAS proteins HIF-1α (or HIF-2α) and the arylhydrocarbon receptor nuclear translocator (ARNT; HIF-1β). All components are constitutively expressed, although HIF-1α and HIF-2α are quickly degraded under normoxia and stabilized under hypoxia (Salceda and Caro 1997; Wiesener et al. 1998), allowing for HIF activity specifically under hypoxic conditions.

Before establishment of a circulatory system capable of delivering oxygenated blood to the embryo, mammalian development occurs in an environment exhibiting O_2 concentrations within the hypoxic range (Rodesch et al. 1992; Fischer and Bavister 1993). In E8.5–E18 mouse embryos, HIF-1α protein is detectable, demonstrating the presence of a hypoxic environment (Iyer et al. 1998). In embryonic stem (ES) cells lacking HIF subunits, the hypoxic transcriptional response is ablated, and animals lacking HIF-1α or ARNT exhibit an embryonic lethality by E9.5 or E10.5, respectively (Kozak et al. 1997; Maltepe et al. 1997; Carmeliet et al. 1998; Iyer et al. 1998; Ryan et al. 1998). Specifically, *Arnt*$^{-/-}$ embryos exhibit defects in blood vessel formation in the yolk sac, branchial arches, and placenta (Kozak et al. 1997; Maltepe et al. 1997). These results suggest that HIF-1-mediated hypoxic gene regulation is important for proper vascular development.

Vascular endothelial cells and hematopoietic stem cells are thought to arise from a bipotential hemangioblast (Choi et al. 1998), based on spatial and temporal association during development, as well as common expression of many cytokines, cytokine receptors, and transcription factors (Ferrara et al. 1996; Shalaby et al. 1997). Because endothelial cells are known to proliferate under hypoxia, we wished to determine whether the proliferation and expansion of hematopoietic progenitors is also stimulated by low O_2. We report here that hematopoietic progenitors proliferate in response to hypoxia in an ARNT-dependent manner. This requirement for ARNT is cell extrinsic, in that *Arnt*$^{-/-}$ ES cells contribute competitively to all hematopoietic lineages in chimeric animals. Importantly, decreased hematopoietic progenitor numbers in *Arnt*$^{-/-}$ embryoid bodies (EBs) can be rescued with exogenous VEGF. These data implicate hypoxic VEGF production as a possible mechanism for both endothelial cell and hematopoietic progenitor cell proliferation in the developing embryo.

Results and Discussion

To determine whether hypoxia stimulates hematopoietic progenitor number, we differentiated ES cells in

[*Key Words*: ARNT; HIF; hypoxia; hematopoiesis; VEGF; embryoid body; ES cells]

[4]Corresponding author. [5]Present address: Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104 USA.
E-MAIL csimon@medicine.bsd.uchicago.edu; FAX (215) 746-5511.

vitro in 3% O_2 to form EBs, from which hematopoietic progenitors were enumerated in colony forming unit (CFU) assays (Keller et al. 1993; Kennedy et al. 1997) (Fig. 1a). Methylcellulose cultures were scored for erythrocyte (E), macrophage (M), granulocyte–erythrocyte–megakaryocyte–macrophage (GEMM), granulocyte–macrophage (GM), and granulocyte (G) CFU (Fig. 1b). As shown in Figure 1a, hypoxia induced a significant increase ($P < 0.006$ to 0.04) in most CFU types generated by wild-type EBs, similar to results seen previously with human bone marrow (Maeda et al. 1986). To test whether the observed hypoxia-induced progenitor expansion is ARNT (HIF-1) dependent, *Arnt*$^{-/-}$ ES cells were used in the same assay. In contrast to wild-type controls, no hypoxic stimulation of *Arnt*$^{-/-}$ progenitor numbers was detected (Fig. 1a). These results suggest that hypoxia, within the range encountered by a developing mammalian embryo, stimulates expansion of hematopoietic progenitors in an ARNT-dependent manner.

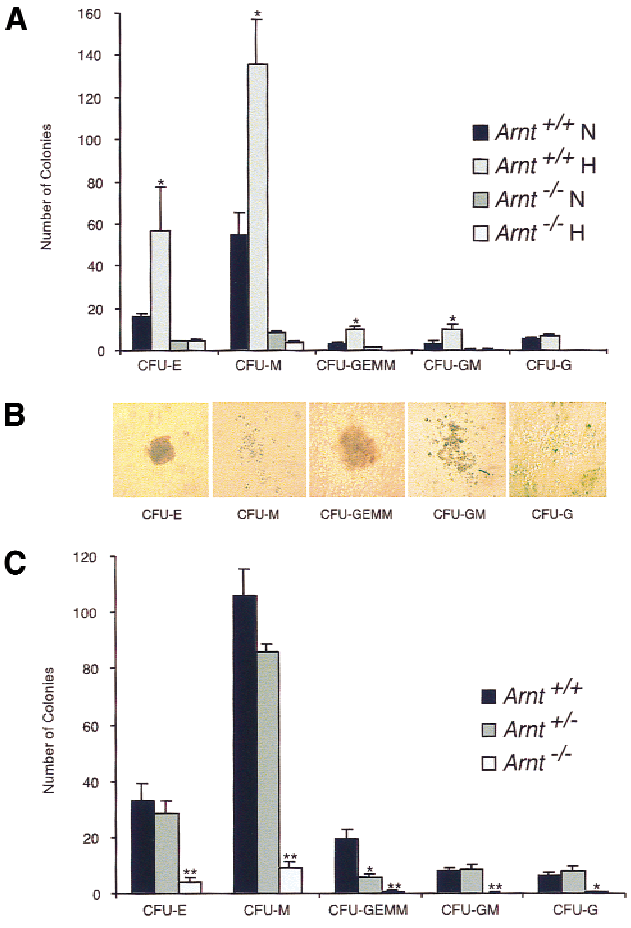

Figure 1. In vitro differentiation of ES cells and replating of hematopoietic progenitors. (*a*) Hypoxia. ES cells were differentiated for 9 days under 20% O_2, 5% CO_2, and 75% N_2 (N) or 3% O_2, 5% CO_2, and 92% N_2 (H) and CFU scored 6–7 days after replating under normoxic conditions. A significant increase (* $P < 0.05$) was seen in *Arnt*$^{+/+}$ CFU-E, CFU-M, CFU-GEMM, and CFU-GM cultured under hypoxia. No increase was seen from *Arnt*$^{-/-}$ EBs under hypoxia. (*b*) Representative CFU in methylcellulose (original magnification; 10x). (*c*) Normoxia. ES cells were differentiated for 9 days and CFU scored 6–7 days after replating. CFU-E, CFU-M, CFU-GEMM, and CFU-GM (** $P < 0.005$) and CFU-G (* $P < 0.05$) were all reduced in *Arnt*$^{-/-}$ EBs. Also, *Arnt*$^{+/-}$ CFU-GEMM showed a significant decrease (* $P < 0.05$) compared to *Arnt*$^{+/+}$ ($n = 4$).

Normoxic progenitor levels from *Arnt*$^{-/-}$ EBs were also significantly decreased as compared to those derived from wild-type EBs (Fig. 1a). EBs are three-dimensional structures that exhibit O_2 gradients due to their relatively large size (Gassmann et al. 1996). Thus, even when cultured under normoxic conditions (20% O_2) EBs can contain regions of mild hypoxia. To confirm that the progenitor defect in *Arnt*$^{-/-}$ EBs was due to an inability to respond to this mild hypoxia, rather than a toxic consequence of gene targeting and subsequent selection procedures, *Arnt*$^{+/-}$ ES cells that had been subjected to the same selection protocol (Mortensen et al. 1992) as the *Arnt*$^{-/-}$ ES cells were also compared in this assay. Because *Arnt*$^{+/-}$ ES cells do not exhibit significant defects in hypoxia responses (Maltepe et al. 1997), these cells provide a control for cell viability. As shown in Figure 1c, *Arnt*$^{-/-}$ ES cells generated significantly fewer CFU-E, CFU-M, CFU-GEMM, CFU-GM, and CFU-G progenitors ($P < 8.7 \times 10^{-5}$ to 0.01) at 20% O_2 than wild-type or heterozygous ES cells. These data suggest a primary hematopoietic defect in *Arnt*$^{-/-}$ embryoid bodies that is not simply a result of in vitro manipulation.

To ascertain whether ARNT-mediated hypoxia responses are also required for the expansion of hematopoietic progenitors in vivo, *Arnt*$^{+/-}$ animals were mated to yield *Arnt*$^{+/+}$, *Arnt*$^{+/-}$, and *Arnt*$^{-/-}$ offspring. Because *Arnt*$^{-/-}$ animals exhibit embryonic lethality by E10.5 (Kozak et al. 1997; Maltepe et al. 1997), E9.5 embryos were analyzed. The production of extraembryonic yolk sac blood islands marks the beginning of hematopoiesis and vasculogenesis in the mouse embryo. Many E9.5 *Arnt*$^{-/-}$ embryos lack blood-filled vitelline vessels, suggesting a possible deficiency in blood cell maturation in addition to the previously described vascular defects (Maltepe et al. 1997). Hematopoietic colony formation assays performed with *Arnt*$^{+/+}$, *Arnt*$^{+/-}$, and *Arnt*$^{-/-}$ E9.5 yolk sacs are shown in Figure 2a. A statistically significant decrease in the number of CFU-E, CFU-M, CFU-GEMM, CFU-GM, and CFU-G progenitors was observed in *Arnt*$^{-/-}$ yolk sacs ($P < 2.2 \times 10^{-5}$ to 0.005). Interestingly, *Arnt*$^{+/-}$ mice also generated significantly fewer (~50%) CFU-GEMM and CFU-GM than *Arnt*$^{+/+}$ animals ($P < 0.02$ to 0.03). This finding was reminiscent of the 50% reduction in CFU-GEMM observed after in vitro differentiation of the *Arnt*$^{+/-}$ EBs (see Fig. 1c). To ensure that the hematopoietic defects seen in the *Arnt*$^{-/-}$ embryos were not due to the stunted growth and apoptotic cell death observed in some E9.5 *Arnt*$^{-/-}$ embryos (data not shown), we performed yolk sac progenitor assays on E8.5 embryos that appear morphologically indistinct from their age-matched *Arnt*$^{+/+}$ and *Arnt*$^{+/-}$ littermates. Only CFU-E could be properly enumerated at this earlier developmental stage as other CFU progenitors are produced at very low numbers (Olson et al. 1995). Like E9.5 embryos, the E8.5 *Arnt*$^{-/-}$ embryos showed a significant

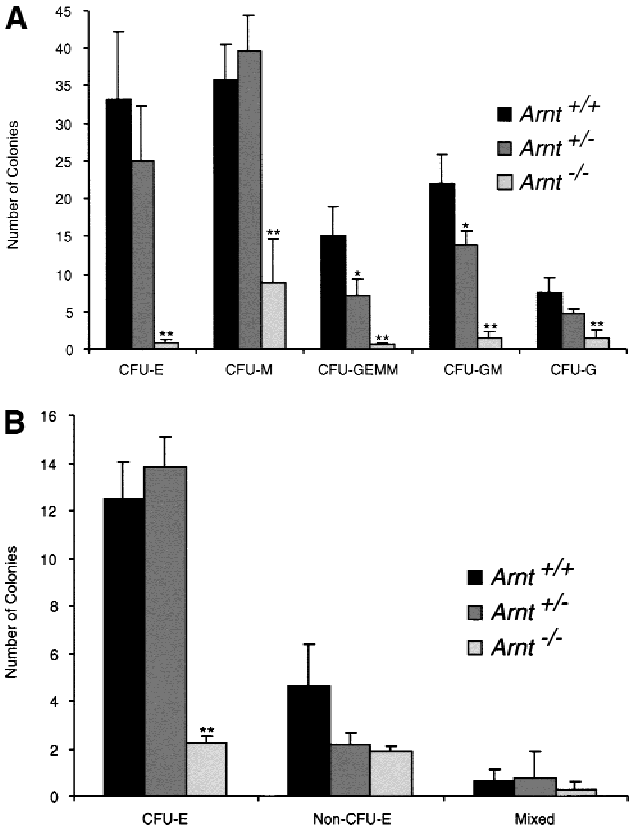

Figure 2. Yolk sac hematopoietic colony formation assays. (*a*) E9.5 yolk sac colonies from *Arnt*$^{+/+}$ (n = 12), *Arnt*$^{+/-}$ (n = 22), and *Arnt*$^{-/-}$ (n = 11) embryos are shown. All *Arnt*$^{-/-}$ CFU showed significant decreases (** $P < 0.005$) when compared to *Arnt*$^{+/+}$ CFU, as did the CFU-GEMM and CFU-GM of *Arnt*$^{+/-}$ yolk sacs (* $P < 0.05$). (*b*) E8.5 yolk sac colonies from *Arnt*$^{+/+}$ (n = 16), *Arnt*$^{+/-}$ (n = 36), and *Arnt*$^{-/-}$ (n = 12) embryos are depicted. *Arnt*$^{-/-}$ CFU-E showed significant decreases (** $P < 0.0005$) when compared to *Arnt*$^{+/+}$ or *Arnt*$^{+/-}$ CFU. Non-CFU-E contain cells of the myeloid lineages, and Mixed contain both erythroid and myeloid cells.

decrease in hematopoietic progenitor number (Fig. 2b; $P < 0.0005$). These data indicate that ARNT is required for the production of normal numbers of yolk sac hematopoietic progenitors in vivo. Furthermore, the in vitro differentiation data confirm the primary nature of this hematopoietic defect, as placental or other vascular defects are circumvented during in vitro EB culture, and therefore, do not induce secondary defects, as may occur in E9.5 *Arnt*$^{-/-}$ embryos.

To establish whether the ARNT-mediated hematopoietic defect was intrinsic to CFU progenitors, we injected *Arnt*$^{+/-}$ and *Arnt*$^{-/-}$ ES cells (129 strain) into wild-type C57BL/6 blastocysts and assayed chimeric animals for CFU formation in adult bone marrow (Robb et al. 1996; Wang et al. 1998) (Fig. 3a). The degree of ES cell contribution was initially estimated by coat color, and more precisely determined by Southern blot analysis of bone marrow DNA. Although the progenitors represent a small subset of total bone marrow cells, this assessment of ES cell contribution is the most technically feasible. Femur and tibial bone marrow was extracted from 6-week-old animals and cultured in methylcellulose in the presence or absence of G418, which selects for neomycin resistance (*neo*^{r}) introduced during the original ES cell targeting. Thus, the number of *neo*^{r} clones derived from these animals reflects the relative contribution of the *Arnt*$^{+/-}$ or *Arnt*$^{-/-}$ hematopoietic progenitors. *Arnt*$^{+/-}$ ES cells behave like *Arnt*$^{+/+}$ ES cells, with the exception of a modest but significant decrease in CFU-GEMM (Fig. 1c), indicating that *Arnt*$^{+/-}$ ES cells should contribute to progenitor populations like *Arnt*$^{+/+}$ ES cells in chimeras. As expected, the number of G418-resistant (G418^{r}) *Arnt*$^{+/-}$ CFUs correlated well with the degree of *Arnt*$^{+/-}$ ES cell contribution to chimeric animals (Table 1). If the *Arnt*$^{-/-}$ progenitor deficiency were due to a cell-intrinsic defect, few to no G418^{r} CFUs should appear in cultures of bone marrow cells derived from *Arnt*$^{-/-}$ chimeric animals. Interestingly, the number of G418^{r} CFUs derived from these animals correlated with the percent chimerism of each animal, indicating a cell-extrinsic defect (Table 1). Southern blot analysis of G418^{r} colonies confirmed they were exclusively derived from *Arnt*$^{-/-}$ ES cells (Fig. 3b).

There is considerable evidence to suggest yolk sac and bone marrow progenitor populations are derived from distinct mesodermal progenitor cells (Medvinsky and Dzierzak 1996). Because *Arnt*$^{-/-}$ embryos display a yolk sac progenitor defect, we wanted to determine whether this population, in addition to the bone marrow progeni-

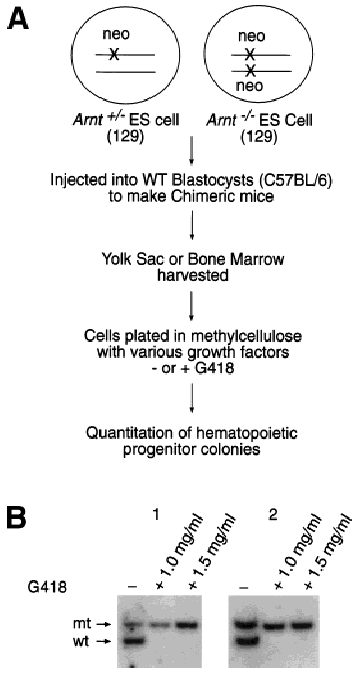

Figure 3. Assay of ES-cell-derived G418^{r} CFU in *Arnt*/wild-type chimeras. (*a*) G418 selection strategy to distinguish wild-type or ES-derived CFU from chimeric animals. (*b*) Southern blot analysis of *Arnt*$^{-/-}$ chimera bone marrow CFU cultured in either 0, 1.0, or 1.5 mg/ml G418. Only the targeted *Arnt* allele (mt) remains after G418 selection, indicating the presence of solely *Arnt*$^{-/-}$-derived G418^{r} CFU.

Table 1. Arnt$^{+/-}$ *and* Arnt$^{-/-}$ *ES cells contribute to bone marrow and yolk sac hematopoietic progenitors*

Tissue	ES cell genotype	Chimera	Percent ES[a]	CFU[b] total	CFU[b] G418^r	CFU[b] %G418^r
Bone marrow		1	0	249	0	0
		2	0	303	0	0
	+/−	3	50	294	62	21
		4	50	165	58	35
		5	60	297	152	51
		1	70	315	195	62
		2	70	352	266	76
	−/−	3	70	385	314	82
		4	70	338	240	71
		5	75	329	259	79
Yolk sac		1	40	732	352	48
		2	40	816	326	40
	−/−	3	55	804	482	60
		4	55	1016	440	43
		5	80	644	578	90

[a]Percentage of ES cell contribution was determined by Southern blot analysis of bone marrow DNA or embryo DNA (for yolk sac analyses).
[b]G418^r CFU were selected in 1.5 mg/ml G418.

tor population, exhibits a cell-extrinsic defect. Toward this end, we generated chimeric animals with *Arnt*$^{-/-}$ ES cells and harvested E10.5 yolk sacs. The level of chimerism of each animal was determined by Southern blot analysis of the embryo proper, and yolk sac cells grown in duplicate methylcellulose cultures in the presence and absence of G418. Similar to results observed from the chimeric bone marrow assays, the number of yolk sac G418^r CFUs correlated with the degree of chimerism, demonstrating a cell extrinsic defect of the yolk sac progenitor population (Table 1). Taken together these studies demonstrate that the *Arnt*$^{-/-}$ hematopoietic defect is not intrinsic to hematopoietic progenitors, but instead due to a specific defect in other cell populations.

One potential explanation that might account for these findings is that *Arnt*$^{-/-}$ yolk sacs produce insufficient levels of extracellular cytokines essential for hematopoietic progenitor proliferation (Wu et al. 1995; Lin et al. 1996). Both EPO and VEGF are critical hematopoietic cytokines (Wu et al. 1995; Ferrara et al. 1996; Lin et al. 1996; Shalaby et al. 1997)and direct targets of ARNT transcriptional activity (via HIF-1) (Bunn and Poyton 1996; Wood et al. 1996; Maltepe et al. 1997; Wenger and Gassmann 1997). To determine whether these cytokines were dysregulated in our in vitro cultures, RNA extracted from *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ EBs differentiated under both normoxic and hypoxic conditions was analyzed by Northern blot. Normoxically cultured *Arnt*$^{+/+}$ EBs produced threefold more VEGF mRNA (including multiple splice variants) than *Arnt*$^{-/-}$ EBs (Fig. 4a), likely due to an inability of the *Arnt*$^{-/-}$ cells to respond to the intrinsic hypoxia of EBs. When *Arnt*$^{+/+}$ EBs were cultured in 3% O_2, they displayed a 4.3-fold increase in VEGF production over normoxic levels. In contrast, and consistent with previous studies, *Arnt*$^{-/-}$ EBs showed only a slight increase (twofold) in VEGF expression in response to hypoxia (Fig. 4a). We were unable to detect EPO expression in any of the EBs with this assay (data not shown). To determine whether inadequate VEGF levels contributed to the *Arnt*$^{-/-}$ progenitor defect, we added exogenous VEGF to EB cultures. As indicated in Figure 4b, exogenous VEGF restored CFU-E, CFU-M, CFU-GEMM, and CFU-GM progenitor numbers to wild-type levels in *Arnt*$^{-/-}$ EBs. In direct contrast, addition of EPO failed to increase progenitor number in the *Arnt*$^{-/-}$ EBs, demonstrating that inadequate EPO production is not responsible for the multilineage hematopoietic defect seen in *Arnt*$^{-/-}$ EBs (Fig. 4c).

Other cytokines, including GM-CSF, IL-1, IL-3, and SCF, are not known to be direct targets of ARNT transcriptional activity, but were also tested in the methylcellulose CFU assay. All failed to rescue a variety of CFU progenitors (data not shown), although SCF did partially rescue the CFU-E precursors (data not shown). Thus, we conclude that VEGF plays a specific and unique role in the proliferation or survival of a broad spectrum of hematopoietic progenitors during EB formation. Low levels of VEGF mRNA detected in *Arnt*$^{-/-}$ EBs is consistent with previous data obtained from *Arnt*$^{-/-}$ embryos exhibiting decreased VEGF mRNA expression (Maltepe et al. 1997). It has been suggested recently that signaling through Flk-1, a known VEGF receptor, is important for hematopoietic progenitor proliferation, but not initial progenitor formation (Schuh et al. 1999), and the requirement for Flk-1 signaling may be dependent on the hematopoietic microenvironment (Hidaka et al. 1999). We propose that ARNT-mediated hypoxic induction of VEGF is a critical developmental signal acting through the Flk-1 receptor to promote proliferation of both vascular endothelial and hematopoietic progenitor cells.

Cytokines, cytokine receptors, and transcription factors represent well-established determinants of hematopoiesis. However, the studies described in this report demonstrate for the first time that hypoxia, a condition normally associated with pathology, is a critical regulator of the proliferation, expansion, or survival of hematopoietic precursors during embryogenesis. Just as embryoid bodies contain mild regions of hypoxia due to the limitations of diffusion, so too, would a developing embryo. These data suggest that subsequent to E8.5, oxygen sensing and responsiveness become critical to the continued expansion and maturation of tissues involved in oxygen and nutrient delivery. Interestingly, this is precisely when *Arnt*$^{-/-}$ embryos begin to show stunted development. Therefore, we propose a molecular pathway in which the hypoxia-inducible factor HIF-1 activates

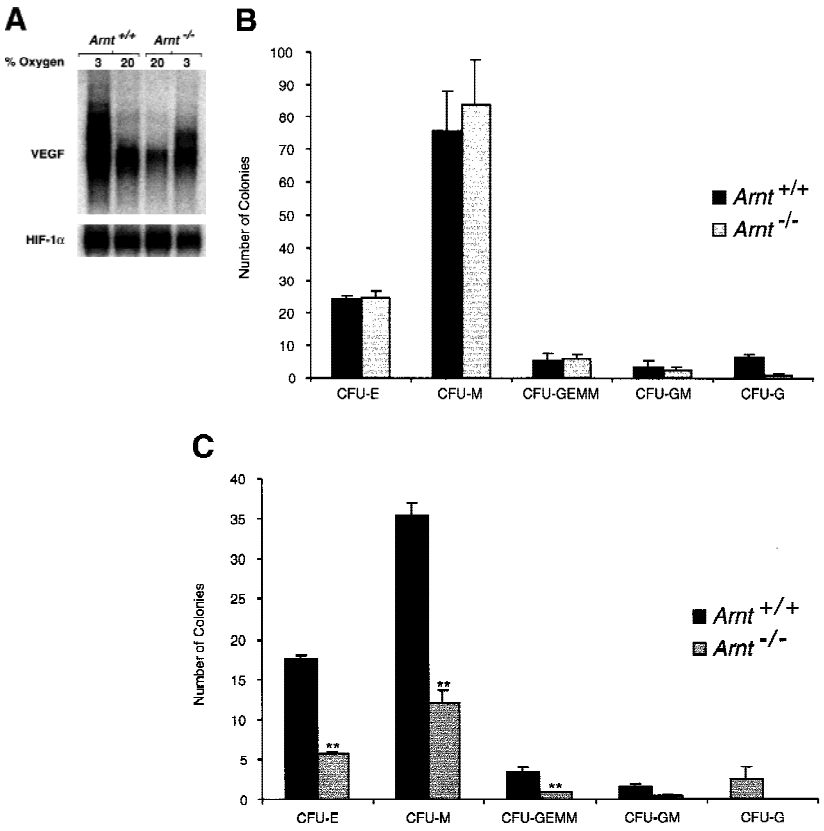

Figure 4. Cytokine rescue experiments. (*a*) Northern analysis of poly(A)-selected RNA (5 µg per lane) derived from $Arnt^{+/+}$ and $Arnt^{-/-}$ EBs, differentiated for 9 days under normoxia (20% O_2) or hypoxia (3% O_2). 3′-UTR probes for VEGF (recognizing multiple splice variants) and HIF-1α (as a loading control) were used. (*b*) Exogenous VEGF (5 ng/ml), when added to the primary differentiation medium, was able to rescue the number of CFU produced by $Arnt^{-/-}$ EBs to $Arnt^{+/+}$ levels ($n = 3$). (*c*) Exogenous EPO (2 U/ml) was not able to rescue progenitor number in $Arnt^{-/-}$ EBs, indicating its deficiency does not lead to the observed phenotype ($n = 3$).

the transcription of *Vegf*, the expression of which stimulates the proliferation or survival (Katoh et al. 1998) of hematopoietic progenitors. The decreased levels of a broad spectrum of hematopoietic progenitors in $Arnt^{-/-}$ embryos suggests a very early defect, possibly involving the hemangioblast, hematopoietic stem cell, or lineage-committed progenitor.

In addition to increasing our understanding of the molecular pathways that regulate embryonic hematopoiesis, our results may have therapeutic relevance. Cytotoxic chemotherapy treatments often leave patients with a decreased ability to regenerate short-lived erythroid and myeloid cells, as hematopoietic stem cells are particularly vulnerable to these agents (Domen and Weissman 1999). Such deficiencies often lead to worsening prognoses, requiring the use of stem cell transplantation. Current transplant protocols are severely curtailed by difficulties in maintaining and expanding hematopoietic stem cells in culture. The hypoxic culture conditions or exogenous VEGF administration discussed in this paper may represent simple and efficient methods of promoting hematopoietic progenitor cell expansion in vitro, thereby enhancing the feasibility of this treatment.

Materials and methods

Generation of ES cells, chimeras, and mice

$Arnt^{+/-}$ and $Arnt^{-/-}$ R1 ES cell clones were generated and genotyped by Southern blot analysis as described (Maltepe et al. 1997). $Arnt^{+/-}$ cells were selected in G418 during the production of $Arnt^{-/-}$ clones (Mortensen et al. 1992). Both heterozygous- and homozygous-targeted ES cells were used to generate chimeric mice, and a colony of $Arnt^{+/+}$ and $Arnt^{+/-}$ animals was maintained by standard $Arnt^{+/-}$ by $Arnt^{+/-}$ crosses. E10.5 yolk sacs or 6-week-old bone marrow were collected for hematopoietic progenitor assays. Chimerism was assessed by agouti coat-color contribution in the adult mice and by Southern blot analysis of the bone marrow, tail, and liver DNA (or E10.5 embryo proper DNA).

Hematopoietic progenitor assays

E8.5, E9.5, and E10.5 yolk sacs were dissected free of the embryo and incubated in 0.25% collagenase (Sigma) in PBS supplemented with fetal bovine serum for 1 hr, followed by mechanical shearing with a 22-gauge needle and syringe. Bone marrow from 6-week-old chimeras was obtained from the femur and tibia. Cells (total number from yolk sac, 2×10^4 from bone marrow) were then plated into methylcellulose medium (Stem Cell Technologies) supplemented with 15% fetal bovine serum, 1% BSA, 10 µg/ml Insulin, 200 µg/ml transferrin (iron-saturated), 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 10 ng/ml rmIL-3, 10 ng/ml rhIL-6, 5 ng/ml rmSCF, 0.5 ng/ml GM-CSF, and 3 U/ml rhEPO. Cultures of cells from chimeric mice were also supplemented with 0, 1.0, or 1.5 mg/ml G418 for selection of $Arnt^{+/-}$ or $Arnt^{-/-}$ progenitors. Hematopoietic colonies were scored by morphology after 6–7 days and confirmed by cytological staining (Olson et al. 1995).

In vitro differentiation and replating of ES cells

Gelatin-adapted R1 ES cells were cultured in medium consisting of highglucose DMEM (GIBCO-BRL) supplemented with 15% fetal calf serum (Hyclone), penicillin–streptomycin (GIBCO-BRL), MEM nonessential amino acids (GIBCO-BRL) and leukemia inhibitory factor. ES cells (~3×10^3) were plated in methylcellulose containing 10% serum, 500 U/ml rhIL-1, 5 ng/ml rmIL-3, 10 µg/ml insulin, 200 µg/ml transferrin, and 10^{-4} M α-monothioglycerol, and allowed to differentiate under normoxic (20% O_2, 5% CO_2, 75% N_2) or hypoxic (3% O_2, 5% CO_2, 92% N_2) conditions. rmVEGF (5 ng/ml), rhEPO (2 U/ml), rmSCF (5 ng/ml), or GM-CSF (0.5 ng/ml) were added as described. After 9 days, cystic EBs were washed free of methylcellulose with PBS and disaggregated with trypsin (GIBCO-BRL) and mechanical shearing, using a 21-gauge needle and syringe. Cells were replated into a secondary methylcellulose medium identical to that used for the hematopoietic progenitor assays and incubated under normoxic conditions. The number of cells plated was equal to the number of cells in 50 embryoid bodies derived from wild-type ES cells differentiated under normoxic conditions. Hematopoietic colonies were scored after 6–7 days.

Northern analysis

Embryoid bodies from ES cells differentiated under 20% and 3% O_2 for 9 days were washed with PBS and RNA was extracted with TRIzol reagent (GIBCO-BRL) according to the manufacturer's instructions. mRNA was isolated using a poly(A)$^+$ selection kit (Invitrogen). The probes for VEGF and HIF-1α were generated with RT–PCR using 3′-UTR-specific primers (Maltepe et al. 1997).

Acknowledgments

We thank Cynthia Clendinin, Michele Hadhazy, Kirsten Sigrist, and Donna Fackenthal for technical assistance; Navdeep Chandel for help with hypoxic cell culture; Jeffrey Leiden and Brian Keith for critically reviewing the manuscript; Cheryl Small for secretarial assistance; and

Denise Wiler for preparing the illustrations. D.M.A. is a fellow of the Medical Scientist Training Program. E.M. is a Nathan and Francis Goldblatt Society Fellow. M.C.S. is an investigator of the HHMI.

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